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# Fylogeneze a evoluce bodlinatých myší rodu *Acomys*
# Phylogenetic relationships and evolution of the genus *Acomys* (Rodentia: Muridae)

Disertační práce

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# Phylogenetic relationships and evolution of the genus *Acomys* (Rodentia: Muridae)

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Doctoral thesis

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Prohlašuji, že jsem se v uvedených rukopisech podílela na všech fázích jejich přípravy a můj celkový podíl na rukopisech odpovídá pořadí a počtu spoluautorů. Zároveň prohlašuji, že jsem nepředložila práci ani její podstatnou část k získaní jiného nebo stejného akademického titulu.

Klára Palupčíková (Průšová)

## Poděkováni

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# 1 Abstrakt

Geografické rozšíření a fylogeneze bodlinatých myší rodu *Acomys* Geoffroy I., 1838 zůstává stále kontroverzním a otevřeným tématem. Tato práce se zabývá vnitrodruhovou strukturou a geografickým rozšířením rodu *Acomys* ze severní, východní, střední a jižní části Afriky, ostrovů Středozemního moře Kypr a Kréta, Tureckého pobřeží, Arabského a Sinajského poloostrova a z Íránu, při využití molekulárních analýz. Molekulární analýzy byly založeny, jak na mitochondriálních genových sekvencích genů D-loop (publikace I) a cytochromu b, tak i na jaderných sekvencích genu intraretinálního vazebného proteinu (IRBP) (publikace III, IV) a rekombinantního aktivačního genu 1 (RAG1) (publikace II.). Dále byla data podrobena fylogenetickým analýzám za použití analýz Maximální pravděpodobnosti (Maximum likelihood, ML), Bayesovské anylýzy (BA), Maximální úspornosti (Maximum parsimony, MP) a metoda nejbližšího souseda (Neighbor-joining, NJ).

Výsledky těchto analýz potvrdily, že afro-středomořské *Acomys cahirinus* a asijské klady *Acomys dimidiatus* jsou jasně oddělené. Velká podobnost mezi haplotypy z kontinentální Afriky a severního Středomoří (klad *A. cahirinus* sensu stricto) podporuje hypotézu, že předci *A. nesiotes, A. cilicicus* a *A. minous* se velmi pravděpodobně rozšířili jako komenzální populace, čímž je zpochybněn jejich status jako platných druhů. Byla nalezena značná genetická variabilita v Asii (publikace I), avšak největší genetická variabilita byla nalezena ve východní Africe (publikace II, III, IV). Multilokusová fylogeneze založená na čtyřech genetických markrech ukazuje přítomnost pěti hlavních skupin bodlinatých myší rodu *Acomys*: *A. subspinosus, A. spinosissimus, A. russatus, A. wilsoni* a *A. cahirinus*. Skupiny *A. spinosissimus, A. wilsoni* a *A. cahirinus* jsou dále strukturovány do fylogenetických subpopulací s převážně parapatrickým rozšířením.

Tato disertační práce přináší informace o existenci nejméně 27 výlučných genetických liniích bodlinatých myší rodu *Acomys* z nichž některé byly poprvé popsány až v této práci.

# 2 Abstract

The geographical distribution and phylogeny of the spiny mice of the genus *Acomys* Geoffroy I., 1838 remains a controversial and open topic. This doctoral thesis deals with the intraspecific structure and geographical distribution of the genus *Acomys* from the northern, eastern, central and southern parts of Africa, the Mediterranean islands of Cyprus and Crete, the Turkish coast, the Arabian and Sinai Peninsula and Iran, using molecular analyzes. Molecular analyzes were based on both the mitochondrial gene sequences of the D-loop genes (publication I.) and cytochrome b, as well as the nuclear sequences of the Intraretinal Binding Protein gene (IRBP) (publication IV) and recombinant activation gene 1 (RAG1) (publication II.). Furthermore, the data were subjected to phylogenetic analyzes using the Maximum Probability, Bayesian, Maximum Parsimony, and Minimum Evolution analysis.

The results of mentioned analyses confirmed that the Afro-Mediterranean *Acomys cahirinus* and Asian *Acomys dimidiatus* are clearly separated. The large similarity between the haplotypes of continental Africa and the northern Mediterranean (*A. cahirinus* sensu stricto) supports the hypothesis that the ancestors of *A. nesiotes*, *A. cilicicus* and *A. minous* are very likely to spread as commensal populations, thereby challenging their status as valid species. Considerable genetic variability was found in Asia (publication I), but the greatest genetic variability was found in East Africa (publications II, III, IV). Multi-locus phylogeny based on four genetic markers shows the presence of five major groups of spiny mice of the genus *Acomys*: *A. subspinosus*, *A. spinosissimus*, *A. russatus*, *A. wilsoni* and *A. cahirinus*. The groups *A. spinosissimus*, *A. wilsoni* and *A. cahirinus* are further structured into phylogenetic sub-populations with predominantly parapatric distributions.

This doctoral thesis provides information on the existence of at least 27 lineages of spiny mice of the genus *Acomys*, some of which were first described in this thesis.

# Obsah

# 3 Úvod

## *3.1* Jedinečnost bodlinatých myší rodu *Acomys*

Bodlinaté myši rodu *Acomys* jsou zvláštní myšovití hlodavci, u kterých se vyvinulo několik jedinečných znaků, z nichž některé nejsou známy u jiných myšovitých hlodavců či dokonce savců. Například Bellofiore et al. (2017) zaznamenal menstruaci u *Acomys cahirinus*. Jde o první záznam menstruačního cyklu u hlodavců, což naznačuje možnost využití bodlinaté myši jako modelový organismus v rámci výzkumu nemocí spojených s menstruací a souvisejících poruch (např. endometrióza či premenstruační syndrom). Nebo je to extrémní schopnost regenerace kůže (Seifert et al. 2012), která v poslední době přitahuje pozornost velkého množství laboratorních vědců (Jiang et al. 2019, Mescher 2017, Simkin et al. 2017, Haughton et al. 2016, Matias et al. 2016, Seifert et al. 2014). Popřípadě nutričně indukovaný diabetes studovaný u *A. cahirinus* (Shafrir 2000, Shafrir et al. 2006), který ukazuje, že bodlinaté myši rodu *Acomys* jsou vhodným modelovým organismem v rámci biomedicínského výzkumu.

V systematicko-ekologickém výzkumu slouží jako model v několika oblastech, např. ke studiu sympatrické speciace. Ta byla zdokumentována u *A. cahirinus* (Hadid et al. 2014) v „Evolučním kaňonu“ v Izraeli, který sám o sobě představuje evoluční laboratoř v přímém přenosu. V „Evolučním kaňonu“ dochází k situaci, kde se na malém území nachází velmi divergentní mikroklimata. Na jednom svahu kopce se setkáváme se suchým tropickým klimatem Afriky a na opačné straně svahu s evropským mírným klimatem. A právě tyto přirozené ostře rozdělené mikro oblasti (vzdálené od sebe pouze 200 m) na hoře Carmel v Izraeli zapříčinily genotypovou i fenotypovou divergenci u skupiny *A. cahirinus* (Hadid et al. 2014).

Stručně se zmíním i o dalších oblatech výzkumu bodlinatých myší – od reprodukční biologie a fyziologie až po chování. Bodlinaté myši mají primárně noční aktivitu, avšak v případě sympatrického výskytu *A. cahirinus* a *A. russatus*, dochází u *A. russatus* k posunutí aktivní doby do časného rána či pozdního odpoledne, a tím nedochází k mezidruhové kompetici (Gutman & Dayan 2005, Haim et al. 1994, Kronfeld-Schor et al. 2001). *A. russatus* je schopen obývat extrémně suché oblasti, kde *A. cahirinus* již často chybí. Obývání velmi teplých stanovišť vyžaduje fyziologické adaptace pro přežití v takto extrémních životních podmínkách. Jednou z nich je snížení klidového metabolismu (Ehrhardt et al. 2005, Merkt &

Taylor 1994, Perrin & Downs 1994, Weissenberg & Shkolnik 1994), který je u *A. russatus* nejnižší v rámci rodu *Acomys* (Degen 1994).

Nedostatek vody v prostředí dokáží překlenout zvýšeným příjmem živočišné stravy, hlavně pojídáním plžů (Degen 1994, Kronfeld-Schor & Dayan 1999, Broza & Nevo 1994, Kam & Degen 1993, Neal 1984). Jejich jedinečné hospodaření s vodou v nehostinném pouštním prostředí je také podpořeno schopností vylučovat koncentrovanější moč (Weissenberg & Shkolnik 1994) a udržení si plazmatické vody i přes celkovou dehydrataci organismu (Horowitz & Borut 1994).

Bodlinaté myši rodu *Acomys* mají na svém dorzálním povrchu nápadné bodliny, které jsou také částečně přizpůsobeny pro potřeby přežití v horkém prostředí. Bodliny mají na svém povrchu hluboké rýhy, kde se během nočního ochlazení sražená vlhkost shromáždí a jemná rosa následně poslouží pro ochlazení organismu při jejím odpařování (Chernova & Kuznetsov 2001).

Bodlinaté myši rodu *Acomys* si neshromažďují potravu, ale dokáží si ukládat značné množství tělesného tuku a snížit rychlost metabolismu v případě, že dojde k omezení přísunu potravy (Ehrhardt et al. 2005). Během dne se střídají dvě behaviorální strategie. První, v době odpočinku, se projevuje jako strnulost / torpor u které dochází ke snížení tělesné teploty za současného snížení rychlosti metabolismu a ukládání energie v podobě tělesného tuku. U druhé dochází ke zvýšení aktivity během doby jejich činnosti pro nalezení potravy (Ehrhardt et al. 2005).

Zástupci rodu *Acomys* podléhají predaci, jak ze strany vzdušných (Edut & Eilam 2003, Eilam et al. 1999, Mandelik et al. 2000, Hendrie et al. 1998), tak i pozemních predátorů (Jones & Dayan 2000). Během svého života se učí rozpoznávat a následně se i vyhýbat podnětům signalizujícím přítomnost predátora (Jones & Dayan 2000, Carere et al. 1999). Rychlý únik mezi kameny se jeví jako nejlepší strategie při útoku predátora, tudíž autotomie ocasu je schopnost, která se považuje za fyziologickou adaptaci vzniklou jako antipredační obranný mechanismus (Shargal et al. 1999).

Bodlinaté myši rodu *Acomys* mají i pozoruhodnou regulaci rozmnožovaní (Wube et al. 2008a, 2008b, 2009, 2016, Medger et al. 2012, Sarli et al. 2016). Tropické i subtropické oblasti mají výrazné změny srážek během roku s jedním či dvěma obdobím dešťů, které následují po období sucha (Nicholson 1993). Ve svém důsledku srážky vedou k vyššímu množství kvalitní potravy ve srovnání s potravní nabídkou během období sucha. *A. spinosissimus* a *A. dimidiatus* využívají období dešťů, jako přírodní faktor k aktivaci své reprodukce (Sarli et al. 2016,

Medger et al. 2012), avšak schopnost reprodukce *A. dimidiatus* neztrácí během celého roku v případě výskytu příznivých podmínek (Sarli et al. 2016).

Mnoho savců využívá pro aktivaci reprodukce sezóně se měnící denní a noční cyklus, což je zvláště účinné ve vysoce předvídatelném prostředí (Bradshaw & Holzapfel 2007), avšak v případě *A. cahirinus* jsou obě pohlaví schopná si udržet rozmnožovací aktivitu, jak v krátké, tak i v dlouhé fotoperiodě. Zatímco u *A. russatus* si dokáže udržet schopnost reprodukce nezávisle na fotoperiodě pouze samice, ale jejich samčí protějšek není schopen udržet testikulární aktivitu během krátké fotoperiody (Wube et al. 2008a). Okolní teplota prostředí společně s dešťovými srážkami je také důležitým faktorem, který je vysoce korelován s reprodukční aktivitou u bodlinatých myší *A. spinosissimus* z jižní Afriky, ale koncentrace testosteronu se zvyšuje již asi dva měsíce před začátkem období dešťů (Medger et al. 2012). V průběhu suchého období dochází ve volné přírodě k výraznému odpaření vody obsažené ve vegetaci a tím dochází ke koncentraci rostlinné tkáně, která je součástí potravní složky bodlinatých myší. Konzumací takovéto rostlinné stravy jsou myši vystaveny stravě se zvýšeným obsahem soli. Wube et al. (2009) zaznamenal, že zvýšený příjem soli v potravě vede u samců xerické populace *A. russatus* k regulaci reprodukce s pravděpodobným signálem o postupujícím období sucha. Avšak v případě mezické populace *A. cahirinus,* žádná regulace reprodukce nebyla pozorována.

Velmi zajímavým znakem v rámci reprodukční strategie u bodlinatých myší rodu *Acomys* je, že rodí prekociální mláďata (Dieterlen 1963, Dewsbury & Hodges 1987, Dempster et al. 1992). Samice mají malý počet mláďat v jednom vrhu, po asi 2x tak delší době gravidity (36-42 dnů) (Brunjes 1990, Nováková et al. 2010), než u běžné myši domácí, což umožňuje mláďatům se velmi dobře vyvinout ještě v prenatálním vývoji. Byl zaznamenán pohlavní dimorfismus ve velikosti těla při narození mláďat. Samčí mláďata jsou signifikantně větší, tento rozdíl se již během života nezvětšuje. Samice tedy v průběhu gravidity více investují do samčího pohlaví svých potomků, přestože sekundární poměr pohlaví se neliší od poměru 1:1 (Nováková et al. 2010). Po porodu dochází u samic k estru postpartum (Dieterlen 1961, Peitz 1981).

Samčí rodičovská péče (Dieterlen 1962, Makin & Porter 1984) pozitivně ovlivňuje růst a fyziologický vývoj mláďat (Elwood & Broom 1978). Samci bodlinatých myší se účastní péče o potomky, kdy pomáhají již při porodu olizováním částečně vytlačeného plodu (Dieterlen 1962). V rozvoji paternálního chování je důležitá předchozí zkušenost. Doba strávená s mláďaty se postupem času prodlužuje, jde o dobu, kdy samice opouští mláďata a samec

mezitím zajišťuje termoregulaci a ochranu mláďat (Szjiarto et al. 1985, Makin & Porter 1984, Porter et al. 1980).

Schopnost samic individuálního rozpoznání vlastních mláďat od cizích je zajištěno přenesením čichové značky na svá mláďata během mateřské péče, jako je olizování, společné choulení či kojení (Porter 1986). Samci také dokáží rozeznat vlastní mláďata od cizích (Makin & Porter 1984). Feromon, který samice bodlinatých myší produkují při kojení, mláďata následují od prvního dne života (Porter & Ruttle 1975), avšak tato preference se snižuje s věkem mláďat až je kolem 25. dne života ignorován (Janus 1988, Porter & Doane 1979).

Sociální stres, u samců a samic různého stáří i sociálního postavení v rámci rodinné skupiny, byl testován na základě stanovení hladin glukokortikoidů (Nováková et al. 2008). Testovány byly komenzální a nekomenzální populace *A. cahirinus*, které se liší jak vzhledem, tak chováním. Bazální hladiny glukokortikoidů se nelišily ani v jednom sociálním postavení. Ať už se jednalo o samce či samice, či různé stáří zvířat, přestože jsou mladí samci, kteří mají snahu se zapojit do reprodukce, terčem agrese dospělým samcem-otcem (Nováková et al. 2008). Avšak při porovnání dvou rozdílných populací se rozdíly ukázaly a bylo zjištěno, že komenzálně žijící myši mají vyšší hladiny glukokortikoidů, což je přičítáno jejich zvýšenému predačnímu tlaku, kterému je každá komenzální populace vystavena v lidských obydlích (Nováková et al. 2008).

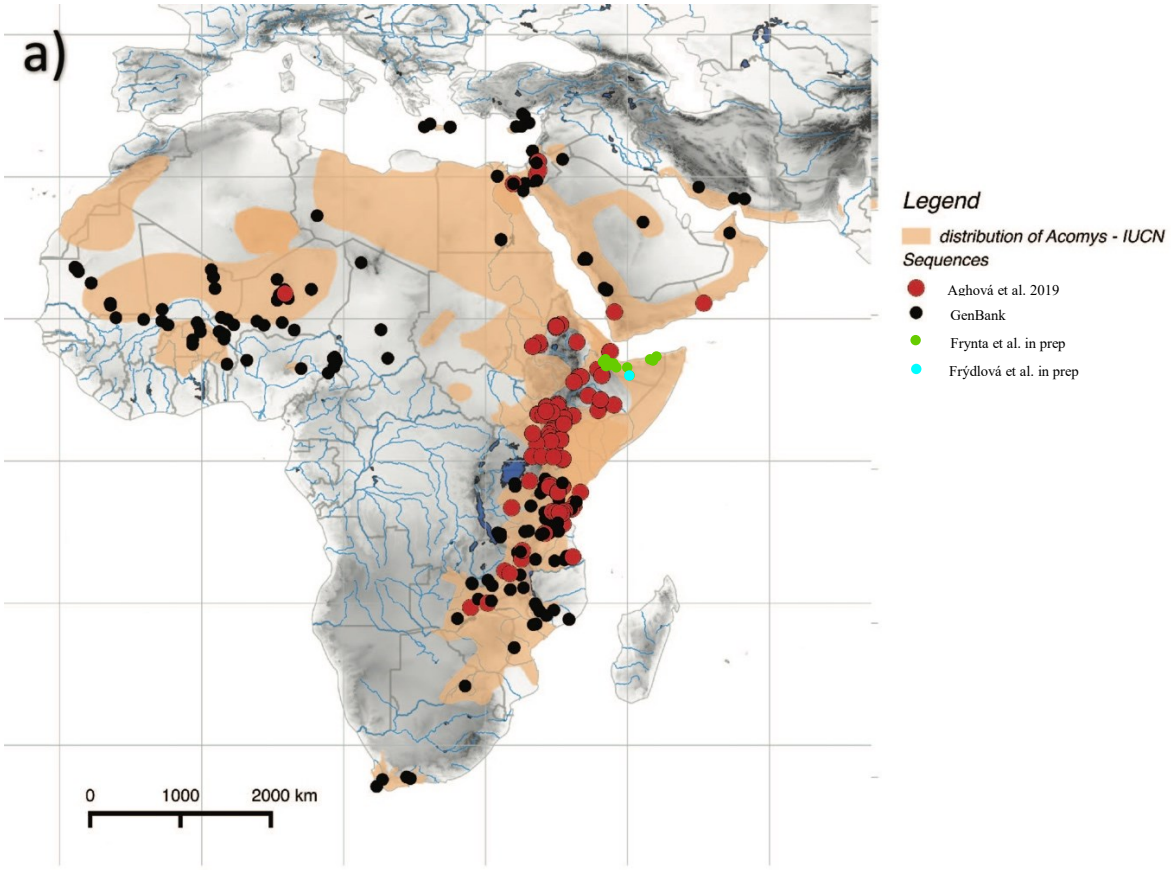
Bodlinaté myši rodí nejčastěji dvě mláďata (shrnutí viz Frynta et al. 2011). Velikost vrhu závisí na hmotnosti samice a počtu juvenilních samic ve skupině. To může být vysvětleno, jako výsledek pozitivního sociálního nastavení ve skupině. Mezipopulační srovnání naznačuje pozitivní korelaci mezi délkou chování zvířat v laboratorních podmínkách a průměrnou velikostí vrhu (Frynta et al. 2011 a nepublikované výsledky). U laboratorně chovaných zvířat po desetiletí patrně dochází k adaptaci na odlišné selekční tlaky, které jsou následně spojeny se změnami plodnosti.

Bodlinaté myši rodu *Acomys* patří k taxonům hlodavců, které byly rozsáhle studovány po celá desetiletí a staly se tak vzorem v mnoha oborech ekologie, fyziologie a evoluce. Nicméně velká většina výše uvedených studií byla provedena na druzích *A. cahirinus*, *A. dimidiatus* či *A. russatus*, což je na těch druzích, které obývají Izrael a sousední oblasti Blízkého východu a představují pouze terminální odnož fylogenetického stromu rodu *Acomys*. Pro správnou interpretaci všech výše uvedených výsledků je velmi důležitá fylogeneze jednotlivých druhů a stejně tak i poznání celého fylogeografického vývojového scénáře.

## 3.2 Biogeografie rodu *Acomys*

Současné fylogenetické hypotézy řadí rod *Acomys* do jasně oddělené podčeledi Deomyinae, která zahrnuje tři další rody: *Uranomys*, *Deomys* a *Lophuromys*, přičemž dvě poslední jmenované představují sesterský klad k *Acomys* (Chevret et al. 2001, Jansa & Wechsler 2004, Alhajeri et al. 2015). Protože tyto tři rody mají výhradně africké rozšíření, je předpoklad, že i původ rodu *Acomys* je na Africkém kontinentu (Alhajeri et al. 2015).

Africký kontinent můžeme rozdělit na sedm dobře definovaných a konzistentních biogeografických regionů: Jihoafrický, Zambijský, Kongský, Somálský, Etiopský, Súdánský a Saharský (Linder et al., 2012). Jednotlivé záznamy o výskytu rodu *Acomys* jsou obecně spojeny s (polo)suchým prostředím (Kingdon 2013) až do výšky 2 500 m nad mořem (Monadjem et al. 2015). Většina druhů *Acomys* se vyskytuje v savanovém, somálsko-masaiském, zambijském a částečně také súdánském regionu. Rozšíření *A. subspinosus* v jižní části Afriky je vázána na vegetaci fynbos. Několik druhů či populací *Acomys* je petrikolní, jejich život je tak těsně spjat s kamenitým stanovištěm. To je obzvlášť patrné u druhů obývajících suché savany, polopouště a pouště. Tyto nároky, na své životní prostředí, vysvětlují, proč druhy *Acomys* typicky chybí v tropické západní a střední Africe (= konžská oblast), ačkoli několik druhů se vyskytuje na severu v oblastech Sahelu a Sahary. Ve srovnání s Jižní Afrikou je druhová bohatost *Acomys* ve východní Africe vyšší, což lze vysvětlit historickým vývojem prostředí. Lorenzen et al. (2012) zjistil překvapivě malou fylogenetickou strukturu u pleistocenních taxonů kopytníků s rozšířením v jižní Africe ve srovnání s populacemi z východní Afriky, což naznačuje, že savany v jižní Africe uchovaly populace / druhy díky stabilnímu životnímu prostředí.




**Obrázek 1**: Analyzované vzorky a geografické rozšíření bodlinatých myší rodu *Acomys*. Obrázek byl převzat a doplněn z Aghová et al. (2019).

Kromě velké části Afrického kontinentu se rod *Acomys* rozšířil do Středomoří (zahrnuje malý pruh podél jižního pobřeží Turecka mezi městy Mersin a Silifke a ostrovy Kypr, Kréta), Asii (od Sinajského poloostrova přes Izrael, Sýrie, Jordánsko, Arabský poloostrov včetně Jemenu) a na přilehlá území v Íránu a Pákistánu podél Perského a Ománského zálivu (Barome et al. 1998; 2000, Kryštufek et al. 2009, Frynta et al. 2010) viz obrázek 1.

Z hlediska biogeografie, která je úzce spjata s ekologií i fylogenetickou biologií (Brown a Lomolino 1998), lze geografické rozšíření rodu *Acomys* rozdělit na Afrotropní a saharskoarabský region (Holt 2012). Afrotropní region je region subsaharské Afriky, toto prostředí se vyznačuje tropickým travním porostem, savany a keři (Sayre et al. 2013).

Biogeografické vztahy mezi těmito bioregiony, zejména mezi Afrikou a Arábií, lze studovat pomocí vyřešené fylogeneze taxonů s velkým rozšířením, založenou na konceptu fylogenetického konzervatismu. Rozšíření některých skupin mezi kontinenty vyžaduje nejen pevninská spojení, ale také vhodné klima v oblasti tohoto spojení v časovém období

předpokládané disperzní události. Současná biologická rozmanitost v Afro-Arábii je výsledkem, jak geomorfologické historie (rift afrických a arabských desek) tak i intenzivní změny klimatu od raného miocénu (např. Steckler et al. 1988).

## 3.3 Bariéry

Současné rozšíření jednotlivých druhů z rodu Acomys jasně naznačuje, že geografické bariéry hrají významnou roli při tvorbě areálů rozšíření a nejspíše i vzniku nových druhů. Ačkoli jsem výše zmínila dobře prostudovaný případ sympatrické speciace v Evolučním kaňonu, geografické scénáře speciace zůstávají hlavním východiskem pro interpretaci speciace bodlinatých myší rodu *Acomys*. Při nejjednodušším z nich, alopatrickém, je areál původního druhu rozdělen a na obou stranách bariéry se populace vyvíjejí odděleně a s postupem času a přibývajícími změnami vznikají dva druhy (Mayr 2009). V následujícím přehledu se proto stručně zabývám vybranými bariérami v historii afrického kontinentu.

### 3.3.1 Pevninské mosty a výměna hlodavců mezi Afrikou a ostatními kontinenty

Během raného miocény (23,0-16,0 Ma), kdy Arabská deska již byla oddělena od Afriky Rudým mořem a rotací proti směru hodinových ručiček došlo nakonec ke srážce s Eurasií, se vytvořil první, avšak dočasný pevninský most mezi Afrikou a Eurasií tzv. Gomphotherium (Rögl, 1999). Během tohoto časového období začínají raní myšovití kolonizovat, jak Afriku, tak i Arábii (Mein et al., 2000, Winkler 1994). Ve středním Miocénu (16,0 - 11,6 Ma) dochází k přerušení pevninského mostu mezi Afrikou a Eurasií díky opětovnému propojení Středo-Indo-Tichomořské cesty (Rögl 1999), která oddělila Afriku od Eurasie, čímž se vytvořily hlavní linie afrotropních a indomalajských hlodavců. Nicméně výměny mezi Afrikou a Arábií přetrvávaly až do středního pliocénu (Denys 1985).

Během středního Miocénu a na začátku messinské salinitní krize cca 13,8 Ma se Středo-Indo-Tichomořská cesta opět uzavřela (Rögl 1999) a současně s globálním ochlazením zapříčinila celkovou aridifikaci prostředí a s tím i spojenou změnu vegetace (Prista et al. 2014). Nově vytvořený pevninský most (Rögl, 1999) umožnil výměnu myšovitých hlodavců mezi Asií, Afrikou a Eurasií (Winkler 1994, Winkler 2002).

Poslední výměna fauny myšovitých taxonů mezi Afrikou, Asií a západní Palearktidou (Benammi et al. 1996, Winkler 2002) je shodná s messinskou salinitní krizí (cca 6 Ma), která probíhala během pozdního miocénu (Hsü a kol. 1973, 1978) a s glaciálními intervaly (Cosentino et al. 2013). Během tohoto období dochází ke globálnímu snižování hladin moří (Haq et al. 1987) a Afrika s Arábií byly znovu propojeny pozemním mostem Negev-Sinaj tzv. Levantským koridorem (Fernandes et al. 2006) a prostřednictvím uzavřené úžiny Bab-el-Mandab 10- 5,3 Ma (Bosworth et al. 2005). Úžina Bab-el-Mandeb, jež vytvořila pozemní most, spojila Afriku a Arábii přes Danakilský blok až do raného středního pliocénu (Redfield et al. 2003). Po znovuotevření průlivu Bab-el-Mandab 5,3 Ma se Arábie definitivně a trvale oddělila od Afriky. Tato výměna fauny je zachycena v rámci afrického podrodu Nannomys, který kolonizoval Afriku cca 5,2 Ma (Bryja et al. 2014). Předpokládá se, že po miocénu již nikdy nedošlo k vzniku dalšího pozemního mostu mezi Afrikou a Arábií (Fernandes et al. 2006). Ale na druhé straně Arabského poloostrova bylo opakovaně navazováno spojení s Asijskou pevninou, když byl Perský záliv vysušen, nebo nanejvýš tvořen řadou sladkovodních jezer (Lambeck 1996; Uchupi et al. 1999).

### 3.3.2 Biogeografické bariéry uvnitř Afriky

Geomorfologickým rysem Afrického kontinentu je převážně malá vertikální členitost reliéfu, která je patrná, jak v nižších, středních, ale i vyšších polohách. Na rozdíl od většiny ostatních kontinentů je Africký reliéf charakterizován spíše povodím, tabulových forem reliéfů (nacházející se vesměs uvnitř kontinentu) a příkopovými propadlinami (Goudie 2005). Příkopové propadliny společně s řekami patří k významným biogeografickým bariérám v Africe. Goudie (2005) potvrdil předchozí studie a dospěl k závěru, že mnoho současných říčních toků je překvapivě velmi mladých. Týká se to také řek oddělujících geografické rozšíření existujících druhů *Acomys*. Je pravděpodobné, že Niger původně pokračoval severovýchodně směrem do Sahary a teprve nedávno se otočil jihovýchodně někam poblíž Tosaye, aby zde vytvořil soutok s řekou Benue. Nil se skládá z několika různých řek, které byly propojeny teprve nedávno (Van Damme & Van Bocxlaer 2009). Její předchůdce Eonile byl založen z reverzně tekoucí řeky Qena v době messinské salinitní krize. V časném pliocénu, dochází k transgresi (zvýšení mořské hladiny, zaplavení pevniny) a rozšíření ústí Eonile až do Asuánu. Řeka Zambezi vznikla až teprve v nedávné době pliocénu až ve středním pleistocénu, spojením dvou říčních toků. Její horní a střední část toku se vyvinuly jako dva samostatné

systémy. Horní tok spolu s řekou Kafue se prapůvodně spojoval s řekou Limpopo, zatímco střední část řeky Zambezi se spojovala s řekou Shire (Goudie 2005). Relativně náhlé změny směru a charakteristika jejího současného kurzu naznačuje, že Zambezi (o rozloze cca 1,33 mil $km^2$) se teprve relativně nedávno stala spojeným jednotným říčním systémem.

Eosahabi a Gabes byly velké řeky severní Sahary, jež byly založené v miocénu a přetrvaly až do období sucha v pliocénu a pleistocénu (Goudie 2005). Pravděpodobně vytvářely přírodní překážku pro šíření z východu na západ v rámci severní Afriky. Čadské jezero, představuje v současné době spolu se svými ztracenými přítoky nejdůležitější bariéru, která brání šíření v tomto směru (Ghienne et al. 2002, Schuster et al. 2009).

Východoafrický riftový systém představuje hlavní geografickou bariéru. První fáze jeho vzniku začala již v raném miocénu (17,0 Ma). Nejvýznamnější však byla jeho druhá fáze v období pozdního miocénu a pliocénu (6,2 - 2,7 Ma; Simon et al. 2017). Krajina východní Afriky se v tomto časovém období dramaticky změnila. Z relativně homogenní oblasti pokryté tropickým smíšeným lesem na heterogenní oblast, kde některé hory dosahují výšky nad 4 000 m a s vegetací od pouštní po vlhkomilnou. K izolaci přispívala nejen odlišná vegetace rostoucí ve vyšších nadmořských výškách riftového systému (Tieszen et al. 1979), ale i přítomnost efemerních jezer, což společně s částečným zaplavením údolí riftu, mohlo představovat přírodní vodní bariéru a zamezit tak možnost volného šíření (Trauth et al. 2010, Junginger & Trauth 2013, Maslin et al. 2014). Velmi proměnlivé prostředí, vzniklé ve východní Africe pod vlivem složité geologie a podnebí ve východoafrickém riftu, mohlo vést ke speciaci a také k následným disperzním událostem (geologická separace, environmentální stres, zrychlený vývoj, rozšiřování specialistů, konkurence mezi druhy) (Maslin et al. 2014).

### 3.3.3 Klimatické změny od dob miocénu spojené se změnami vegetace (23-5,32 Ma)

Charakteristickým rysem klimatu na počátku miocénu bylo oteplování, s kterým bylo spojené i tání ledovců. Tato teplá fáze dosáhla svého klimatického optima v pozdním středním miocénu (17-15 Ma) (Zachos et al. 2001). Počasí se vyznačovalo sezónním rozložením srážek s výrazným rozdílem mezi obdobím dešťů a sucha (Bonnefille 2010). V pozdním miocénu dochází díky výše uvedeným klimatickým podmínkám, ke globálnímu šíření rostlin, typu C4 (zejména tropických trav). Rostliny typu C4 požadují pro svou expanzi vlhko, teplo a nízký $CO_2$ (Huang et al. 2001). Bonnefille (2010) popsal výraznou variabilitu složení lesů, kdy v

Etiopii byl zdokumentován listnatý les, zatímco v západní Keni rostly vlhčí stálezelené lesy. Po tomto klimatickém optimu došlo k postupnému ochlazování a obnově hlavní ledové pokrývky (Holbourn et al. 2005, Zachos et al., 2001). Od středního miocénu bylo ve východní Africe vytvořeno mnoho ekosystémů (Cerling et al. 1997). Ve východní Africe se začaly šířit pastviny / savany zhruba před 10 Ma (Cerling, 1992; Cerling et al., 1993), ale v západní Africe dochází k šíření až o 3 Ma později (Bonnefille 2010), kdy nejvyšších hodnot bylo dosaženo mezi 8,5 - 6,5 Ma (Cerling et al. 1997). Ochlazování pokračovalo až do počátku pliocénu (6 Ma) (Zachos et al. 2001), avšak na začátku pliocénu dochází k mírnému oteplení a sice až do 3,2 Ma (Poore & Sloan 1996, Maslin a kol. 1998).

S výrazným orogenickým vzestupem Himalájského pásma 8 Ma dochází k vývoji monzunů a tím i k výrazné změně klimatu (Partridge et al. 1995). Saharská poušť vzniká mezi 7 Ma a 2,5 Ma (Schuster et al. 2006, Swezey 2009). V průběhu období mio-pliocénu došlo v rámci třech epizod (10,5 Ma, 7 Ma a 5,5 Ma) k nárůstu vegetace a to, jak trav, tak i lesů, což naznačuje všeobecné vlhčí kontinentální klima (Bonnefille 2010). Rozšíření vlhkomilného lesa bylo zdokumentováno v Čadu cca 7 Ma (Brunet et al. 2005), v povodí řeky Niger (Morley 2000) a ve východní Africe 6,8 Ma (Bonnefille 2010). Někdy těsně kolem počátku messinské salinitní krize došlo k výrazné změně vegetace. V celé tropické oblasti panovalo velmi aridní klima, které se projevovalo významným poklesem stromového porostu současně v západní i východní Africe (6,5 až 5,5 Ma; Duggen et al. 2003, Schuster et al. 2006). V celé tropické Africe dochází současně s prudkým poklesem lesního pokryvu k masivnímu rozšíření stepi a pouště (Bonnefille 2010). V severní a jižní Keni existovala zalesněná území až do 3,7 Ma (Sepulcher et al. 2006), která naznačují výskyt vlhkého klimatu v severovýchodní Africe (Hill et al. 2002). Tektonická činnost a probíhající vrásnění ve východoafrickém riftu 3,2 Ma v Keni (Veldkamp et al. 2007), zvyšovala celkovou ariditu prostředí, ale tyto děje umožnily vytvořit vhodné stanoviště pro horské lesy ve východní Africe při okrajích východoafrického riftu.

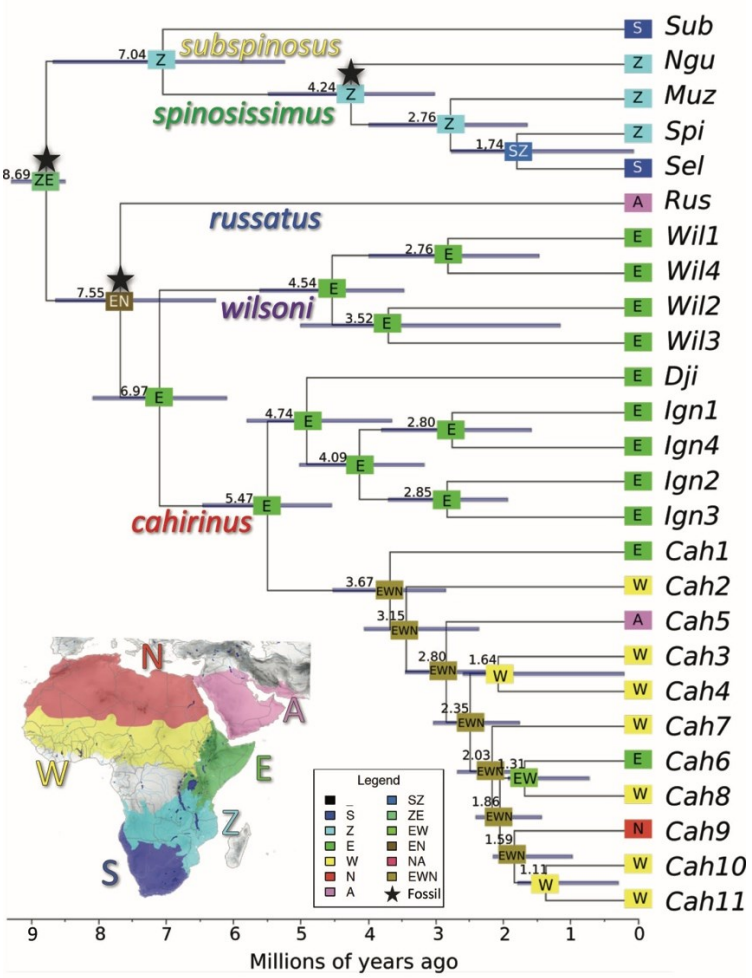
## 3.4 Fylogenetické vztahy mezi druhy / populacemi *Acomys*

Pro rozsáhlou studovanost bodlinatých myší mnoha obory, se zvyšuje důležitost poznání fylogenetických vztahů jednotlivých druhů. Znalost těchto vztahů je nutným podkladem pro rekonstrukci ancestrálních stavů znaků, jinak řečeno pro určení toho, kdy a ve kterém kladu

příslušné znaky, přesněji jejich stavy vznikly resp. zanikly. Věrohodná fylogenetická hypotéza (strom) se tak stává nepostradatelnou pro všechny studie provedené na bodlinatých myších, jako na modelovém organismu. Bez ní není správná interpretace jednotlivých výsledků v evolučním pohledu (např. zda jde skutečně o adaptaci) vůbec možná (cf. Harvey a Pagel 1991).

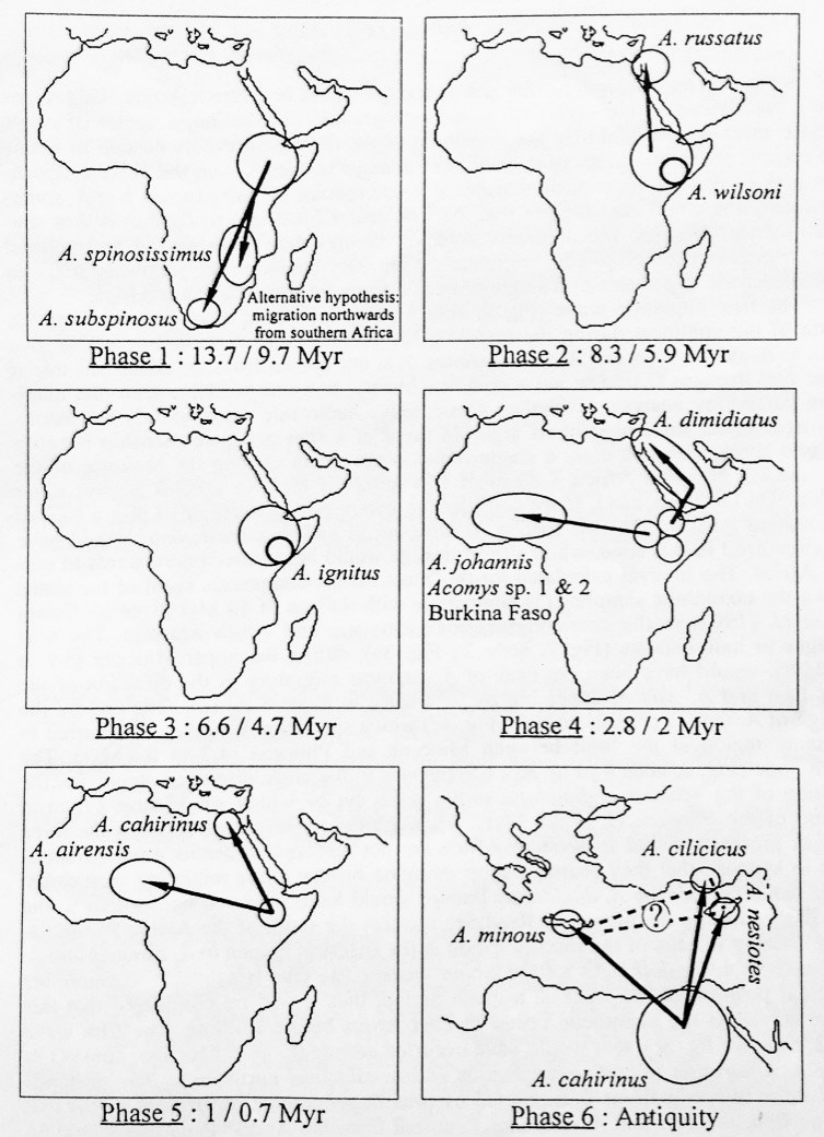
Rod *Acomys* (Geoffroy, 1838) byl popsán na začátku devatenáctého století. Existuje mnoho popsaných druhů, nicméně některé z nich nelze podle morfologických znaků snadno rozlišit. Je to zejména díky jejich velké vnitrodruhové variabilitě a shodné generalizované morfologii. Opakované snahy o revizi fylogenetické systematiky byly založeny na morfologických znacích (Ellerman 1941), chromozomech (Matthey 1968, Macholán 1995) a molárním vzoru zubů (Chevret 1993). Kromě *A. dimidiatus*, který má svou oblast výskytu v Asii, je v současné době velmi složité určit, které z afrických druhů rodu *Acomys* jsou právě uznávané. Kingdon (2013) a Monadjem et al. (2015) uvádějí 16 afrických druhů: *A. cahirinus, A chudeaui, A. muzei, A. cineraceus, A. ngurui, A. ignitus, A. johannis, A. kempi, A. louisae, A. mullah, A. percivali, A. russatus, A selousi, A. spinosissimus, A. subspinosus a A. wilsoni*. Avšak Denys et al. (2017) uvádí celkem již 21 druhů bodlinatých myší rodu *Acomys* s 12 podruhy. Tento historicky velmi nejednotný pohled na celkový počet a vymezení exitujících druhů bodlinatých myší rodu *Acomys* vyústil ve velmi rozsáhlou práci, která rekonstruuje fylogenetické vztahy v rámci rodu *Acomys* na základě největšího dostupného multilokusového datového souboru obsahující čtyři genetické markery ze 700 genotypizovaných jedinců z 282 lokalit (Aghová et al. 2019). Výsledky ukázaly přítomnost pěti hlavních skupin v rámci *Acomys*, které byly označeny jako *subspinosus*, *spinosissimus*, *russatus*, *wilsoni* a *cahirinus*. Kdy tři z těchto skupin (*spinosissimus*, *wilsoni* a *cahirinus*) jsou dále strukturovány do dalších fylogenetických linií s uvedením celkového počtu 26 existujících kryptických druhů *Acomys* viz obrázek 2 (Aghová et al. 2019) respektive 27 druhů (Frýdlová et al. in prep.) až 28 druhů *Acomys* (Frynta et al. in prep.). Teprve v pracích Frynta et al. (in prep.) a a Frýdlová et al. (in prep.) byla popsána oblast Somalindu. *A. louisae*, jakožto endemit Somalilandu, je jasně oddělenou skupinou od ostatních linií *Acomys* a samotná populace je rozdělena do dvou podskupin (Frynta et al. in prep.). Od středního Somalilandu na východ se nachází jedna „Somalilandská“ podskupina, která se v nedávné době rozšířila na své nynější území a její velikost populace se rychle zvětšila. Severozápadní Somaliland společně s Džibuti a východní Etiopií obývá „Džibutská“ podskupina a má extrémně vysokou sekvenční diverzitu (Frynta et al. in prep.), která bude spojená s tamní složitou geologickou historií území. Výskyt *A. louisae*

v Somalilandu během posledního interglaciálu (120-140 ka) byla pravděpodobně velmi fragmentovaná, jak napovídají výsledky z bioklimatického modelovaní (Aghová et al. 2019). Model neukázal širší oblast možného výskytu bodlinatých myší (Aghová et al. 2019), což je v souladu s nalezeným genetickým vzorcem (Frynta et al. in prep.). Velmi pravděpodobně došlo během posledního interglaciálu k zalesnění v centrální a východní části Somalilandu, nastolení nevhodných ekologických podmínek pro *A. louisae*, a tím i k částečnému nebo úplnému vymření bodlinatých myší s následnou rekolonizací z místního refugia, či oblasti východoafrické příkopové propadliny po interglaciálním období (Frynta et al. in prep.). Frýdlová et al. (in prep.) nalezli v jižním Somalilandu bodlinatou myš, která obývá červené písky bez skal (což je neobvyklé u většiny myší rodu *Acomys*). Molekulární znaky ukázaly, že jde o novou samostatnou linii sesterskou ke kladu zahrnujícímu předpokládané druhy Ign2 a Ign3 (=*A. ignitus*).




**Obrázek 2:** Rekonstrukce historické biogeografie a divergenčních událostí v rámci rodu *Acomys* (převzato z Aghová et al. 2019).

Avšak i nadále zůstává nutnost taxonomické revize hlavně u východoafrických taxonů, kde se nachází největší variabilita celého rodu *Acomys*. Většina současných molekulárních prací trpí zejména malým počtem vzorků získaných pro vlastní analýzu a / nebo nedostatečným geografickým pokrytím odebraných vzorků. Starší práce jsou založené na mitochondriálních sekvencích genu cytochromu *b* nebo D-loop. Barome et al. (1998, 2000, 2001a), který zkoumal vztahy mezi hlavními druhy myší rodu *Acomys* a navrhl následující fylogenetickou hypotézu (*subspinosus*, (*spinosissimus*, (*russatus*, *wilsoni*, (*ignitus*, ((*cahirinus, chudeaui*), (*dimidiatus, johannis*))))))). Následně Barome et al. (2000) navrhl odpovídající fylogeografický scénář (obrázek 3), kde předpokládané místo šíření je východní Afrika. Během středního miocénu došlo k bazálnímu rozdělení 9,7–13,7 Ma a první migrační událostí do jižní Afriky, kde vzniká *A. subspinosus a A. spinosissimus*, respektive 8,69 Ma (Aghová et al. 2019). Následné migrační vlny ve svrchním miocénu 5,9 – 8,3 Ma byly směrem na Blízký Východ (*A. russatus*), respektive 7,55 Ma (Aghová et al. 2019) a do východní Afriky (*A. wilsoni*), respektive 6,97 Ma (Aghová et al. 2019). Na stejném území jako dříve *A. wilosni* speciuje i *A. ignitus* v rozmezí mio-pliocenu 4,7 – 6,6 Ma (Barome et al. 2000). Skupina *cahirinus-dimidiatus* vzniká někdy na konci pliocénu 07-2,8 Ma (Barome et al. 2000) respektive 2,8 Ma (Aghová et al. 2019) a protože sdílela při šíření společnou historii, má velmi složité vnitřní uspořádání. Do severní Afriky se rozšířil *A. cahirinus*, do západní Afriky *A. johannis* zanedlouho následovaná *A. airensis* a přes úžinu Bab Al-Mandab na Arabský poloostrov *A. dimidiatus* (Barome et al. 2000). Pravděpodobně člověkem došlo k rozšíření bodlinatých myší z Egypta na přilehlé ostrovy Kypr, Kréta (Kunze et al. 1999) i jižní pobřeží Turecka a jejich pozdějšímu popsání jako *A. nesiotes*, *A. minous* a *A. cilicicus* (Barome et al. 2000).




**Obrázek 3**: Fylogeografický scénář disperze rodu *Acomys* (převzato z Barome et al. 2000).

Barome et al. (2001b) zkoumal varianty mitochondriálních haplotypů u ostrovní populace *A. minous* z Kréty a odhalil nedávný původ všech středomořských populací rodu *Acomys*. Tyto populace, původně uznávané jako odlišné druhy *A. minous*, *A. nesiotes* a *A. cilicicus*, jsou pravděpodobně konspecifické s *A.cahirinus* a představují potomky myší, které se rozšířily z Afriky prostřednictvím staroegyptského obchodu. To bylo později podpořeno úspěšným křížením *A. cahirinus* (nikoli však *A. dimidiatus*) s *A. cilicicus* a *A. nesiotes* chovaných v zajetí (Frynta & Sádlová 1998 a nepublikovaná data). Frynta et al. (2010) potvrdili, pomocí rychle se vyvíjejícího mitochondriálního kontrolního regionu D-loop, že středomořské haplotypy *A. nesiotes* a *A. cilicicus* se shlukují s pevninským *A. cahirinus* (Egypt, Libie, Čad). Kromě toho byla také zdokumentována mitochondriální divergence mezi *A. dimidiatus* ze Sinaje, Jemenu, Spojených arabských emirátů a Íránu. Tato data potvrdila, že *A.*

*dimidiatus* má své rozšíření pouze v asijské části celkové distribuce *Acomys* s jasnou genetickou odlišností od výhradně afrického (+ středomořského) *A. cahirinus* o kterém dříve psal Volobouyev et al. (2007).

Jižní část rozšíření *Acomys* byla důkladně zkoumána Verheyenem et al. (2011). Demonstroval skrytou mitochondriální a morfologickou divergenci v rámci *A. spinosissimus* sensu lato, skládající se z pěti odlišných kladů, u kterých nedochází k překrývaní v rámci jejich jednotlivých geografických oblastí výskytu. Kromě znovu uznaného druhu *A. selousi* a dalšího nepopsaného taxonu z oblasti severně od řeky Zambezi popsal *A. ngurui* a *A. muzei*.

Nedávno došlo k přezkoumání fylogenetických vztahů v rámci celého kladu Gerbilinae-Deomyinae, včetně 16 druhů *Acomys* (Alhajerim et al. 2015). Jejich časová kalibrace naznačuje mírně kratší chronologii 15,9–17,6 Ma než předchozí studii Chevret & Dobigny (2005), která uvedla rozštěpení Gerbilinae od Deomyinae někdy kolem 17 Ma. Avšak obecný kladogenetický vzor výsledného fylogenetického stromu potvrdil předchozí práce molekulárních studií (viz výše), i v případě analýzy zahrnující 900 druhů hlodavců (Steppan & Schenk 2017).

# 4 Cíle práce

Tato práce je zaměřená na fylogenezi a molekulární charakteristiku nově získaných vzorků bodlinatých myší rodu *Acomys,* především ze široké druhové skupiny *cahirinus*. Za pomocí molekulárně genetických metod jsem se snažila vyřešit fylogenetické vztahy uvnitř sledované skupiny a v návaznosti i fylogeografické vztahy a populační parametry populací z oblasti Afrického rohu.

Konkrétní cíle práce byly:

1. Vytvořit relevantní fylogenetickou hypotézu o evoluci bodlinatých myší rodu *Acomys*
2. molekulárně charakterizovat vzorky pocházející z následujících oblastí:
   a) neafrického rozšíření rodu *Acomys.* Cílem bylo především ověřit, a jak došlo ke kolonizaci Středomořských ostrovů Kypr, Kréta a pobřeží Turecka druhy *A. nesiotes A. minous a A. cilicicus*. a Arabského poloostrova s Iránem druhem *A. dimidiatus*.
   b) Afrického rohu. Somaliland je velmi neprobádanou zemí, která má za sebou velmi složitou historii, jak geologickou, tak klimatickou. Molekulární data o bodlinatých myších z této oblasti dosud zcela chyběla. Zaměřila jsem se na druh *Acomys louisae*, který je endemitem této oblasti.
   c) Afrického rozšíření – kde byla snaha shromáždit všechna dostupná data i z předchozích studií o fylogenezi bodlinatých myší rodu *Acomys* a spojit je do jedné datové sady. Tato data byla doplněna materiálem z mnoha nezávislých expedic uskutečněných několika vědeckými týmy. V mnoha případech se jednalo o oblasti dosud neprovzorkované.

# 5 Komentář k jednotlivým rukopisům

1) **Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula**. (Frynta et al. 2010).

   Afro-středomořská linie *Acomys cahirinus* a asijská *Acomys dimidiatus* jsou jasně oddělené. Velká podobnost mezi mitochondriálními haplotypy z kontinentální Afriky a severního Středomoří (linie *A. cahirinus* sensu stricto) podporuje hypotézu, že předci *A. nesiotes, A. cilicicus* a *A. minous* se velmi pravděpodobně rozšířily jako komenzální populace. Byla nalezena značná genetická variabilita v Asii. Kromě haploskupiny ze Sinaje a Jordánska (odpovídající *A. dimidiatus* sensu stricto) jsme objevili dvě dříve neznámé haploskupiny, jednu z Jemenu a druhou z Íránu a Spojených arabských emirátů.

2) **Multiple radiations of spiny mice (Rodentia: Acomys) in dry open habitats of Afro- Arabia: evidence from a multi-locus phylogeny. (**Aghová et al. 2019).

   Bodlinaté myši rodu *Acomys* jsou rozšířené v suchých a polosuchých areálech Afriky, Arábie a Středního východu. Na základě dosud největšího multilokusového genetického datového souboru z více než 200 lokalit, který v zásadě kopíruje celý areál rozšíření rodu *Acomys,* došlo k revizi fylogenetických vztahů *Acomys*. Fylogenetická analýza odhalila pět hlavních kladů: *subspinosus, spinosissimus, russatus, wilsoni a cahirinus* v rámci nichž se ukázalo celkem 26 geneticky odlišných linií, kdy největší rozmanitost byla nalezena ve východní Africe.

3) **The first report of spiny mouse belonging to Acomys ignitus group in Somaliland: Phylogenetic affinities of a new distinct mitochondrial lineage. (**Frýdlová et al. in prep.).

   Africký roh je velmi nepřístupnou oblastí, hned z několika pohledů a brání tak výzkumu místní fauny. Veškerý materiál zde získaný je pro vědu velmi cenný. Myši rodu *Acomys* ze Somálska jsou velmi málo známé. V tomto článku uvádíme první nález *Acomys*

*ignitus* – Ignitus 5, který obývá rozdílné prostředí než většina ostatních druhů *Acomys*. Nový záznam značně rozšiřuje distribuci celé skupiny na východ.

4) **Molecular characterization of *Acomys louisae* from Somaliland: A deep divergence and contrasting genetic patterns in a rift zone.** (Frynta et al. in prep.)

V tomto článku jsme se zaměřili na bodlinatou myš *Acomys louisae*, která je endemitem Somalilandu. Fylogenetická analýza ukázala jasnou genetickou odlišnost *A. louisae* od ostatních linií *Acomys*, ale také ukázala výrazné rozdělení do dvou podskupin v rámci skupiny *A. louisae*. Vzorky ze středního a východního Somalilandu, včetně vzorků z typové lokality, tvoří první jasně odlišnou „Somalilandskou" podskupinu. Druhou „Džibutskou" podskupinu tvoří zbývající vzorky ze severozápadního Somálska s 5 dříve publikovanými sekvencemi z Džibuti a východní Etiopie. Populační výpočty i haplotypová síť naznačují, že populace „Somaliland" se v nedávné době rozšířila na své nynější území a její velikost populace se rychle zvětšila. Naproti tomu „Džibutská" podskupina vykazuje extrémně vysokou sekvenční diverzitu, vysvětlitelnou dlouhodobě stabilní a početnou populací. Tato práce byla sepsána na základě nově dovezených vzorků z celkem tří expedic do Somalilandu včetně z léta roku 2019.

# 6 Publikace

## 6.1 Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula.

Frynta D., Palupčíková K., Bellinvia E., Benda P., Skarlantová H., Schwarzová L., Modrý D.

*Zootaxa* (2010) 2660: 46-56.

Frynta D., **Palupčíková K.**, Bellinvia E., Benda P., Skarlantová H., Schwarzová L., Modrý D. (2010). Phylogenetic relationships within the cahirinus-dimidiatus group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula. *Zootaxa*. 2660: 46-56.
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**Příspěvek autora:**

Prohlašuji, že Klára Palupčíková přispěla k publikaci:

- KP provedla veškeré laboratorní práce, fylogenetické analýzy a podílela se na sepisování rukopisu.

V Praze dne

prof. RNDr. Daniel Frynta, Ph.D.

(první i korespondenční autor a školitel)

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# Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula

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## Abstract

Spiny mice belonging to the *cahirinus-dimidiatus* group of the genus *Acomys* have become a widely used model in physiology and behaviour. To improve current knowledge concerning the phylogeny of this taxon, we analysed 24 samples from Libya, Chad, Egypt, Jordan, Cyprus, Crete, Turkey, Yemen and Iran. We sequenced the whole mitochondrial control region and part of the flanking tRNA genes for a total length of 986 to 996 bp and described 22 haplotypes. Our results confirmed that the Afro-Mediterranean and Asian clades are clearly distinct (p-distance = 6-8.1%). The former clade corresponds to *A. cahirinus sensu lato* (i.e. including also the Cretan *A. minous*, Cypriot *A. nesiotes* and Turkish *A. cilicicus*). Haplotypes of *A. cahirinus* from the E Sahara (S Egypt, SW Libya, N Chad) grouped with those of *A. cilicicus* and *A. minous* (p-distance ≤ 2.2%), while haplotypes of *A. nesiotes* grouped with one haplotype representing the commensal *A. cahirinus* from Cairo (p-distance = 1.2%). Close similarity among haplotypes from mainland Africa and NE Mediterranean (clade *A. cahirinus sensu stricto*) support the hypothesis that ancestors of *A. nesiotes*, *A. cilicicus* and *A. minous* dispersed most probably as commensal populations, thus questioning their status of valid species. The most surprising finding was the considerable genetic variation in Asia. In addition to a haplogroup from Sinai and Jordan (corresponding to *A. dimidiatus sensu stricto*), we detected two previously unknown haplogroups, from Yemen and Iran + United Arab Emirates. These clades are fairly distinct and separate species/subspecies status of these animals might be further considered.

**Key words:** spiny mice, mitochondrial DNA, mitochondrial control region, D-loop, phylogeography, commensalism, Yemen, Libya, Cyprus, Persian Gulf

## Relazioni filogenetiche all'interno del gruppo *cahirinus-dimidiatus* nel genere *Acomys* (Rodentia: Muridae): nuove linee mitocondriali identificate nella regione del Sahara, in Iran e nella penisola araba

## Sommario

I topi spinosi appartenenti al gruppo *cahirinus-dimidiatus* nel genere *Acomys* sono diventati animali modello ampiamente usati in studi fisiologici e comportamentali. Per migliorare le conoscenze attuali riguardanti la filogenesi di questo taxon, abbiamo analizzato 24 esemplari di topo spinoso provenienti da Libia, Chad, Egitto, Giordania, Cipro,

46 *Accepted by P. Gaubert: 21 Aug. 2010; published: 28 Oct. 2010*

Creta, Turchia, Yemen e Iran. Abbiamo sequenziato l'intera regione di controllo del mitocondrio e parte degli adiacenti geni per la sintesi di tRNA, per una lunghezza totale tra i 986 e i 996 bp, descrivendo 22 diversi aplotipi.

I nostri risultati hanno confermato la presenza di considerevoli differenze tra il clade afro-mediterraneo e il clade asiatico (distanza p = 6-8,1%). Il primo clade corrisponde ad *A. cahirinus sensu lato* (compresi *A. minous*, presente in Creta, e *A. nesiotes,* presente a Cipro). Gli aplotipi di esemplari di *A. cahirinus* provenienti dal Sahara orientale (Egitto meridionale, Libia sud-occidentale, Chad settentrionale) risultano simili a quelli di individui di *A. cilicicus* e *A. minous* (distanza p ≤ 2,2%). Mentre gli aplotipi di animali identificati come a *A. nesiotes* sono simili a quelli di topi commensali originari dal Cairo (*A. cahirinus* propriamente detto). La stretta somiglianza tra gli aplotipi proveneinti dall'Africa continentale e la regione meditterranea orientale (*A. cahirinus sensu strictu*) conferma l'ipotesi che gli antenati di *A. nesiotes, A. cilicicus* e *A. minous* si dispersero come popolazioni commensali, mettendo quindi in discussione la validità di questi taxa come specie.

Sorprendente è stata la scoperta di una notevole variabilità genetica presente in Asia. Oltre ad un chiaro raggruppamento di aplotipi, corrispondente ad esemplari della penisola del Sinai e della Giordania (appartenenti ad *A. dimidiatus sensu stricto*), abbiamo identificato due gruppi finora sconosciuti: un primo gruppo in Yemen e un secondo in Iran e negli Emirati Arabi. Questi due cladi sono chiaramente distinti, per essi dovrà essere preso in considerazione un possibile status di specie o subspecie.

**Parole chiave:** topo spinoso, DNA micondriale, regione mitocondriale di controllo, D-loop, filogeografia, commensalismo, Yemen, Libia, Cipro, Golfo persico

## Introduction

Spiny mice belonging to the *cahirinus-dimidiatus* group of the genus *Acomys* have become a widely used model for physiological (e.g., Frynta *et al.* 2009), behavioural (Nováková *et al.* 2010 and references herein) and evolutionary (e.g., Krasnov *et al.* 2005) studies (also see Van der Straeten 1994). Although this complex is morphologically distinct from other *Acomys* species groups (Denys *et al.* 1994), there is no agreement among traditional taxonomists concerning the relationships among populations or species (Wilson & Reeder 2005). Consequently, nearly all experimental animals that come from somewhere within the region of the Fertile Crescent or the Levant were reported as *A. cahirinus* (Desmarest, 1819), irrespective of their precise taxonomic status (e.g., Carere *et al.* 1999; Hefner *et al.* 2001; Weber & Hohn 2005).

Barome *et al.* (1998; 2000; 2001), analysing variation in the cytochrome *b* (mtDNA) in 14 *Acomys* species, revealed the existence of two distinct subclades within the *cahirinus-dimidiatus* clade. One comprises *A. dimidiatus* (Cretzschmar, 1826) from Sinai, Israel, Jordan and Saudi Arabia, its sister branch including unnamed forms from Cameroon and Burkina Faso. The other one includes *A. cahirinus* (Desmarest, 1819) from Egypt and *A. airensis* Thomas et Hinton, 1921 from Niger and Mali. Currently, a thorough study examining samples from SW Sahara revealed that the populations from Mauretania, Mali and Niger form a distinct clade clearly separated from both the *cahirinus* and *dimidiatus* groups (Nicolas *et al.* 2009). This subclade includes not only *A. airensis* but also Mauretanian populations of *A. chudeaui* (Kollman, 1911), therefore the former species should be further considered as a junior synonym of the latter (Nicolas *et al.* 2009).

Surprisingly, two Mediterranean species, *A. nesiotes* Bate, 1903 from Cyprus, and *A. minous* Bate, 1906 from Crete, had cyt *b* sequences almost identical with those of *A. cahirinus* from the type locality (Cairo, Egypt). The remaining species of the Mediterranean area, *A. cilicicus* Spitzenberger, 1978 from Cilicia (SE coast of Anatolia, Turkey) in addition to some *A. minous,* shared a somewhat different haplotype lineage (labeled B) of unknown origin, but were still unequivocally closely related to those of Egyptian *A. cahirinus* (Barome *et al.* 2000; 2001). The above findings may suggest that these Mediterranean species are not endemic survivors from the Tertiary period, but rather are descendants from recent, most probably commensal colonists transferred to these areas by humans.

Recently, Volobouev *et al.* (2007) formally elevated *dimidiatus* to the rank of species and reviewed karyological (cf. Nevo 1985; Sokolov *et al.* 1993; Macholán *et al.* 1995; Volobouev *et al.* 1996a; b; 2002; Kivanc *et al.* 1997; Kunze *et al.* 1999; Zima *et al.* 1999), morphological and biogeographical evidence suggesting clear differences between *A. cahirinus* from Africa (including Egypt) and *A. dimidiatus* from the

Asian part of the range including Sinai. They also hypothesized a phylogeographic scenario including immigration of *dimidiatus* from Africa to south of the Arabian Peninsula through the Red Sea. Nevertheless, in spite of the extensive distribution of *A. dimidiatus* in Asia (Bates 1994), ranging from Sinai, throughout the Arabian Peninsula, the Iranian coast of the Persian Gulf region and the Gulf of Oman to W Pakistan (Bobrov & Neronov 1998), only the populations from Sinai, Israel and Jordan have been examined by molecular methods, so far.

Species rank of *A. dimidiatus* may be supported also by the fact that fertile hybrids between *A. dimidiatus* and *A. cahirinus*, neither natural nor artificial, have been described. Jordan (2000), however, reported sterile hybrids between the dark commensal population of *A. cahirinus* from Cairo and a large pale form from Giza (suburb of Cairo) referred to as *A. dimidiatus megalodus* Setzer, 1959. As the type locality of the latter taxon is the Suez region (Wadi Sayal), species identity of the latter population was not assessed via molecular methods, and according to Volobouev *et al.* (2007), not *A. dimidiatus* but rather *A. cahirinus* has to be expected at the locality situated in the African part of Egypt.

The aim of this paper is to sequence fragments of rapidly evolving mitochondrial genes providing high resolution for recent evolutionary history, to reconstruct the phylogeny of the *cahirinus-dimidiatus* group with a special focus on understudied regions including eastern Sahara, Cyprus, Yemen and the Persian Gulf. We then discuss our results in terms taxonomic and phylogeographic implications.

## Material and methods

**Specimens.** For the present study, 24 individuals belonging to the *cahirinus-dimidiatus* group of the genus *Acomys* were analysed. Our specimens or their maternal ancestors were live-trapped from natural populations in Egypt (2 samples), Libya (1), Cyprus (2), Crete (1), Turkey (1), Sinai Peninsula (4), Jordan (3), Yemen (2), United Arab Emirates (1), and Iran (3). Other samples came from laboratory populations in Egypt (1), Chad (1), and zoological parks (2; original localities unknown). The tip of the tail or a finger were taken from sampled animals and stored in Eppendorf tubes with 96% ethanol. Alternatively, as concerned deceased animals, kidney or muscle tissues were used. Origins of the specimens are detailed in Table 1.

**DNA extraction and sequencing.** Total genomic DNA was isolated with DNAeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines.

The entire mitochondrial Control Region (CR) and the flanking tRNA genes were PCR-amplified in two overlapping segments for a total length of about 1000 bp, using primer pairs 5' ATAAACATTACTCTGGTCTTGTAAAC 3' – 5' CACAGTTATGTTGRTCATGG 3' and 5' CGTTCCCCTAAATAAGACA 3' – 5' TAATTATAAGGCCAGGACCA 3' (Bellinvia, 2004).

PCR reactions were carried out in 50 µl volume including 2.5 µl of each 10 µM primer, 5 µl of 10X PCR buffer (Fermentas), 5 µl of 10 mM dNTP, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of 5 U/ml Fermentas Taq DNA polymerase, 5 µl of DNA and 27 µl of $ddH_2O$. The PCR amplification protocol consisted of 31 cycles of denaturation at 95ºC for 30 s, annealing at 50ºC for 1 min, and extension at 72ºC for 1 min; a further 15 min elongation step at 72ºC followed the last cycle. Concentration and composition of the reaction mix were similar for both pairs of primers. The protocol used followed Bellinvia (2004). For some of the samples the temperature of annealing had to be decreased to 47ºC to obtain usable PCR products. All PCR products were purified with the Qiaquick® purification kit (Qiagen, Hilden, Germany) and directly sequenced using the same primers used for amplification.

Sequence and phylogenetic analyses. Sequences were aligned and manually checked using BioEdit (Hall 1999), Clustal X 1.81 (Thompson *et al.* 1997) and GENEDOC version 2.6.003 (Nicholas & Nicholas 1997). Three individuals of *A. russatus* were included as outgroup.

Neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed under PAUP* version 4.0b10 (Swofford 2002), and Bayesian analysis (BA) was conducted with MrBayes 3.1 (Huesenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). For MP we conducted heuristic search analyses with 100,000 random taxon addition replicates using tree-bisection and reconnection (TBR)

branch swapping. The branch support was evaluated using 1000 bootstrap pseudoreplicates (Felsenstein 1985). All characters were equally weighted and unordered. Tree search with NJ algorithm was done with Jukes – Cantor distance and node support within the final topology was assessed through 1000 bootstrap pseudoreplicates.

**TABLE 1.** Sample set considered in the present study, with geographic origin of samples.

| No. | Num. map | Species | Locality | Geographic origin |
|---|---|---|---|---|
| NES1 | 1 | *A. nesiotes* | Ağirdağ, Cyprus (leg. M. Macholán) | 35º 18' N, 33º 15' E |
| NES2 | 2 | *A .nesiotes* | Cinarli 4km SE, NE Cyprus (leg. D. Frynta) | 35º 19' 06 N, 33º 47' 26 E |
| CAIR | 3 | *A. cahirinus* | Cairo, Egypt, laboratory colony at Charles University, Praha (founders provided by P.-O. Barome) | 30º 04' N, 31º 14' E |
| SIM1, SIM2 | 4 | *A. cahirinus* | Abu Simbel archaeological site, Egypt, (founders captured by J. Borek) | 22º 22' N, 31º 38' E |
| LIB | 5 | *A. cahirinus* | Mts Akakus, Libya (colony founders captured by D. Frynta and L. Schwarzová) | 25º 44' 562 N, 12º 08' 211 E |
| CHAD | 6 | *A.cahirinus* | Tibesti, Chad, laboratory colony in ZOO Plzeň | – |
| CIL | 7 | *A. cilicicus* | E of Silifke, Turkey, 2 samples laboratory colony at Charles University, Praha (colony founders captured by J. Sádlová) | 36º 26' N, 34º 06' E |
| MIN | 8 | *A. minous* | Crete, laboratory colony in ZOO Plzeň | – |
| EMIR | 9 | *A. cf. dimidiatus.* | Jabal Hafit, United Arab Emirates, laboratory colony in ZOO Plzeň (colony provided through Breeding centre for endangered Arabian wildlife, Sharjah, UAE, founders captured by Peter Arras, Al Ain) | 24º 04' N, 55º 47' E |
| IRA1 | 10 | *A. cf. dimidiatus* | Khos Hangan, N of Bandar Abbas, Iran; 500 m a.s.l. (colony founders captured by D. Frynta, L. Schwarzová and P. Kunzová) | 27º 38' 362 N, 56º 13' 226 E |
| IRA2 | 11 | *A. cf. dimidiatus* | Zagros, Iran (colony founders captured by D. Frynta, L. Schwarzová and P. Kunzová) | 28º 55' 892 N, 52º 31' 770 E |
| IRA3 | 12 | *A. cf. dimidiatus* | Dehbarez, Iran (leg. P. Benda and P. Nová) | 27º 27' 745 N, 57º 19' 197 E |
| YEM1,YEM2 | 13 | *A. cf. dimidiatus* | Hawf, Yemen (leg. P. Benda) | 16º 39' N, 53º 03' E |
| SIN1,SIN2 | 14 | *A. dimidiatus* | Wadi Gharandal, Sinai, Egypt (leg. R. Lučan) | 29º 08' N, 31º 51' E |
| JOR3 | 15 | *A.dimidiatus* | Wadi Ramm, Jordan (colony founders captured by D. Modrý) | 29º 36' N, 35º 24' E |
| JOR4 | 15 | *A.dimidiatus* | Wadi Ramm, Jordan, ZOO Plzeň (founders captured by D. Modrý a T. Peš) | – |
| JOR1 | 15 | *A.dimidiatus* | Wadi Ramm, Harab Antar, Jordan (leg. D. Modrý) | 29º 36' N, 35º 24' E |
| JOR2 | 15 | *A.dimidiatus* | Wadi Ramm, Lawrence spring, Jordan (leg. D. Modrý) | 29º 36' N, 35º 24' E |
| BRONX | | *A. dimidiatus* | Lab. strains, 2 samples, ZOO Bronx and ZOO Prague | – |
| RUS1 | | *A. russatus* | Lab. strain, Charles University, Prague | – |
| RUS2 | | *A. russatus* | Wadi Ramm, Harab Antar, Jordan (leg. D. Modrý) | 29º 36' N, 35º 24' E |
| LEW | | *A.russatus „lewisi"* | laboratory colony, Al Wisad-Heber, Jordan (leg. D. Modrý) | 31° 50' N, 38° 08' N |

Optimal model of studied mtDNA sequence evolution was selected using the AIC criterion in Modeltest 3.7 (Posada & Crandall 1998). For ML analysis we used heuristic search with 300 random taxon addition replicates and TBR branch swapping. Node support within the ML tree topology was assessed by bootstrap analysis with 750 pseudoreplicates (in each 10 random addition replicates only).

For the Bayesian analysis, we partitioned our alignment into tree domains: (i) the Central domain (CD), which is the most conserved region of CR; (ii) the Extended terminal-associated sequence (ETAS) domain; and (iii) the Conserved sequence block (CSB) domain, adjacent to CD (see Larizza et *al.* 2002). Two

independent runs of analyses were conducted with a random starting tree and for $6x10^6$ generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 15000 trees (1,500,000 generations).

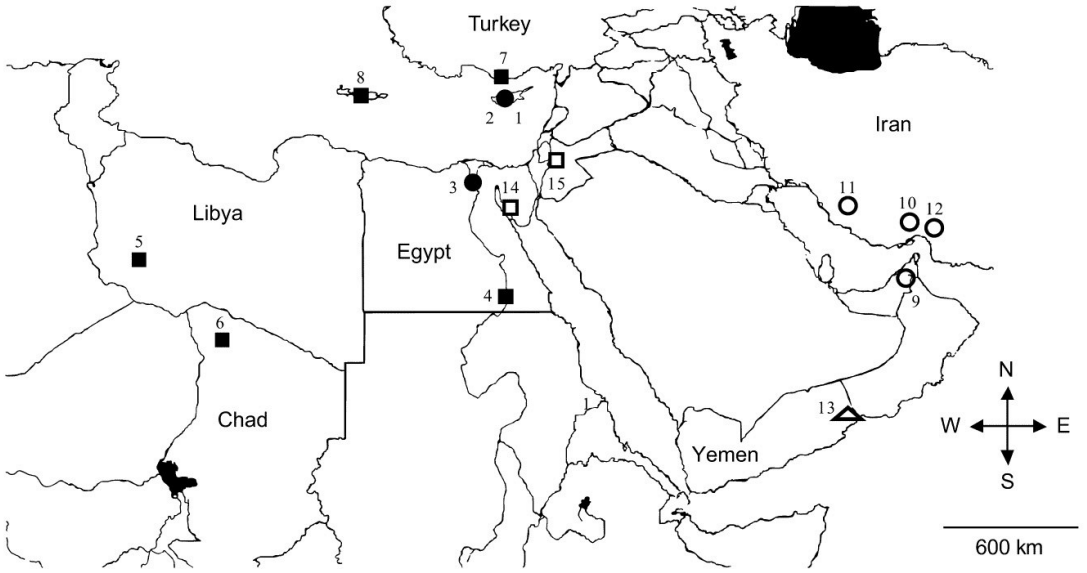


**FIGURE 1.** Map of sampled localities in North Africa, the Mediterranean, the Arabian Peninsula and Iran within the *cahirinus-dimidiatus* group of the genus *Acomys*. Black circles and squares show *cahirinus* lineages from Cairo-Cyprus and eastern Sahara-Turkey-Crete, respectively; white circles show *dimidiatus* lineages from Iran-Emirates; white triangle shows Yemen; white squares show the Sinai-Jordan lineage. Numbers refer to Table 1.

## Results

We analyzed 24 ingroup samples representing 22 haplotypes. We obtained a nucleotide alignement of 1006 nucleotide positions, of which 219 were variable and 195 were parsimony-informative. The CR itself ranged in length from 837 to 839 bp.

Most of the recovered topologies agreed substantially (MP, NJ and BA), although ML somewhat differed by placing haplotypes from Iran and Emirates as basal offshoots of the *cahirunus-dimidiatus* group. MP, NJ and BA revealed revealed two clearly distinct clades. The first clade, further referred to as "Afro-Mediterranean", contained haplotypes from North Africa, Crete, Cyprus and Turkey. The second clade, further referred to as "Asian", contained haplotypes from Sinai, Jordan, the Arabian Peninsula as well as those from Iran (*dimidiatus*). Uncorrected p-distances between haplotypes belonging to the Afro-Mediterranean and Asian clades varied within the range of 6.0–8.1% (Table 2).

Haplotypes belonging to the Afro-Mediterranean clade were very similar to each other (uncorrected p-distances varied within the range of 0.2–2.2 %). Phylogenetic relationships within this clade were poorly supported. However, haplotypes from eastern Sahara (S Egypt, S Libya, N Chad), Turkey and Crete formed a distinct, monophyletic group.

The Asian clade was less homogenous than the Afro-Mediterranean one (within-group uncorrected p-distances = 0.3–5.6 %). It split into three distinct and geographically localised, well-supported lineages: (1) Sinai-Jordan, (2) Yemen, (3) Iran-Emirates. The relative position of these lineages in the tree was not resolved.

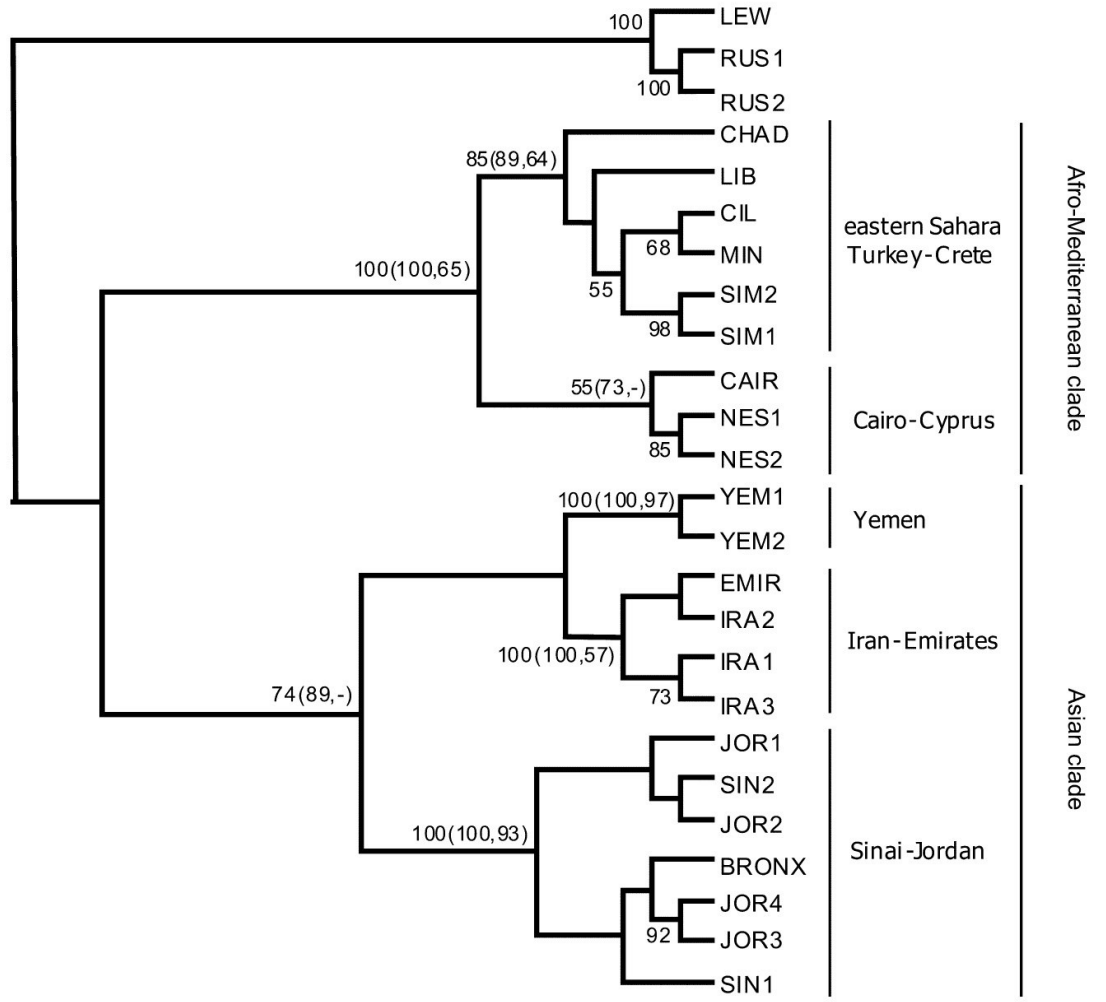


**FIGURE 2.** Strict consensus parsimony tree of the *A. cahirinus-dimidiatus* group (22 maximum parsimony trees). Tree length = 326, CI = 0.7761, RI = 0.9189, RC = 0.7131. Bootstrap values (in percentage) are indicated at nodes. Bootstrap supports of the main clusters obtained after Neighbour Joining and Maximum Likelihood analyses are provided in parentheses. Codes of samples refer to Table 1.

## Discussion

Surprisingly, in spite of there being 2200 km distance between the Akakus Mts (Libya) and Abu Simbel (Egypt), the haplotypes that we found in these two sites and at Tibesti Mts (Chad) were nearly identical (p-distance = 0.3%). This may suggest that the range of the species *A. cahirinus* also encompasses most of the central Sahara region. Because the type locality of *A. airensis* in Air, another south Saharan mountain region that shares with our localities almost the same ecological conditions, is situated just 700 km from the Akakus Mts and 800 km from the Tibesti Mts, the validity of *A. airensis* as a species may be questioned (but see Denys *et al.* 1994; Sicard & Tranier 1996). The populations of *A. airensis* sequenced to date and that were sharply distinct from *A. cahirinus,* came from the lowlands of Mali and Niger (e.g., Barome *et al.* 1998; 2000; 2001, Nicolas *et al.* 2009).

**TABLE 2.** Uncorrected p-distances between studied haplotypes.

| | SIN1 | SIN2 | JOR1 | JOR2 | BRONX | JOR4 | JOR3 | YEM1 | YEM2 | EMIR | IRA1 | IRA2 | IRA3 | LIB | CHAD | CIL | MIN | SIM2 | SIM1 | NES1 | NES2 | CAIR | RUS1 | LEW | RUS2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SIN1 | | 0,012 | 0,009 | 0,008 | 0,008 | 0,007 | 0,010 | 0,051 | 0,051 | 0,052 | 0,048 | 0,047 | 0,048 | 0,070 | 0,068 | 0,069 | 0,070 | 0,069 | 0,073 | 0,065 | 0,067 | 0,069 | 0,140 | 0,137 | 0,138 |
| SIN2 | 0,012 | | 0,011 | 0,010 | 0,013 | 0,014 | 0,017 | 0,053 | 0,054 | 0,059 | 0,056 | 0,054 | 0,057 | 0,073 | 0,071 | 0,074 | 0,073 | 0,072 | 0,076 | 0,068 | 0,070 | 0,072 | 0,141 | 0,136 | 0,139 |
| JOR1 | 0,009 | 0,011 | | 0,009 | 0,013 | 0,011 | 0,014 | 0,051 | 0,051 | 0,053 | 0,049 | 0,048 | 0,049 | 0,069 | 0,067 | 0,068 | 0,069 | 0,066 | 0,070 | 0,066 | 0,068 | 0,070 | 0,138 | 0,138 | 0,135 |
| JOR2 | 0,008 | 0,010 | 0,009 | | 0,010 | 0,009 | 0,012 | 0,049 | 0,049 | 0,055 | 0,051 | 0,050 | 0,051 | 0,071 | 0,069 | 0,072 | 0,071 | 0,070 | 0,074 | 0,066 | 0,068 | 0,070 | 0,139 | 0,134 | 0,137 |
| BRONX | 0,008 | 0,013 | 0,013 | 0,010 | | 0,008 | 0,011 | 0,052 | 0,052 | 0,055 | 0,053 | 0,050 | 0,053 | 0,069 | 0,067 | 0,070 | 0,069 | 0,068 | 0,072 | 0,064 | 0,066 | 0,068 | 0,141 | 0,136 | 0,139 |
| JOR4 | 0,007 | 0,014 | 0,011 | 0,009 | 0,008 | | 0,003 | 0,050 | 0,050 | 0,055 | 0,051 | 0,050 | 0,051 | 0,065 | 0,063 | 0,066 | 0,065 | 0,064 | 0,068 | 0,060 | 0,062 | 0,064 | 0,140 | 0,137 | 0,138 |
| JOR3 | 0,010 | 0,017 | 0,014 | 0,012 | 0,011 | 0,003 | | 0,053 | 0,053 | 0,056 | 0,052 | 0,051 | 0,052 | 0,068 | 0,066 | 0,069 | 0,068 | 0,067 | 0,071 | 0,063 | 0,065 | 0,067 | 0,143 | 0,140 | 0,141 |
| YEM1 | 0,051 | 0,053 | 0,051 | 0,049 | 0,052 | 0,050 | 0,053 | | 0,002 | 0,059 | 0,053 | 0,052 | 0,054 | 0,070 | 0,070 | 0,073 | 0,072 | 0,072 | 0,075 | 0,073 | 0,071 | 0,073 | 0,141 | 0,138 | 0,139 |
| YEM2 | 0,051 | 0,054 | 0,051 | 0,049 | 0,052 | 0,050 | 0,053 | 0,002 | | 0,059 | 0,053 | 0,052 | 0,054 | 0,070 | 0,070 | 0,073 | 0,072 | 0,072 | 0,075 | 0,073 | 0,071 | 0,073 | 0,141 | 0,138 | 0,139 |
| EMIR | 0,052 | 0,059 | 0,053 | 0,055 | 0,055 | 0,055 | 0,056 | 0,059 | 0,059 | | 0,010 | 0,007 | 0,010 | 0,078 | 0,076 | 0,077 | 0,078 | 0,075 | 0,079 | 0,077 | 0,077 | 0,079 | 0,138 | 0,134 | 0,136 |
| IRA1 | 0,048 | 0,056 | 0,049 | 0,051 | 0,053 | 0,051 | 0,052 | 0,053 | 0,053 | 0,010 | | 0,003 | 0,002 | 0,080 | 0,078 | 0,079 | 0,080 | 0,077 | 0,079 | 0,077 | 0,077 | 0,081 | 0,135 | 0,131 | 0,133 |
| IRA2 | 0,047 | 0,054 | 0,048 | 0,050 | 0,050 | 0,050 | 0,051 | 0,052 | 0,052 | 0,007 | 0,003 | | 0,003 | 0,077 | 0,075 | 0,076 | 0,077 | 0,074 | 0,078 | 0,074 | 0,074 | 0,078 | 0,134 | 0,130 | 0,132 |
| IRA3 | 0,048 | 0,057 | 0,049 | 0,051 | 0,053 | 0,051 | 0,052 | 0,054 | 0,054 | 0,010 | 0,002 | 0,003 | | 0,080 | 0,078 | 0,079 | 0,080 | 0,077 | 0,079 | 0,077 | 0,077 | 0,081 | 0,136 | 0,132 | 0,134 |
| LIB | 0,070 | 0,073 | 0,069 | 0,071 | 0,069 | 0,065 | 0,068 | 0,070 | 0,070 | 0,078 | 0,080 | 0,077 | 0,080 | | 0,004 | 0,008 | 0,006 | 0,008 | 0,009 | 0,019 | 0,017 | 0,017 | 0,136 | 0,135 | 0,136 |
| CHAD | 0,068 | 0,071 | 0,067 | 0,069 | 0,067 | 0,063 | 0,066 | 0,070 | 0,070 | 0,076 | 0,078 | 0,075 | 0,078 | 0,004 | | 0,008 | 0,006 | 0,008 | 0,009 | 0,015 | 0,013 | 0,015 | 0,134 | 0,131 | 0,134 |
| CIL | 0,069 | 0,074 | 0,068 | 0,072 | 0,070 | 0,066 | 0,069 | 0,073 | 0,073 | 0,077 | 0,079 | 0,076 | 0,079 | 0,008 | 0,008 | | 0,002 | 0,007 | 0,009 | 0,019 | 0,017 | 0,015 | 0,135 | 0,136 | 0,135 |
| MIN | 0,070 | 0,073 | 0,069 | 0,071 | 0,069 | 0,065 | 0,068 | 0,072 | 0,072 | 0,078 | 0,080 | 0,077 | 0,080 | 0,006 | 0,006 | 0,002 | | 0,005 | 0,007 | 0,017 | 0,015 | 0,013 | 0,135 | 0,134 | 0,135 |
| SIM2 | 0,069 | 0,072 | 0,066 | 0,070 | 0,068 | 0,064 | 0,067 | 0,072 | 0,072 | 0,075 | 0,077 | 0,074 | 0,077 | 0,008 | 0,008 | 0,007 | 0,005 | | 0,002 | 0,018 | 0,016 | 0,016 | 0,135 | 0,135 | 0,134 |
| SIM1 | 0,073 | 0,076 | 0,070 | 0,074 | 0,072 | 0,068 | 0,071 | 0,075 | 0,075 | 0,079 | 0,079 | 0,078 | 0,079 | 0,009 | 0,009 | 0,009 | 0,007 | 0,002 | | 0,022 | 0,020 | 0,020 | 0,136 | 0,137 | 0,136 |
| NES1 | 0,065 | 0,068 | 0,066 | 0,066 | 0,064 | 0,060 | 0,063 | 0,073 | 0,073 | 0,077 | 0,077 | 0,074 | 0,077 | 0,019 | 0,015 | 0,019 | 0,017 | 0,018 | 0,022 | | 0,002 | 0,012 | 0,137 | 0,132 | 0,137 |
| NES2 | 0,067 | 0,070 | 0,068 | 0,068 | 0,066 | 0,062 | 0,065 | 0,071 | 0,071 | 0,077 | 0,077 | 0,074 | 0,077 | 0,017 | 0,013 | 0,017 | 0,015 | 0,016 | 0,020 | 0,002 | | 0,012 | 0,137 | 0,132 | 0,137 |
| CAIR | 0,069 | 0,072 | 0,070 | 0,070 | 0,068 | 0,064 | 0,067 | 0,073 | 0,073 | 0,079 | 0,081 | 0,078 | 0,081 | 0,017 | 0,015 | 0,015 | 0,013 | 0,016 | 0,020 | 0,012 | 0,012 | | 0,140 | 0,137 | 0,140 |
| RUS1 | 0,140 | 0,141 | 0,138 | 0,139 | 0,141 | 0,140 | 0,143 | 0,141 | 0,141 | 0,138 | 0,135 | 0,134 | 0,136 | 0,136 | 0,134 | 0,135 | 0,135 | 0,135 | 0,136 | 0,137 | 0,137 | 0,140 | | 0,017 | 0,006 |
| LEW | 0,137 | 0,136 | 0,138 | 0,134 | 0,136 | 0,137 | 0,140 | 0,138 | 0,138 | 0,134 | 0,131 | 0,130 | 0,132 | 0,135 | 0,131 | 0,136 | 0,134 | 0,135 | 0,137 | 0,132 | 0,132 | 0,137 | 0,017 | | 0,015 |
| RUS2 | 0,138 | 0,139 | 0,135 | 0,137 | 0,139 | 0,138 | 0,141 | 0,139 | 0,139 | 0,136 | 0,133 | 0,132 | 0,134 | 0,136 | 0,134 | 0,135 | 0,135 | 0,134 | 0,136 | 0,137 | 0,137 | 0,140 | 0,006 | 0,015 | |

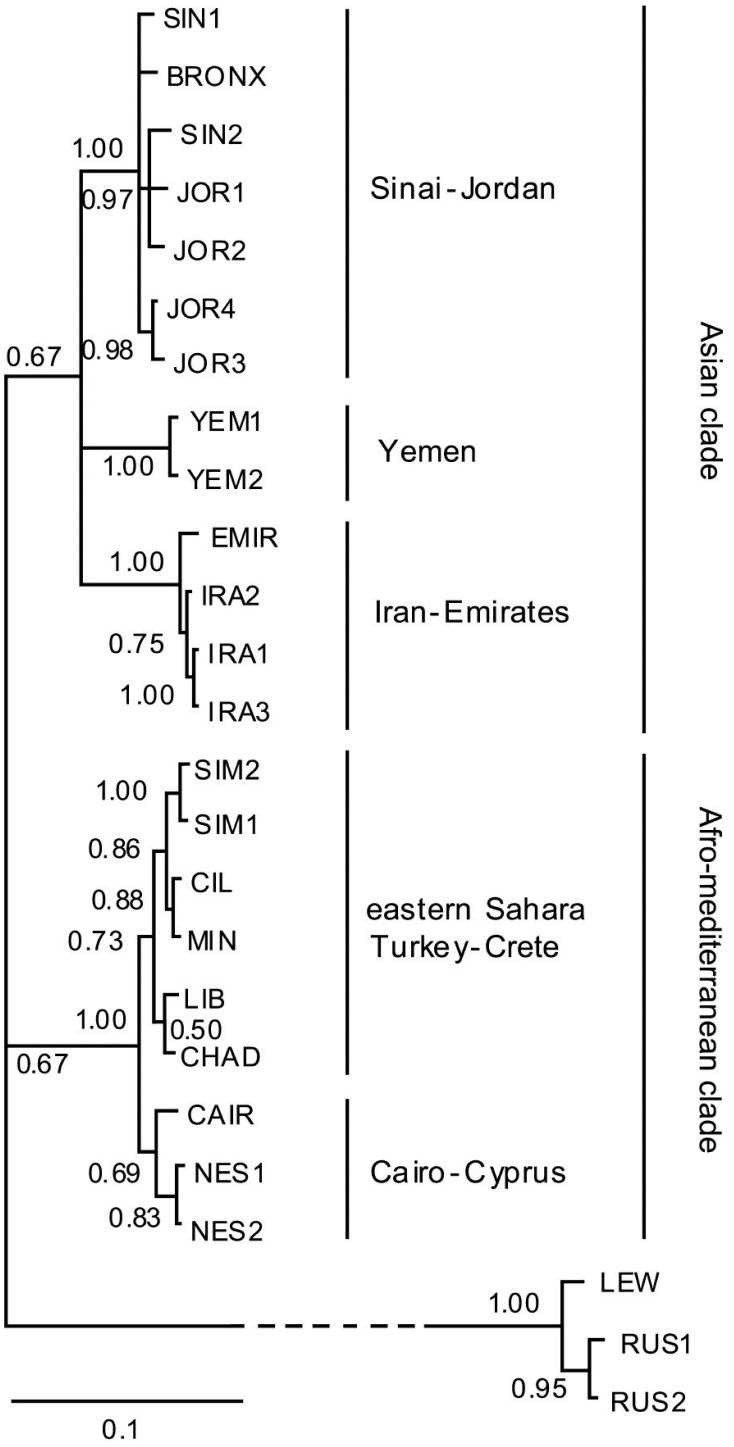


**FIGURE 3.** Bayesan phylogenetic tree of the *A. cahirinus-dimidiatus* group. Posterior probabilities are given at nodes. For visual convenience, the length of the branch leading to the outgroup has been divided by two. Codes of samples refer to Table 1.

As expected, our results also clearly supported previous studies' that suggested the paraphyly of the North African *A. cahirinus* with respect to other species of *Acomys* coming from the Mediterranean islands and northern coast (*A. nesiotes*, *A. cilicicus* and *A. minous*). Our data provide further evidence supporting the recent dispersal of spiny mice to the NE Mediterranean region (see Barome et al. 1998; 2000). As CR is non-coding sequence evolving even faster than cyt *b*, it provides a somewhat more sensitive test to examine the hypothesis supporting the anthropogenous origin of the Mediterranean species of spiny-mice. Close similarity among particular haplotypes from mainland Africa and those from the NE Mediterranean suggests that ancestors of *A. nesiotes*, *A. cilicicus* and *A. minous* dispersed most probably as commensal populations following ancient trade routes. Their status as valid species may thus be considered questionable.

Barome *et al.* (2001) identified two clearly distinct mitochondrial groups (A and B) within Cretan populations of *A. minous*. The authors reported that haplotypes of *A. nesiotes* from Cyprus and *A. cahirinus* from type locality (Cairo) belonged to group A, while those of *A. cilicicus* from Cilicia coast grouped with B. Given that we recovered the same haplotype attributions, we may tentatively conclude that our lineages from Cyprus and Cairo correspond to group A, while those from Crete and Cilicia belong to group B. We also found haplotypes belonging apparently to group B in the above-mentioned three localities from the eastern Sahara. We can speculate that bearers of group B haplotypes that colonized Crete and Cilicia in antiquity were transferred by Egyptian and/or Phoenician trade ships from southern Egypt. Nevertheless, sampling in North Africa is still too low to allow the exclusion of other mainland regions as potential geographical sources for the group B haplotypes found in NE Mediterranean.

Bearers of both A and B haplotype group contributed to contemporary *A. minous* (Barome *et al.* 2001), and both the *A. cilicicus* from S Turkish coast and *A. cahirinus* populations from Abu Simbel (belonging to B group) hybridised in our laboratory with *A. nesiotes* and *A. cahirinus* from Cairo (belonging to A group; Frynta & Sádlová 1998 and Frynta et al., in prep.). Therefore we warn against premature taxonomic splitting of mainland populations of *A. cahirinus* according to haplotype group.

We found a close similarity between *A. dimidiatus* haplotypes from Sinai and Jordan, i.e., regions known to be inhabited by different chromosomal forms of this species (Nevo 1985), supporting the current view that 36 and 38 chromosome forms interbreed freely and thus obviously belong to a single species (Volobouev *et al.* 2007).

The similarity between Persian haplotypes and a haplotype from the Emirates on the opposite side of Persian Gulf and the Gulf of Oman may be explained by the presence of a land bridge that allowed free faunal dispersal across the Persian Gulf during the last glacial period, between the south of the Arabian Peninsula and Iran (Anderson 1999).

Considerable sequence divergence within the Asian clade is probably our most surprising finding. Obviously, the southern part of the Arabian Peninsula is a territory with high haplotype diversity and not just a peripheral area of the Asian clade expansion. Thus, it is unlikely that the Arabian Peninsula was colonized by *A. dimidiatus* from north-eastern Africa via Sinai and Jordan. More likely is that the colonisation event could result from prehistoric marine transgression through land bridge across the Red Sea, as suggested by Barome *et al.* (2000) and Volobouev *et al.* (2007). This southern route scenario was previously suggested for other mammals (Bailey 2009) including carnivores, hyrax, oryx (Harrison & Bates 1991), *Hamadryas* baboons (Wildman *et al.* 2004, Winney *et al.* 2004) and even humans (White *et al.* 2003). Nevertheless, recent geological surveys in the Bab al-Mandab revealed that the Red Sea never completely disappeared in this area during the Quaternary period (Fernandes *et al.* 2006). Thus, the occurrence of a land bridge between Africa and the Arabian Peninsula should be considered impossible since at least 2 millions years (Bailey 2009) or even the end of the Miocene (Fernandes *et al.* 2006).

Our results suggest that haplotypes from Iran and Emirates as well as those from Yemen are only distantly related to those from Sinai and Jordan. As *A. dimidiatus* was described from Sinai, the Sinaitic-Jordan lineage might correspond to the nominotypic subspecies *A. d. dimidiatus*. Scientific names for bearers of the Yemeni and Persian haplotype lineages (if their taxonomic distinctness is proven) should be searched among older geographically congruous descriptions, including *A. whitei* Harrison, 1980 from Oman—supposedly matching our Iran-Emirates lineage, *A. d. homericus* Thomas, 1923 from Yemen (type locality El Khaur, Aden Protectorate [= SW Yemen]), and *A. flavidus* Thomas, 1917 described from southern Pakistan. In addition, there are two older descriptions from an unspecified part of Arabia: *A. hispidus* (Brants, 1827) and

*A. megalotis* (Lichtenstein, 1829). Nevertheless, additional morphological, hybridization and behavioural data are necessary to clarify taxonomic statuses these populations. In addition, the consideration of CR sequences from their African relatives is needed to evaluate genetic variability and possibilities of additional spreading routes (from Africa to Asia, around Africa, etc).


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## Literature


Anderson, S.C. (1999) *The Lizards of Iran*. Society for the Study of Amphibians and Reptiles, Ithaca, New York, 442 pp.

Bailey, G. (2009) The Red Sea, Coastal Landscapes, and Hominin Dispersals. *In*: Petraglia, M.D. & Rose, J.I. (Eds), *The Evolution of Human Populations in Arabia: Paleoenvironments, Prehistory and Genetics*. Springer Dordrecht Heidelberg London New York, 15–38.

Barome, P.-O., Lymberakis, P., Monnerot, M. & Gautun, J.-C. (2001) Cytochrome b sequences reveal *Acomys minous* (Rodentia, Muridae) paraphyly and answer the question about the ancestral karyotype of *Acomys dimidiatus*. *Molecular Phylogenetics and Evolution,* 18, 37–46.

Barome, P.-O., Monnerot, M. & Gautun, J.-C. (1998) Intrageneric phylogeny of *Acomys* (Rodentia, Muridae) using mitochondrial gene cytochrome b. *Molecular Phylogenetics and Evolution,* 9, 560–566.

Barome, P.-O., Monnerot, M. & Gautun, J.-C. (2000) Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. *Mammalia,* 64, 423–438.

Bates, P.J.J. (1994) The distribution of *Acomys* (Rodentia: Muridae) in Africa and Asia. *Israel Journal of Zoology,* 40, 199–214.

Bellinvia, E. (2004) A phylogenetic study of the genus *Apodemus* by sequencing the mitochondrial DNA control region. *Journal of Zoological Systematics and Evolutionary Research,* 42, 289–297.

Bobrov, V.V. & Neronov, V.M. (1998) On boundary between the Palaearctic and Indo-malayan faunal realms in India and Pakistan (from data on distribution of Rodentia). *Zoologicheskij Zhurnal,* 77, 1162–1167.

Carere, C., Casetti, R., de Acetis, L., Parretta, G., Cirulli, F. & Alleva, E. (1999) Behavioural and nociceptive response in male and female spiny mice (*Acomys cahirinus*) upon exposure to snake odour. *Behavioural Processes,* 47, 1–10.

Denys, C., Gautun, J.-C., Tranier, M. & Volobouev, V. (1994) Evolution of the genus *Acomys* (Rodentia, Muridae) from dental and chromosomal patterns. *Israel Journal of Zoology,* 40, 215–246.

Ellerman, J.R. (1941) *The families and genera of living rodents*. Vol. 2. British Museum (Natural History), London.

Felsenstein, J. (1985) Confidence limits on phylogeny: an approach using the bootstrap. *Evolution,* 39, 783–789.

Fernandes, C.A., Rohling, E.J. & Siddal, M. (2006) Absence of post-Miocene Red Sea land bridges: biogeographic implications. *Journal of Biogeography,* 33, 961–966.

Frynta, D. & Sádlová, J. (1998) Hybridisation experiments in spiny-mice from the Eastern Mediterranean: systematic position of *Acomys cilicicus* revisited. *Zeitschrift für Säugetierkunde,* 63 (Suplementum), 18.

Frynta, D., Nováková, M., Kutalová, H., Palme, R. & Sedláček, F. (2009) Apparatus for Collection of Fecal Samples from Undisturbed Spiny Mice (*Acomys cahirinus*) Living in a Complex Social Group. *Journal of the American Association for Laboratory Animal Science*, 48, 1–6.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 41, 95–98.

Harrison, D.L. & Bates, P.J.J. (1991) *The Mammals of Arabia*. Kent: Harrison Zoological Museum, Sevenoaks, 354 pp.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics,* 17, 754–755.

Jordan, M.J.R. (2000) Hybridisation research into the specific status of Egyptian spiny mouse (*Acomys cahirinus*) and Arabian spiny mouse (*Acomys dimidiatus*). *Federation Research Newsletter* (The Federation of Zoological Gardens

of Great Britain and Ireland. Paignton), 1 (4 Suplementum).
Kivanc, E., Sözen, M., Colak, E. & Yigit, N. (1997) Karyological and phallic aspects of the spiny mouse *Acomys cilicicus* Spitzenberger, 1978 (Rodentia: Muridae) in Turkey. *Turkish Journal of Zoology,* 21,167–169.
Krasnov, B.R., Morand, S., Hawlena, H., Khokhlova, I.S. & Shenbrot, G.I. (2005) Sex-biased parasitism, seasonality and sexual size dimorphism in desert rodents. *Oecologia,* 146, 209–217.
Kunze, B., Dieterlen, F., Traut, W. & Winking, H. (1999) Karyotype relationship among four species of spiny mice (*Acomys*, Rodentia). *Zeitschrift für Säugetierkunde,* 64, 220–229.
Larizza, A., Pesole, G., Reyes, A., Sbisá, E. & Saccone, C. (2002) Lineage specificity of the evolutionary dynamics of the mtDNA D-loop region in Rodents. *Journal of Molecular Evolution,* 54, 145–155.
Macholán, M., Zima, J., Červená, A. & Červený, J. (1995) Karyotype of *Acomys cilicicus* Spitzenberger, 1978 (Rodentia: Muridae). *Mammalia,* 59, 397–402.
Nevo, E. (1985) Genetic differentiation and speciation in spiny mice, *Acomys*. *Acta Zoologica Fennica,* 170, 131–136.
Nicholas, K.B. & Nicholas, H.B., Jr (1997): GENEDOC: a tool for editing and annotating multiple sequence alignments, 2.5.000 edn. Distributed by the authors.
Nicolas, V., Granjon, L., Duplantier, J.-M., Cruaud, C. & Dobigny, G. (2009) Phylogeography of spiny mice (genus *Acomys*, Rodentia: Muridae) from the south-western margin of the Sahara with taxonomic implications. *Biological Journal of the Linnean Society,* 98, 29–46.
Nováková, M., Vašáková, B., Kutalová, H., Galeštoková, K., Průšová, K., Šmilauer, R., Šumbera, R. & Frynta, D. (2010) Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: *Acomys*). *Behavioral Ecology & Sociobiology,* 64, 371–379.
Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics,* 14, 817–818.
Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* 19, 1572–1574.
Sicard, B. & Tranier, M. (1996) Caractères et répartition de trois phénotypes d'*Acomys* (Rodentia, Muridae) au Burkina Faso. *Mammalia,* 60, 53–68.
Sokolov, V.E., Orlov, V.N., Baskevich, M.I., Bekele, A. & Mebrate, A. (1993) A caryological study of the spiny mouse *Acomys* Geoffroy 1838 (Rodentia Muridae) along the Ethiopian Rift Valley. *Tropical Zoology,* 6, 227–235.
Swofford, D.L. (2002) *PAUP**. Phylogenetic analysis using parsimony. Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.
Thompson, D.J., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tool. *Nucleic Acids Research,* 24, 4876–4882.
Van der Straeten, E. (1994) A species- and subject-coded bibliography of *Acomys* from Africa and the Middle East. *Israel Journal of Zoology,* 40, 265–289.
Volobouev, V., Aniskin, V.M., Lecompte, E. & Ducroz, J.-F. (2002) Patterns of karyotype evolution in complexes of sibling species within three genera of African murid rodents inferred from the comparsion of cytogenetic and molecular data. *Cytogenetic and Genome Research,* 96, 261–275.
Volobouev, V., Auffray, J.C., Debat, V., Denys, C., Gautun, J.C. & Tranier, M. (2007) Species delimitation in the *Acomys cahirinus-dimidiatus* complex (Rodentia, Muridae) inferred from chromosomal and morphological analyses. *Biological Journal of the Linnean Society,* 91, 203–214.
Volobouev, V., Gautun, J.-C. & Tranier, M. (1996a) Chromosome evolution in the genus *Acomys* (Rodentia, Muridae): Chromosome banding analysis of *Acomys cahirinus*. *Mammalia,* 60, 217–222.
Volobouev, V., Gautun, J.-C., Sicard, B. & Tranier, M. (1996b) The chromosome complement of *Acomys* spp. (Rodentia, Muridae) from Oursi, Burkina Faso – the ancestral karyotype of the *cahirinus-dimidiatus* group? *Chromosome Research,* 4, 526–530.
Weber, E.T. & Hohm, V.M. (2005) Circadian activity rhythms in the spiny mouse, *Acomys cahirinus*. *Physiology and Behavior,* 86, 427–433.
White, T.D., Asfaw, B., DeGusta, D., Gilbert, H., Richards, G.D., Suwa, G. & Howell, F.C. (2003) Pleistocene *Homo sapiens* from Middle Awash, Ethiopia. *Nature,* 423, 742–747.
Wildman, D.E., Bergman, T.J., al-Aghbari, A., Sterner, K.N., Newman, T.K., Phillips-Conroy, J.E., Jolly, C.J. & Disotell, T.R. (2004) Mitochondrial evidence for the origin of hamadryas baboons. *Molecular Phylogenetics and Evolution,* 32, 287–296.
Wilson, D.E. & Reeder, D.M. (Ed.) (2005) *Mammal Species of the World: A Taxonomic and Geographic Reference. Third Edition. Volume 2.* The Johns Hopkins University Press, Baltimore, pp. 1191–1210.
Winney, B.J., Hammond, R.L., Macasero, W., Flores, B., Boug, A., Biquand, V., Biquand, S. & Bruford, M.W. (2004) Crossing the Red Sea: phylogeography of the hamadryas baboon, *Papio hamadryas hamadryas*. *Molecular Ecology,* 13, 2819–2827.
Zima, J., Macholán, M., Piálek, J., Slivková, L. & Suchomelová, E. (1999) Chromosomal banding pattern in the Cyprus spiny mouse, *Acomys nesiotes*. *Folia Zoologica,* 48, 149–152.

## 6.2 Multiple radiations of spiny mice (Rodentia: *Acomys*) in dry open habitats of Afro- Arabia: evidence from a multi-locus phylogeny.

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V Praze dne

prof. RNDr. Daniel Frynta, Ph.D.

(školitel)

"Authors' contributions

DF, RS and JB conceived and designed the study; TA, DF, RS, LL, YM, JS, JV, JM, DM, JB collected important part of samples; TA and KP performed laboratory analysis; TA analysed data; TA, JB, KP drafted the first version of the manuscript. All authors made substantial contribution on acquisition of data, revised the draft, gave final approval of the version to be published and agreed to be accountable for all aspects of the work."

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# Multiple radiations of spiny mice (Rodentia: *Acomys*) in dry open habitats of Afro-Arabia: evidence from a multi-locus phylogeny

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## Abstract

**Background:** Spiny mice of the genus *Acomys* are distributed mainly in dry open habitats in Africa and the Middle East, and they are widely used as model taxa for various biological disciplines (e.g. ecology, physiology and evolutionary biology). Despite their importance, large distribution and abundance in local communities, the phylogeny and the species limits in the genus are poorly resolved, and this is especially true for sub-Saharan taxa. The main aims of this study are (1) to reconstruct phylogenetic relationships of *Acomys* based on the largest available multilocus dataset (700 genotyped individuals from 282 localities), (2) to identify the main biogeographical divides in the distribution of *Acomys* diversity in dry open habitats in Afro-Arabia, (3) to reconstruct the historical biogeography of the genus, and finally (4) to estimate the species richness of the genus by application of the phylogenetic species concept.

**Results:** The multilocus phylogeny based on four genetic markers shows presence of five major groups of *Acomys* called here *subspinosus, spinosissimus, russatus, wilsoni* and *cahirinus* groups. Three of these major groups (*spinosissimus, wilsoni* and *cahirinus*) are further sub-structured to phylogenetic lineages with predominantly parapatric distributions. Combination of alternative species delimitation methods suggests the existence of 26 molecular operational taxonomic units (MOTUs), potentially corresponding to separate species. The highest genetic diversity was found in Eastern Africa. The origin of the genus *Acomys* is dated to late Miocene (*ca.* 8.7 Ma), when the first split occurred between spiny mice of eastern (Somali-Masai) and south-eastern (Zambezian) savannas. Further diversification, mostly in Plio-Pleistocene, and the current distribution of *Acomys* were influenced by the interplay of global climatic factors (e.g., Messinian salinity crisis, intensification of Northern Hemisphere glaciation) with local geomorphology (mountain chains, aridity belts, water bodies). Combination of divergence dating, species distribution modelling and historical biogeography analysis suggests repeated "out-of-East-Africa" dispersal events into western Africa, the Mediterranean region and Arabia.

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**Conclusions:** The genus *Acomys* is very suitable model for historical phylogeographic and biogeographic reconstructions of dry non-forested environments in Afro-Arabia. We provide the most thorough phylogenetic reconstruction of the genus and identify major factors that influenced its evolutionary history since the late Miocene. We also highlight the urgent need of integrative taxonomic revision of east African taxa.

**Keywords:** *Acomys*, Savanna, Biogeography, Africa, Arabia, Sahara, Somali-Masai, Zambezian savanna, Plio-Pleistocene

## Background

The Old-World savanna biome spans the tropical grasslands, scrublands and wooded savannas of sub-Saharan Africa [1]. These open non-forested habitats represent the most widespread terrestrial environment in Africa [2] and they harbour one of the most abundant and diverse mammalian communities on Earth [3]. In Africa, four major biogeographic regions can be distinguished, which are defined by the geographical distribution of vascular plants and terrestrial vertebrates, where savanna-like ecosystems predominate (Zambezian, Somali, Sudanian and South African [3]).

Numerous geological and climatic events have affected the biological diversity of contemporary savanna-like ecosystems in Africa. In Eastern Africa, the East African Rift System (EARS) started to develop *ca.* 45–33 Ma [4], which led to a change in the region's topography and the consequent aridification of East Africa, most intensively since late Miocene [5–8]. These climatic changes are best documented by the shift from C3 (moisture-adapted plants) to C4 (tropical arid-adapted grasses) plants [9–11]. The climatically turbulent Pliocene and especially Pleistocene periods, when arid and humid conditions alternated, resulted in a series of expansions and contractions of climatic zones [12, 13] that influenced the distribution and diversification of biodiversity in this region [14, 15].

Small mammals, especially rodents, are very good model organisms for phylogeographic reconstructions. Most of them are usually habitat specialists, exhibit low dispersal ability and have relatively high substitution rates, at least at mitochondrial DNA (mtDNA). Spiny mice of the genus *Acomys* I. Geoffroy Saint-Hilaire, 1838 inhabit seasonally dry open habitats in large regions of sub-Saharan Africa, the Eastern Mediterranean and the Arabian Peninsula [16]. Because they usually constitute abundant parts of local small mammal communities and their samples are easy to collect, they potentially represent a suitable group for testing hypotheses pertaining to the biogeography of dry open habitats in Africa and Arabia. *Acomys* belongs to a handful of rodent taxa that have been extensively studied for decades, and they have been used in several fields of study (e.g. ecology [17–22], physiology [23–26] and evolutionary biology [27–29]). Nevertheless, the vast majority of these studies was performed on taxa from Israel and neighbouring areas of the Middle East, representing only small fragment of the phylogenetic diversity of the genus [30–33].

The genus *Acomys* was described as a separate taxon at the beginning of the nineteenth century, but there is still no synthesis of diversity across the genus, even though species names and descriptions abound [16, 34–38] (see Additional file 1). There were repeated attempts for systematic classification of *Acomys* using morphological characters [34, 39] and chromosomes [38, 40] (see Additional file 1). However, many currently recognized species cannot be easily distinguished using morphological characters due to significant intraspecific variability and generalized morphology. Available molecular studies [30–33, 41–46], are all based on limited taxon sampling and/or geographic coverage. Furthermore, earlier studies largely relied only on sequences of mitochondrial genes, which can be misleading in species delimitations (e.g., [47, 48]). To conclude, the estimation of the total number and delimitation of extant *Acomys* species and their biogeographical history would benefit from a more extensive study based on multiple molecular markers.

### Aims

In this study, we focus on phylogeography and biogeography of the genus *Acomys* by phylogenetic analysis of the largest available dataset and substantially improved geographic and taxon sampling. The aims of this study are (1) to reconstruct the phylogeny of the genus using multilocus dataset; (2) to identify the main biogeographical divides in the distribution of *Acomys* diversity in seasonally dry open habitats in Afro-Arabia; (3) to test proposed hypotheses of historical biogeography of the genus *Acomys* (i.e. to disentangle the role of geomorphology and climate changes on their diversification), with particular focus on dispersal events among major dry regions in Africa and between Africa, the Arabian Peninsula and the Eastern Mediterranean; (4) to estimate the species richness of the genus by applying of the phylogenetic species concept to identify the genetic groups and geographical regions that are worth further integrative taxonomic studies.

## Results

### Phylogeny, species delimitation, and distribution of genetic variability of Acomys

Both Bayesian inference (BI) and Maximum Likelihood (ML) analyses of concatenated multilocus data provided very similar phylogenetic reconstructions and revealed five major groups that we will hereafter call *subspinosus*, *spinosissimus*, *russatus*, *wilsoni* and *cahirinus* (Fig. 1a). If the number of nodes that are supported by PP ≥ 0.95 or BP ≥ 70 are considered, BI analyses yielded a slightly more robust topology (79 supported nodes; topology shown at Fig. 1a) compared to the ML tree (64 supported nodes; topology not shown). Five major groups are also supported in separate mitochondrial (not shown) and nuclear (Additional files 2 and 3) gene trees.

Three of these major groups (*spinosissimus*, *wilsoni*, and *cahirinus* groups) are further sub-structured to phylogenetic lineages (Fig. 1, Additional files 2 and 3) with predominantly parapatric distributions (Fig. 2). Based on the topology and the shape of the phylogenetic trees and the geographical distribution of genetic variability, we identified 26 distinct genetic lineages as our

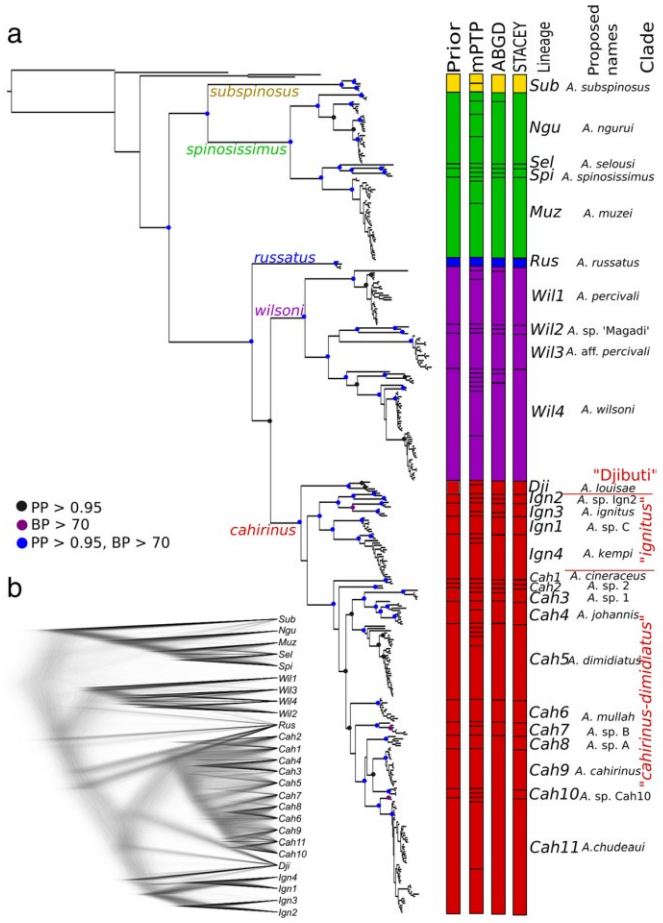


**Fig. 1** Multilocus phylogeny of the genus *Acomys*. **a** Bayesian phylogeny of the concatenated multi-locus matrix calculated in MrBayes. The support from Bayesian analysis in MrBayes (posterior probability, PP) and maximum likelihood analysis in RAxML (bootstrap probability, BP) is indicated by different colours on the nodes (black PP > 0.95, BP < 70; violet PP < 0.95, BP > 70; blue PP > 0.95, BP > 70). Five main *Acomys* groups (*subspinosus*, *spinosissimus*, *russatus*, *wilsoni* and *cahirinus*) are shown by different colours. The results of four different species delimitation approaches ("by-eye" prior; two delimitation approaches based on mtDNA: mPTP, ABGD; and a multilocus species delimitation in STACEY - see more details in the text) are shown in columns on the right, where individual "species" are separated by black lines. Additional information for 26 delimited taxa are provided, abbreviation of the lineages, and previously used taxonomic assignments. **b** DensiTree cloudogram of coalescent species trees from STACEY (for MCC species tree with PP see Additional file 4)

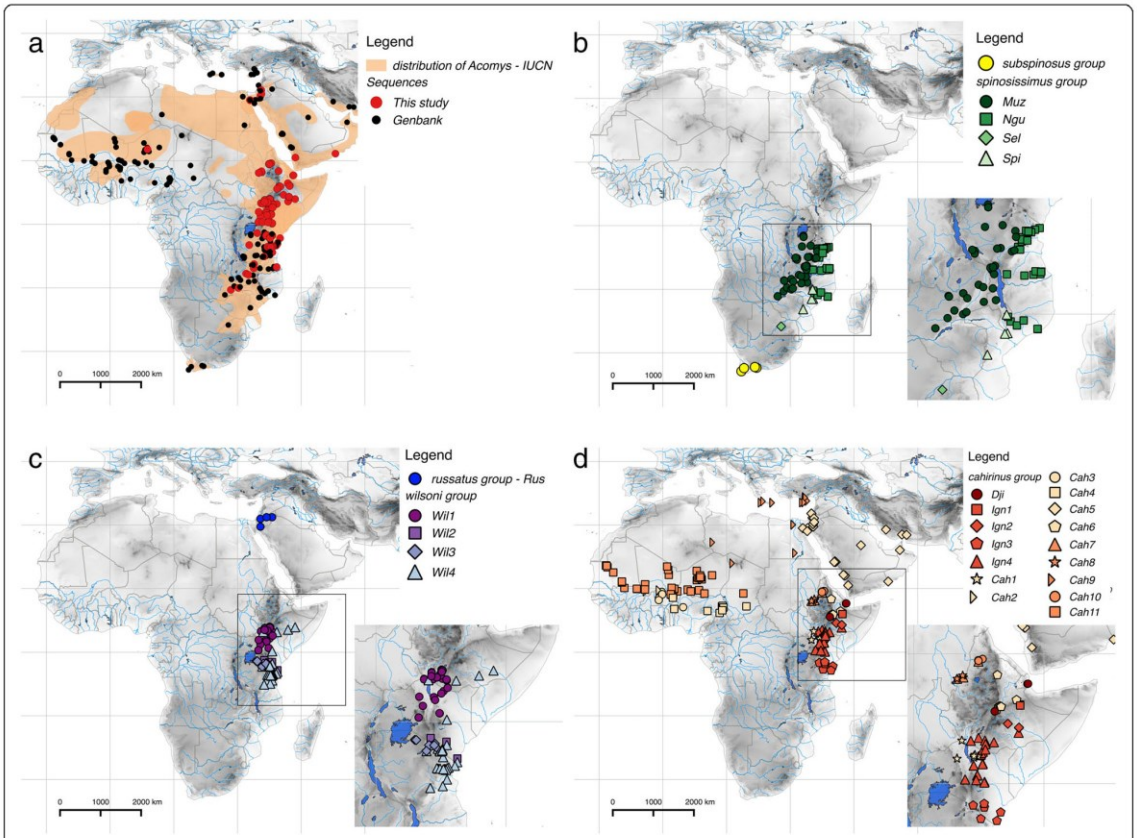


**Fig. 2** Analysed samples and the distribution of genetic variability in the genus *Acomys*. **a** Geographical distribution of the genus *Acomys* according IUCN (orange background); the origin of newly genotyped individuals is shown by red circles, while georeferenced sequences from GenBank are shown by black circles; **b** distribution of genetic lineages in the *subspinosus* and *spinosissimus* groups; **c** distribution of genetic lineages in the *russatus* and *wilsoni* groups; **d** distribution of genetic lineages in the *cahirinus* group

prior candidate species for next steps of genetic species delimitation (Fig. 1a). Their mutual relationships are relatively well resolved (with three well-defined clades: "Djibuti", "*ignitus*" and "*cahirinus-dimidiatus*", in the *cahirinus* group), with the important exception of lineages *Cah1-Cah11* representing probably a rapid radiation of the "*cahirinus-dimidiatus*" clade (Fig. 1a). The multispecies coalescent species tree from Species Tree And Classification Estimation, Yarely (STACEY) also revealed very similar topology to that reconstructed from the concatenated super-matrix (Fig. 1b, Additional file 4). The main differences are in unresolved positions of the *russatus* group and the "Djibuti" clade and a weakly resolved topology within the three major groups (*spinosissimus*, *wilsoni*, *cahirinus*; Additional file 4).

Species discovery approaches based on mitochondrial cytochrome *b* gene (*CYTB*) variability split the genus *Acomys* into 57 putative species in multi-rate Poisson Tree Process (mPTP) and 32 putative species in Automatic Barcode Gap Discovery (ABGD; Fig. 1), but ABGD did not find any clear gap between intra- and interspecific distances ("barcoding gap"; not shown). On the other hand, results from multispecies coalescent analysis in STACEY supported all 26 a priori defined species as separate gene pools (Fig. 2a). Taking into account the fact that multispecies coalescent does not statistically distinguish structure associated with population isolation vs. species boundaries [49], we will therefore use the term "species" for genetically distinct lineages or molecular operational taxonomic units (MOTUs).

The *subspinosus* group with only one lineage (*Sub*) is limited to South Africa (Fig. 2b). The separate species status was confirmed by all species delimitation analyses, and mPTP even suggested two different mitochondrial sublineages as two separate species (Fig. 1a). Mean intraspecific genetic distance is 1.5%, the distance to the nearest lineage is 21.3% (Table 1).

**Table 1** Genetic distances calculated from BI phylogenetic tree by the Species Delimitation algorithm in Geneious. Intraspecific distances and interspecific distances from the nearest lineage in percents (%)

| Group | Lineage | Nearest lineage | Intra Dist | Inter Dist - Closest |
|---|---|---|---|---|
| *subspinosus* | *Sub* | *Ngu* | 1.5 | 21.3 |
| *spinosissimus* | *Ngu* | *Muz* | 2.9 | 10.7 |
| | *Sel* | *Spi* | 0.0 | 7.1 |
| | *Spi* | *Sel* | 3.2 | 7.1 |
| | *Muz* | *Spi* | 1.1 | 8.0 |
| *russatus* | *Rus* | *Ign1* | 0.5 | 14.4 |
| *wilsoni* | *Wil1* | *Wil2* | 1.5 | 12.3 |
| | *Wil2* | *Wil3* | 4.7 | 10.5 |
| | *Wil3* | *Wil2* | 1.0 | 10.5 |
| | *Wil4* | *Wil2* | 3.1 | 9.6 |
| *cahirinus* | *Dji* | *Ign1* | 2.2 | 9.6 |
| | *Ign2* | *Ign3* | 2.9 | 6.4 |
| | *Ign3* | *Ign2* | 2.9 | 6.4 |
| | *Ign1* | *Ign4* | 0.7 | 5.9 |
| | *Ign4* | *Ign1* | 1.6 | 5.9 |
| | *Cah1* | *Cah2* | 0.6 | 7.5 |
| | *Cah2* | *Cah6* | 0.8 | 6.5 |
| | *Cah3* | *Cah4* | 0.8 | 6.1 |
| | *Cah4* | *Cah5* | 2.1 | 6.0 |
| | *Cah5* | *Cah4* | 1.4 | 6.0 |
| | *Cah6* | *Cah9* | 0.8 | 5.5 |
| | *Cah7* | *Cah9* | 1.3 | 5.5 |
| | *Cah8* | *Cah9* | 1.2 | 4.0 |
| | *Cah10* | *Cah9* | 0.6 | 3.0 |
| | *Cah9* | *Cah10* | 0.6 | 3.0 |
| | *Cah11* | *Cah9* | 1.2 | 4.5 |

The *spinosissimus* group inhabits the eastern part of the Zambezian bioregion (Fig. 2b). The STACEY approach confirmed four distinct species (*Muz, Ngu, Spi, Sel*), while mPTP and ABGD suggested 10 and six species, respectively (Fig. 1a). The intraspecific *CYTB* distances in four species range from 1.1 to 3.2% (excluding *Sel*, where only a single sequence was available). Interspecific distances to the nearest neighbour are 7.1-27.0% (Table 1).

The distribution of sequenced samples from the *russatus* group is restricted to arid regions of the Levant (Jordan, Israel; Fig. 2c). A single species (*Rus*) was supported by all species delimitation analyses. The mean intraspecific distance is low (0.5%), while interspecific distance to the nearest neighbour is 14.4% (Table 1).

The *wilsoni* group is divided into four well supported lineages, suggested as separate species by STACEY, and they are predominately distributed in the Somali-Masai savanna (Fig. 2c): *Wil1* lives on both sides of the Great Rift Valley (GRV) in Kenya and Ethiopia, *Wil2* and *Wil3* are two lineages with parapatric distribution in southern Kenya and northern Tanzania, and, finally, *Wil4* was found east of GRV from north-eastern Tanzania to southern Ethiopia, where it overlaps with *Wil1*. The mPTP split the *wilsoni* group into 13 putative species, and ABGD into eight species. The intraspecific distances of four STACEY species ranged from 1.0 to 4.7%, interspecific distances among them are from 10.5 to 12.3% (Table 1).

The highest genetic diversity was found within the *cahirinus* group (16 lineages delimited as species by STACEY). The group is composed of three significantly supported clades (with unresolved relationships among them), distributed parapatrically, with only small overlap (Fig. 2d): (i) the clade "Djibuti" with a single species (*Dji*) recorded from Djibuti and Dera National Park in Ethiopia, geographically neighbouring the Ethiopian Afar province; (ii) the clade "*ignitus*" distributed south-east of GRV with four lineages (*Ign1-Ign4*); (iii) the clade "*cahirinus-dimidiatus*" widespread north-west of GRV, including Sahel and Sudanian savanna, eastern Mediterranean, Middle East and Arabian peninsula, with 11 species (*Cah1-Cah11*) delimited by STACEY (Fig. 1). Intraspecific distances within the *cahirinus* group range from 0.6 to 2.9%, interspecific from 3.0 to 9.6% (Table 1).

### Historical biogeography and divergence dating

Based on the Dispersal-Extinction Cladogenesis model and the time-calibrated tree, the TMRCA of the genus *Acomys* is dated to 8.69 Ma (95% HPD = 8.51–9.29 Ma). The ancestral area is predicted to be in Eastern Africa, but it is not resolved whether in the Zambezian or the Somali region (Fig. 3). The first split in the Late Miocene separated southern groups (*subspinosus* + *spinosissimus*) from northern groups (*russatus* + *wilsoni* + *cahirinus*). Around 7.04 Ma (95% HPD = 5.25–8.67 Ma) the ancestor of the *subspinosus* group in South Africa diverged from the ancestor of the *spinosissimus* group in the Zambezian region, where the latter group started to diverge around 4.24 Ma (95% HPD = 3.02–5.48 Ma). The ancestor of the *russatus* group in Arabian region separated from *Acomys* in Somali region around 7.55 Ma (95% HPD = 6.27–8.64 Ma). The split between *cahirinus* and *wilsoni* groups occurred in Somali region around 6.97 Ma (HPD = 6.10–8.08 Ma). The *wilsoni* group started to diversify around 4.54 Ma (95% HPD = 3.48–5.61 Ma), while the first split in the *cahirinus* group is dated to 5.47 Ma (95% HPD = 4.55–6.46 Ma), in both cases the beginning of radiation is predicted in the Somali-Masai savanna. Subsequent splits in the *cahirinus* group occurred around 5 Ma, when the ancestors of clades "Djibuti", "*ignitus*" and

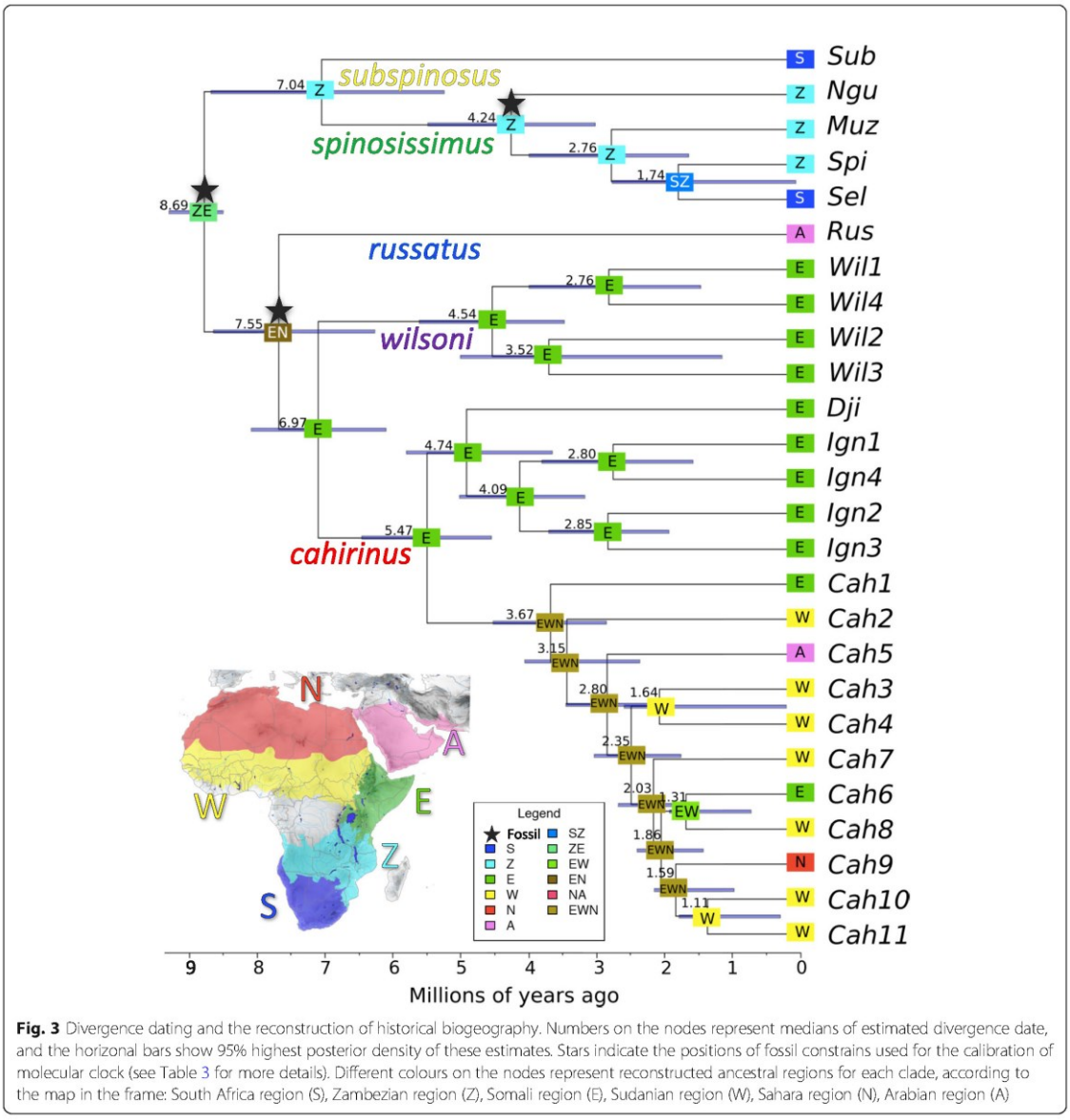


**Fig. 3** Divergence dating and the reconstruction of historical biogeography. Numbers on the nodes represent medians of estimated divergence date, and the horizonal bars show 95% highest posterior density of these estimates. Stars indicate the positions of fossil constrains used for the calibration of molecular clock (see Table 3 for more details). Different colours on the nodes represent reconstructed ancestral regions for each clade, according to the map in the frame: South Africa region (S), Zambezian region (Z), Somali region (E), Sudanian region (W), Sahara region (N), Arabian region (A)

"*cahirinus-dimidiatus*" diverged most likely in Eastern Africa. The biogeographic history of the "*cahirinus-dimidiatus*" clade was more complex. While *Cah1* stayed in Somali region, the ancestor of lineages *Cah2-Cah11* likely dispersed to the north-west (especially Sudanian savanna), where most genetic lineages currently occur. The results suggest one Pleistocene migration back to the Somali region (*Cah6* in Afar region, ~ 1.31 Ma) and two independent dispersals to the north, either to the Arabian region (*Cah5* dated at 2.80 Ma) and to the Sahara region (*Cah9* dated at 1.59 Ma).

### Species distribution modelling

The bioclimatic MaxEnt model for the present shows that the sampling in this study covers almost the complete range of suitable climatic conditions for genus *Acomys* (Fig. 4a). The most important variables predicting the modelled geographic distribution of spiny mice were the annual range of temperature and annual precipitation. The predicted distribution is relatively continuous in eastern Africa, mainly in the Somali-Masai and eastern Zambezian savanna. On the contrary, climatically unsuitable are moist mountains in Ethiopia,

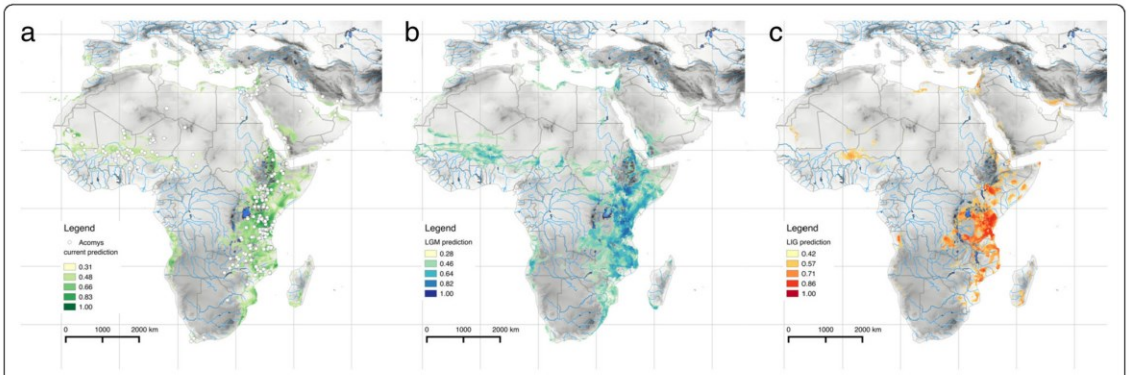


**Fig. 4** The probability of *Acomys* occurrence based on the MaxEnt modelling of bioclimatic niches. More intensive colour indicates higher probability of suitable conditions. **a** The model for the present; white dots indicate the sampled localities; **b** prediction for the last glacial maximum (21 ka); **c** prediction for the last interglacial period (120–140 ka)

Kenya and Albertine Rift, as well as very arid regions of Horn of Africa and Masai xeric scrublands in north-eastern Kenya. The belt of suitable climatic conditions occurs in West Africa, on the boundary between Sudanian savanna and Sahel, as well as along Mediterranean sea and Arabian Peninsula and southern Iran. Isolated suitable areas are predicted in western Angola and northern Namibia, despite the absence of present-day occurrence of *Acomys* species. The model for LGM predicts very similar distribution of spiny mice, with more continuous belt of suitable conditions in Sudanian region (Fig. 4b). The predicted distribution during LIG was more fragmented, with highly suitable conditions in southern Ethiopia, Kenya and Tanzania, and isolated patches in the Horn of Africa, southern part of the Sahara desert, and Mozambique (Fig. 4c). Altogether, it seems very likely that the climatic conditions in the core of the present day distribution, in open seasonally dry habitats in East Africa, were apparently favourable at least during the last glacial cycle.

## Discussion

Spiny mice of the genus *Acomys* represent a speciose group of rodents, widely distributed in seasonally dry savanna of Africa, Arabia and Middle East. They often are a dominant component of small mammal assemblages and in some habitats, e.g. in rocky outcrops, they can be even the only rodents captured (our unpubl. data). They were able to colonize wide spectrum of non-forested habitats, from miombo woodlands to rocks in the middle of Sahara, from the sea coast up to 2500 m above sea level (a.s.l.) [e.g., 36]. Despite *Acomys* abundance and practical importance (e.g., as model taxa in behavioural or biomedical research; [50]), the knowledge of their evolutionary history, taxonomy and biogeography has been very limited and biased to particular regions or intrageneric clades [28, 30, 31, 33, 41, 43, 45]. Here we compiled the largest multilocus genetic dataset to date for the genus *Acomys* (genotypes of 699 individuals from more than 280 localities covering a majority of the distribution of this genus), reconstructed phylogenetic relationships, described biogeographical patterns and evolutionary history, and estimated the spiny mice species diversity.

### Phylogeny and biogeographical patterns in the genus *Acomys*

Phylogenetic analysis revealed clear evidence for the existence of five major groups, *subspinosus, spinosissimus, russatus, wilsoni* and *cahirinus* (Figs. 1 and 2, Additional files 2 and 3), which diverged in the late Miocene (Fig. 3). For the first time we provide nuclear genetic data for the *subspinosus* group and we refer its sister position with the *spinosissimus* group, which has never been confirmed [31, 41, 45]. This sister relationship is supported also by the fact that *subspinosus* and *spinosissimus* groups share the same triplicate-type X chromosome that is very rare among mammals [51]. For the first time we also sequenced the nuclear markers of the *russatus* group, but its phylogenetic relationships with the *wilsoni* and *cahirinus* groups remained unresolved (Fig. 1), which suggests fast divergence in the late Miocene (see similar results in [45]). Using multilocus genetic data, we unequivocally identified that the first split within the genus occurred between south-eastern and eastern Africa, i.e. (*subspinosus* + *spinosissimus*) vs. (*russatus* + *wilsoni* + *cahirinus*) (Fig. 3). Further diversification of the five major groups is dated to Plio-Pleistocene and, interestingly, the diversification rate within them is very unequal. While *subspinosus* and *russatus* remained monotypic at the margins of *Acomys* distribution (in the Cape Region and Middle East, respectively), *spinosissimus*

and *wilsoni* clades diversified by a comparable rate in Eastern Africa, either in Zambezian or Somali-Masai savannas. The most intensive spatial and cladogenetic expansion occurred in the *cahirinus* group that colonized large areas of Africa, Arabia and eastern Mediterranean region, even if the most genetic variation is still observed in eastern Africa.

Our phylogenetic analysis revealed at least three independent "out-of-Africa" events, differing significantly by the date of divergence from their African counterparts. The oldest disperser was the ancestor of the *russatus* group, currently distributed only in the Sinai Peninsula and the Middle East, which split from the ancestors of the *wilsoni* and *cahirinus* groups already in late Miocene (Fig. 3, see also [30]). Much more recently, at the beginning of Pleistocene, the ancestors of *Cah5* colonized the Arabian Peninsula (and South Iran). It is unclear whether it was through the Bab al-Mandab connection between Africa and Yemen [33, 52] or through Sinai and around the Red Sea coastline [53]. Lastly, the western Mediterranean islands and south coast of Turkey were colonized during Antiquity by possibly commensal populations from the lineage *Cah9*, likely from Egypt [31, 33, 54].

Spiny mice of the *cahirinus* group (specifically "*cahirinus-dimidiatus*" clade) are widely distributed also in the Sudanian savanna. Results of our biogeographical analysis suggest multiple colonization and diversification waves, directed usually from eastern to western Africa. The first split between *Cah1* (which stayed restricted in Somali region along the Lake Turkana) and remaining lineages occurred in the late Pliocene/early Pleistocene. The ancestors of lineages *Cah2*, *Cah 3*, and *Cah4* diverged in southern (more humid) part of Sudanian savanna probably simultaneously with the colonization of Arabian Peninsula by *Cah5*. Much later (*ca.* 1–1.5 Mya), the split between northern (*Cah9*) and southern (*Cah10* and *Cah11*) resulted in current diversity in large arid areas in northern part of the Sudanian region, Sahel and Sahara desert. At the same time the Ethiopian highlands caused the diversification of *Cah8* (living in easternmost Sudanian region) and *Cah6* (currently limited to Afar triangle). Faunal exchange between Somali-Masai and Sudanian savanna in the Plio-Pleistocene have been described also in other rodents and very often the oldest lineages of Sudanian taxa are found in Eastern Africa [55–57], which well corresponds with the pattern found in spiny mice.

The current distribution of genetic diversity of *Acomys* is affected by major biogeographic divides, represented by (1) extremely dry open habitats, (2) mountain blocks and (3) large water bodies, e.g. Rift Valley lakes or rivers. Even if the spiny mice are considered as typical inhabitants of (semi-)arid environments [58], they avoid extremely dry habitats. Distribution modelling based on bioclimatic data (Fig. 4) suggests low suitability of habitats in most of Saharan and Arabian deserts, as well as in Masai xeric bushland and shrublands in northernmost Kenya and Horn of Africa. However, even in such habitats the spiny mice can occur, but they have very patchy distribution with isolated populations in rocky areas, considered as remains of more continuous distribution in past humid periods, when savanna-like habitats prevailed [43, 59–63]. Sahara desert seems to work as a barrier between clades *Cah9* and *Cah11* (albeit the data are missing e.g. from southern Algeria).

The expansion of forests during interglacials [64, 65], known as Pleistocene breathing model [66], repeatedly fragmented savanna biome, especially in eastern Africa. The forested mountain blocks thus formed an important biogeographical divide for savanna-dwelling organisms and supported allopatric diversification in this nowadays relatively continuous ecosystem [67]. In agreement with this hypothesis, we can see the coincidence of genetic structure in the *spinosissimus* group with Eastern Arc Mountains (EAM) and Southern Rift Mountains (SRM). These mountain ranges, even if permeable today for taxa living in open habitats, clearly separate *Ngu* and *Muz* lineages (Fig. 2b; see more details in [44]). Similar structure was recently observed in numerous other taxa of sympatric murid and bathyergid rodents living in open habitats of Zambezian region [67–70]. Similarly, the mountains in north-eastern Tanzania (Kilimanjaro, Pare, Usambara) currently delimit distribution for some *Acomys* lineages (*Wil2* vs. *Wil3* or *cahirinus* vs. *spinosissimus* groups), and, again, this pattern was found in other savanna rodents (e.g. *Saccostomus* [71], *Gerbilliscus* [72]). Further to the north, Kenyan highlands could have worked in similar way as they seem to limit distribution of several *Acomys* lineages (*Wil1* vs. *Wil2* + *Wil3*, *Ign3* vs. *Ign4*). Lastly, Ethiopian Highlands, delimit distributions of lineages especially in the *cahirinus* group. For example lineages *Dji* and *Cah6* are restricted to the Afar province in north-eastern Ethiopia (see also very similar pattern in gerbils of the genus *Gerbilliscus*, often sympatric with spiny mice; [72]).

Rivers and other more or less linear water bodies (e.g., contemporary rift lakes, or palaeolakes; [73]) are known to play an important role in shaping genetic structure and diversification patterns in organisms living in open habitats. For example the Zambezi-Kafue river system delimits southern border of distribution of *Ngu* and *Muz* lineages (Fig. 2b; [44]) and have been implicated in forming genetic diversity in savanna-dwelling organisms as diverse as killifishes [74], gerbils [75], African pouched mouse [71], baboons [76] and several species of antelopes [77]. The largest river in Sudanian savanna is the Niger River, which makes a border between *Cah11*

and other West African *Acomys* lineages, is also dominant biogeographical divide in many other rodents (e.g., [43, 55, 67, 78]). On the other hand, the Nile Valley seems to serve rather as a suitable migration corridor for northward spreading of savanna taxa from eastern Africa (see [56] for grass rats, or [79] for shrews). In *Acomys*, it very likely allowed the lineages *Cah5, Cah9* and *Rus* to colonize the northern Africa and Arabian Peninsula. Trauth et al. [73] suggested that during the humid periods of Pleistocene, the bottom of GRV was filled by water (much more than today), and the so-called palaeolakes formed an important biogeographic barrier causing allopatric diversification or even speciation. The phylogeographic pattern concordant with this hypothesis was recently described in Eastern African gerbils [72] and the role of GRV on genetic structure is visible also in *Acomys*. For example, *Cah1* is separated from "*ignitus*" clade by GRV and, similarly, more records of *Wil1* occur west of GRV, while other lineages of the *wilsoni* group were found predominantly east of GRV.

#### Evolutionary scenario of the genus *Acomys* – interplay of geomorphology and climatic changes

The origin of *Acomys* is dated in late Miocene, which is in concordance with the first occurrence of savannas that appeared as a result of rifting activity in eastern Africa and climatic changes [4]. Spiny mice belong to the subfamily Deomyinae, whose other members occur mostly in forest or forest margins (i.e. genera *Deomys* and *Lophuromys*) or moist savanna (*Uranomys*) [16]. The phylogenetic relationships among genera of Deomyinae are not sufficiently resolved [46, 80, 81], but it seems likely that the ancestor of *Acomys* colonized dry open habitats early after their late Miocene appearance profiting from empty niches in this ecosystem. Barome et al. [31] proposed the origin of *Acomys* ca. 13.7 Ma in East Africa or in South Africa, while Alhajeri et al. [46] placed it more generally to sub-Saharan Africa at 10 Ma. Other authors [40, 82] suggested the origin for the genus in Eastern Africa, mainly in the Ethiopian region [82]. Our historical biogeography reconstruction is in partial agreement with previous studies as the origin of the genus is placed either in Somali and/or Zambezian regions (Fig. 3), i.e. the regions with the highest contemporary genetic diversity of spiny mice. Even if the oldest fossil records of *Acomys* ancestors are found in Zambezian savanna (†*Preacomys griffini* and †*Preacomys karsticus* ca. 9 Ma from Namibia; [83]), they occurred in the late Miocene also in East Africa (†*Preacomys kikiae* 8.5 Ma from Chorora, Ethiopia; [84]).

Periods of warm humid climate in Late Miocene caused the last occurrence of coast-to-coast belt of tropical forest, which is evidenced by phylogenetic analyses of plants and animals living in nowadays fragmented forests of Congo basin and eastern African montane and coastal forests [85, 86]. This continuous forest was likely one of the most important factors in early evolution of savanna inhabitants, because it separated northern (= Somali-Masai) and southern (= Zambezian) savannas. In *Acomys*, this resulted in allopatric divergence between the ancestor of *subspinosus* + *spinosissimus* group in the Zambezian savanna and the ancestor of *wilsoni* + *russatus* + *cahirinus* in the Somali-Masai savanna (estimated in this study at 8.7 Ma). The same geographical and temporal pattern, i.e. late Miocene split of northern and southern taxa, was observed also in other savanna mammals, e.g. gerbils [57], pouched mice [71], several genera of antellopes [87], warthogs [88] and giraffes [89, 90] .

Later on, but still in Late Miocene, the evolution of savanna's fauna was significantly influenced mainly by the Messinian salinity crisis (MSC, [91]), dated to 6.0–5.3 Ma. Very little is known about the effect of the MSC on eastern African climate [6], but it is generally held that overall aridification at the Miocene/Pliocene boundary promoted the expansion of very dry habitats [92]. Inhospitable very arid (desert) areas in north-eastern Africa expected at MSC period thus likely interrupted the connection of *Acomys* populations between Somali-Masai and eastern Mediterranean area. This period corresponds to the split of the *russatus* group, which remained effectively isolated in the north.

Plio-Pleistocene period (starting 5.3 Ma) is characterized by intensive climatic oscillations. There are several well-known climatic transitions, like the intensification of Northern Hemisphere glaciation (iNHG; 3.2–2.5 Ma, [93, 94]), the development of the Walker circulation (2.0–1.7 Ma; [95]) and the early-middle Pleistocene transition (1.2–0.8 Ma; [96]). These periods of pronounced climate variability significantly affected the distribution of forests, palaeolakes and savannas [97]. For example during more humid periods the currently fragmented montane forests in Eastern Arc Mountains and Kenyan highlands probably expanded into lower altitudes, became more continuous and formed significant barriers to gene flow for taxa living in open dry habitats [67, 71, 72, 98]. The bottom of GRV was filled by the palaeolakes, which prevented gene flow of savanna-dwelling species, leading to diversification, or even speciation [73]. Because of wide confidence intervals of our estimates of divergence times, it is not possible to link particular splits to specific climatic events. However, it is highly probable that a majority of current genetic diversity in *spinosissimus*, *wilsoni* and *cahirinus* groups is a result of repeated fragmentation of savannas in Plio-Pleistocene, caused by climatic changes. This is further supported by parapatric distribution of lineages within major clades, where the distribution borders often correspond to predicted barriers of the gene flow (i.e. too arid or forested areas, and palaeolakes).

### Species richness of *Acomys* – the need of further integrative taxonomic studies

The number of species crucially depends on adopted species concept. Rapidly increasing amount of genetic data now allows to apply the so-called integrative taxonomic approach, which usually complements the widely used typological or biological species concepts by genetic [99] and/or phylogenetic [100] species delimitations. The taxonomy of *Acomys* has been unresolved since the second half of the twentieth century. In Additional file 1 we listed several taxonomic alternatives, based mostly on morphological traits, used recently for the genus *Acomys*. The most comprehensive list was provided by Ellerman [34] with 25 species and 17 subspecies. On the other extreme, Setzer [38] recognized only five species that partly correspond to major genetic clades recovered in our study: *A. cahirinus* (7 subspecies), *A. dimidiatus* (12 subspecies), *A. russatus* (one subspecies), *A. spinosissimus* (one subspecies) and *A. subspinosus* (9 subspecies, including *A. wilsoni*). Widely accepted list of Musser and Carleton [37] contained 18 species. Monadjem et al. [36] lists 15 species in sub-Saharan Africa, including three newly delimited species in the *spinosissimus* group [45]. The most recent and comprehensive Handbook of the Mammals of the World [16] listed 21 species and 12 subspecies.

We used several genetic species delimitation methods and their estimates of spiny mice species richness differ significantly from 57 species (mPTP) to 26 species (STACEY as well as our prior delimitation based on geographical distribution of genetic diversity). The species delimitation based only on mitochondrial markers (ABGD and mPTP) have the tendency to overestimate the number of revealed species and they often identify as separate species also genetic lineages that are traditionally considered as intraspecific phlyogeographic structure. For example ABGD revealed four species within *Ngu* lineage, while Petružela et al. [44] recently showed on multi-locus dataset that they represent only phylogeographic structure of *A. ngurui*. Below we use the most conservative estimate (26 MOTUs, here considered as "genetic lineages" and named according Fig. 1a) and compare these species delimitations with previous taxonomic work. Multi-species coalescent approaches to species delimitation (like STACEY) in fact diagnose the genetic structure, with no distinction between structure due to populations or due to species [49]. Therefore, the aim of the following part is not to perform a formal taxonomic revision, but to show the genetic clades and geographic regions where further integrative taxonomic analyses (employing combination of genetic, morphological, ecological and other data) could lead either to new descriptions or synonymization of *Acomys* taxa.

#### *The subspinosus group*

This group is monotypic and contains a single lineage *Sub*.

*(1) Sub*

*Distribution:* South Africa (Cape Province).

*Available name: Acomys subspinosus* (Waterhouse, 1838).

*Type locality:* Western Cape Province, Cape of Good Hope, South Africa.

*Karyotype:* 2n = 64, NF = 70 [101, 102].

*Additional information:* Based on its unique dental and skull morphology, *A. subspinosus* has been placed in its own subgenus *Subacomys* with an "ancestral" karyotype (2n = 64, NF = 70; [51, 101–104]). Its separation from other *Acomys* was also indicated by phylogenetic analyses of *CYTB* [31, 32, 45]. Using for the first time the combination of mitochondrial and nuclear markers, we unequivocally showed its sister relationship with *spinosissimus* group, i.e. it does not represent the first cladogenetic split of *Acomys*. As a consequence, the validity of the subgenus *Subacomys* (mentioned erroneously as *Preacomys* in Denys et al. [16]) is questionable.

#### *The spinosissimus group*

This strongly supported monophyletic group has been revised repeatedly [41, 44, 45] and four genetic lineages were distinguished and named. However, the genetic data from the southern part of its distribution are still very limited and especially the taxonomy and distribution of *A. selousi* and *A. spinosissimus* should be further explored.

*(2) Ngu*

*Distribution:* Lineage *Ngu* is distributed in three well supported parapatric sublineages from Tanzania (East of EAM) to central Mozambique (north of the Zambezi River; [44]).

*Available name: Acomys ngurui* Verheyen et al., 2011.

*Type locality:* Nguru Ya Ndege, Tanzania.

*Karyotype:* 2n = 60, NFa = 68 [45].

*Additional information:* This species is very similar to *A. muzei* from which it differs by relatively shorter tail and non-overlapping distribution [44, 45]. Barome et al. [41] reported it as *A.* cf. *selousi* from Berega. Three genetically distinct sublineages probably represent intraspecific variation [44].

*(3) Sel*

*Distribution:* Northern part of South Africa, only one genetically confirmed locality from the Kruger National Park [45]. Northern limits of its distribution are not fully resolved.

*Available name: Acomys selousi* De Winton, 1896.

*Type locality:* Essex Farm, Zimbabwe.

*Karyotype:* 2n = 58–62, NFa = 68 [101, 102] .

*Additional information:* According some authors [35, 37, 38]; *A. transvaalensis* and *A. selousi* are synonyms of *A. spinosissimus*. Here we follow the view of Verheyen et al. [45] and Monadjem et al. [36] and consider this

lineage as a separate species, but further taxonomic investigation of the *spinosissimus* group in South African region is required.

*(4) Spi*
*Distribution:* Mozambique, Zimbabwe, southern Malawi.
*Available name: Acomys spinosissimus* Peters, 1852.
*Type locality:* Tette and Buio, Mozambique.
*Karyotype:* 2n = 60, NFa = 68 [101].
*Additional information:* We follow the view of Verheyen et al. [45] and Monadjem et al. [36] and include only populations from central Mozambique and southern Malawi into this species. Petružela et al. [44] recently showed that its distribution north of the Zambezi River is much more restricted compared to maps in Monadjem et al. [36] and the two well distinct genetic sublineages of this species seem to be separated by the Zambezi river.

*(5) Muz*
*Distribution:* Central and western Tanzania, Malawi (west of the Lake Malawi), Zambia.
*Available name: Acomys muzei* Verheyen et al., 2011.
*Type locality:* Muze, Tanzania.
*Karyotype:* 2n = 58–62, NFa = 68 [45].
*Additional information:* Recent analyses showed that the populations in Malawi and Zambia (reported as *A. spinosissimus* in Monadjem et al. [36]) belong to this species [44]. Further, the highest genetic diversity of the species was recorded west of the Lake Malawi, while Tanzanian populations represent only relatively recent colonization event [44].

***The russatus group***
The *russatus* group is monotypic with only one lineage *Rus*. The phylogenetic relationships with its sister groups *cahirinus* and *wilsoni* are not fully resolved (Fig. 1).

*(6) Rus*
*Distribution:* Egypt (separate subspecies *aegyptiacus* was described in Eastern Desert), Sinai, Jordan, Israel, Saudi Arabia, Yemen and Oman. Genotyped material in this study originates only from Jordan and Israel.
*Available name: Acomys russatus* (Wagner, 1840).
*Type locality:* Sinai, Egypt.
*Karyotype:* 2n = 66, NF ≥ 66 [40].
*Additional information:* Denys et al. [51] referred that *A. russatus* is very distinctive in its molar morphology and chromosomal traits and previous *CYTB* [31, 41, 45] as well as our multilocus genetic analyses showed that it is not closely associated with any other *Acomys* taxon. *Acomys russatus* and *A. dimidiatus* (= *Cah5* in this study) can live in sympatry, and their differences in ecology, physiology, and activity patterns (especially in Israel) have been extensively documented (see references in [105]).

***The wilsoni group***
This group is well supported in all phylogenetic analyses (Fig. 1 in this study, [31, 41]), but its relationships with *russatus* and *cahirinus* groups are not completely resolved. Distribution of the *wilsoni* group is limited to the Somali region [sensu 3]. Based on multilocus species delimitation we recognize four genetic lineages, but the species limits must be further investigated.

*(7) Wil1*
*Distribution:* South Ethiopia, Kenya (along GRV).
*Available name: Acomys percivali* Dollman, 1911.
*Type locality:* Chanler Fall, Nyiro, Kenya.
*Karyotype:* 2n = 36 and NF = 68 [40].
*Additional information:* Phylogenetically the most distinct MOTU within the *wilsoni* group (Fig. 1). It was not included in previous phylogenetic studies. Distribution of *A. percivali* reported by Monadjem et al. [36] and Denys et al. [16] is very similar to that of *Wil1*. Janecek et al. [103] regarded *A. percivali* as the species genetically most closely related to *A. wilsoni* (= *Wil4*). Both clades were found sympatric at several localities in southern Ethiopia, where they can be distinguished by external morphology (our unpublished data) and karyotypes [16].

*(8) Wil2*
*Distribution:* Southern Kenya.
*Available name:* None. Based on Barome et al. [31], we use in Fig. 1 the name *A.* sp. 'Magadi'.
*Type locality:* Not relevant.
*Karyotype:* Not known.
*Additional information:* Known only from three localities from southern Kenya, all of them reported by Barome et al. [31]. They mentioned this MOTU as two different species, *A.* sp. 'Magadi' and *A. wilsoni*, and this structure was reflected also by mPTP and ABGD analyses in our study. The conspecificity with *Wil3* and/or *Wil4* are plausible hypotheses and should be tested.

*(9) Wil3*
*Distribution:* NW Tanzania, southern Kenya, most localities in the bottom of GRV.
*Available name:* None. Based on Mgode [42], we use in Fig. 1 the name *Acomys* aff. *percivali.*
*Type locality:* Not relevant.
*Karyotype:* 2n = 58 [42].
*Additional information:* Mgode [42] named 13 spiny mice from northern Tanzania (localities Tingatinga, Longido, Mt. Gelai-Olikisima and Kilimamoja-Karatu) belonging to this lineage as *Acomys* cf. *percivali*. They differ from *Wil4* (= *A. wilsoni*) in skull morphology and karyotype. Because the type locality of *A. percivali* (Mt. Nyiro, Kenya) is very far from the distribution of *Wil3*, the name *A. percivali* more probably belongs to *Wil1*,

while *Wil3* likely deserves a formal description as a new species.

*(10) Wil4*
*Distribution:* Southern Ethiopia (Somali region), Kenya (east of GRV), north-eastern Tanzania.
*Available name: A. wilsoni* Thomas, 1892.
*Type locality:* Mombasa, Kenya.
*Karyotype:* 2n = 62 and NF = 76 [42, 106].
*Additional information:* Verheyen et al. [45] suggested that *A. wilsoni* (meaning *Wil2* and *Wil4* included in their study of mitochondrial variation) is probably a species complex, which is confirmed by our data. The conspecificity of *Wil4* with *Wil2* and/or *Wil3* should be further investigated as they might represent intraspecific phylogeographic structure (see similar patterns in savanna-dwelling rodent species in southern part of Somali region, e.g. in *Gerbilliscus vicinus*; [72] or *Saccostomus umbriventer*; [71]). If *Wil2* and *Wil4* are different species, the analysis of the type material of *A. wilsoni* will be required to decide, which of them is true *A. wilsoni* (both are distributed around the type locality of *wilsoni*).

#### *The cahirinus group*

This is the most diversified *Acomys* group comprising of three main clades, "Djibuti" (one MOTU *Dji*), "*ignitus*" (four MOTUs *Ign1* – *Ign4*) and "*cahirinus-dimidiatus*" (11 MOTUs *Cah1* – *Cah11*), with unresolved mutual relationships.

*(11) Dji*
*Distribution:* Djibuti, Afar province in Ethiopia, probably also Somalia (from where no genetic data are available).
*Available name: Acomys louisae* Thomas, 1896.
*Type locality:* 65 km S of Berbera, Somalia.
*Karyotype:* 2n = 68 and NF = 68 ([107]; our unpubl. data).
*Additional information: Acomys louisae* was placed in a separate subgenus *Peracomys* based on dental characters [51]. We have not checked the skull morphology of our material from *Dji*, but the recently collected animals from eastern Ethiopia (Dire Dawa region) likely assigned to *A. lousiae* by morphological characters (by C. Denys, unpubl. data) clustered with *Dji* at *CYTB* (not included in this study). According to Petter [108], *A. louisae* cannot be distinguished from the "*cahirinus-dimidiatus* complex" (sensu [109, 110]) on the basis of skull or external characteristics. This species may co-occur with *A. mullah* (= *Cah6*) in the Afar triangle (larger, HB > 100 mm with grey or greyish-brown dorsal pelage). *A. louisae* should have a bright rufous or brown dorsal pelage and relatively very long tail (> 100% of HB; [36]). In our pilot analysis we were not able to find significant external size differences between individuals from lineages *Dji* and *Cah6* (considered as *A. mullah*, see below), but more detailed morphological investigation is needed.

*(12) Ign1*
*Distribution:* Eastern part of Ethiopia (Babile).
*Available name:* None. Based on Lavrenchenko et al. [111], we use the name *Acomys* sp. C in Fig. 1.
*Type locality:* Not relevant.
*Karyotype:* 2n = 44, NF = 68 [111, 112].
*Additional information:* This presumably new species was mentioned for the first time as genetically and cytogenetically very divergent lineage (*Acomys* sp. C) by Lavrenchenko et al. [111] from the Babile Elephant Sanctuary in Eastern Ethiopia. This lineage is only known from the Babile Elephant Sanctuary, where it is very common and abundant species. It can have wider distribution in poorly sampled regions of southeastern Ethiopia and Somalia (see similar pattern in gerbils, [72]). The conspecificity with other lineages of the *ignitus* clade should be further tested. The comparison with the type material of *A. mullah*, described from nearby town Harar, is necessary (see also below).

*(13) Ign2*
*Distribution:* South-eastern Ethiopia.
*Available name:* None.
*Type locality:* Not relevant.
*Karyotype:* Not known.
*Additional information:* This lineage is reported here for the first time. It is known only from two localities in the south-eastern slope of Ethiopian Highlands (Sof Omar caves and Imi; each locality has very distinct mitochondrial haplotypes). It might be more widespread in poorly sampled Somali region of Ethiopia, and in Somalia. Its sister lineage *Ign3* (= *A. ignitus*) is geographically distant, but the conspecificity with other lineages of the *ignitus* clade are worth of further taxonomic work.

*(14) Ign3*
*Distribution:* Southern Kenya, northernmost Tanzania.
*Available name: Acomys ignitus* Dollman, 1919. In Alhajeri et al. [46] was this species incorrectly mentioned as *A. percivali*.
*Type locality:* Voi, Kenya.
*Karyotype:* 2n = 50, NF = 66–68 [102].
*Additional information: A. ignitus* has been recorded in and around Tsavo National Park [36], which corresponds to the distribution of this MOTU. Whether or not other lineages of the *ignitus* clade (especially *Ign2*) are conspecific with *A. ignitus* must be investigated by integrative taxonomy approach.

*(15) Ign4*
Distribution: Kenya and southernmost Ethiopia (east of GRV).

*Available name: Acomys kempi* Dollman, 1911.
*Type locality:* Chanler Falls, N Guaso Nyiro, Kenya.
*Karyotype:* Not known.
*Additional information:* This species was previously listed as subspecies of *A. ignitus* (Hollister, 1919) [113] or *A. cahirinus* (Setzer, 1975) [38], but rehabilitated as clearly distinct species by Janecek [103]. *Acomys kempi* was found sympatric with *A. percivali* (= *Wil1*) at several localities in Kenya and southern Ethiopia and these two taxa can be easily distinguished, e.g. by coat coloration (the latter being usually greyish and darker). There is no evidence of distributional overlap with *Ign3* (*A. ignitus*), but further sampling in southern Kenya would be desirable.

*(16) Cah1*
*Distribution:* North-west Kenya (the only region from where the genetic data is available), Sudan, South Sudan. Very probably also in Uganda.
*Available name: Acomys cineraceus* Heuglin, 1877.
*Type locality:* Doka, Sudan.
*Karyotype:* 2n = 48–50 [114].
*Additional information:* Formerly included in *A. cahirinus* [38, 108], but Dieterlen (in litt.) noted that *A. cineraceus* is a distinct species [37]. Separation of *A. cineraceus* from *A. cahirinus* is supported by chromosomal data (2n = 48 or 50 for *A. cineraceus*, 2n = 36 for *A. cahirinus*; [37, 114]). Limits of the geographic range of *A. cineraceus* are unresolved, especially its western part [37] . Compared to the distribution maps in Denys et al. [16] and Happold [115], we were not able to confirm its occurrence in western Ethiopia.

*(17) Cah2*
*Distribution:* Burkina Faso, Mali.
*Available name:* None. Based on Barome et al. [30], we call this MOTU as *Acomys* sp. 2 in Fig. 1.
*Type locality:* Not relevant.
Karyotype: 2n = 66–68, NF = 66–72 [116].
*Additional information:* Barome et al. [30] called this MOTU as *Acomys* sp. 2, Granjon and Duplantier [116] included it in *A. johannis* species complex. The distribution of *Cah2* in West Africa is overlapping with *Cah3* and *Cah4*, and its specific status should be investigated by integrative taxonomy approach using larger material and multi-locus genetic analysis.

*(18) Cah3*
*Distribution:* Burkina Faso.
*Available name:* None. Based on Barome et al. [30], we call this MOTU as *Acomys* sp. 1 in Fig. 1.
*Type locality:* Not relevant.
*Karyotype:* 2n = 66–68, NF = 66–72 [116].
*Additional information:* Barome et al. [30] called this MOTU as *Acomys* sp. 1, Granjon and Duplantier [116] included it in *A. johannis* species complex. The distribution of *Cah3* in West Africa is overlapping with *Cah2*, *Cah4* and *Cah11*. The West African *A. johannis* species complex (paraphyletic in our study, grouping *Cah2*, *Cah3* and *Cah4*; see also [116]) requires taxonomic revision.

*(19) Cah4*
*Distribution:* Chad, Cameroon, Niger, Nigeria, Benin.
*Available name: A. johannis* Thomas, 1912.
*Type locality:* Bauchi Plateau Kabwir, North Nigeria.
*Karyotype:* 2n = 66–68, NF = 66–72 [116].
*Additional information:* This MOTU was included in previous phylogenetic studies as *A. johannis* [30, 31, 37, 43, 46]. Formerly it was included in *A. cahirinus* [38] or *A. cineraceus* [104]. Sicard and Tranier [63] provided a detailed report on the geographic distribution of three pelage colour phenotypes of *Acomys* occurring in Burkina Faso [117], assigned them to *A. johannis*, and contrasted their external, cranial, and dental morphology with *A. chudeaui* (= *Cah11*). Using *CYTB* sequences, Barome et al. [31] reported the specimens from Burkina Faso and Mali as *Acomys* sp. 1 (= *Cah3*) and *Acomys* sp. 2 (= *Cah2*) and specimens from Niger, Benin, Cameroon, and Niger as *A. johannis* (= *Cah4*), but their conspecificity has never been tested by the combination of multi-locus genetic and phenotypic data.

*(20) Cah5*
*Distribution:* Egypt (Sinai only), Arabian Peninsula, South Iran.
*Available name: Acomys dimidiatus* Cretzschmar, 1826.
*Type locality:* Sinai, Egypt.
*Karyotype:* 2n = 36–38, NF = 68–70 [110].
*Additional information: Acomys dimidiatus* is nearly indistinguishable from *A. cahirinus* with regard to external morphology, which resulted in great confusions relative to its classification [16]. With few exceptions (e.g., [34, 37, 38, 46, 118, 119]) *A. dimidiatus* usually has been listed in the synonymy of *A. cahirinus* [36, 37, 82, 120]. A morphological and cytogenetical review of *Acomys* species made by Denys et al. [51], who provided the external, skull and dental characteristics of all the type specimens available, validated *A. cahirinus* as distinct from *A. dimidiatus*. Frynta et al. [33] referred two major lineages in northern Africa and Middle East, which should represent *A. cahirinus* and *A. dimidiatus*, respectively. The type localities of these two species are very close each other (Cairo and Sinai) but their distributions seem to be separated by the Isthmus of Suez.

*(21) Cah6*
*Distribution:* Horn of Africa (S Eritrea, Djibouti, E Ethiopia and N Somalia).
*Available name: Acomys mullah* Thomas, 1904.
*Type locality:* Harar, Ethiopia.

Karyotype: Not known.
*Additional information:* Awaiting more detailed taxonomic revision, we assigned the name *A. mullah* to the lineage *Cah6* distributed in the margins of the Afar triangle in Ethiopia. This species is considered a member of the "*cahirinus-dimidiatus*" complex [16, 121], which is confirmed by our phylogenetic study. This species may co-occur with *A. louisae* (= *Dji*). It should be also taken in consideration that the type locality of *A. mullah* (Harar) is very close to the only known locality of *Ign1* and is separated from the Afar lowland by the Chercher Mts. Comparison of *Ign1* and *Cah6* with the type material of *A. mullah* (and *A. brockmani* considered as its synonym) is necessary.

*(22) Cah7*
*Distribution:* North-west Ethiopia (Mai Temen and Alatish NP).
*Available name:* None. Based on Lavrenchenko et al. [112], we use the name *Acomys* sp. B in Fig. 1.
*Type locality:* Not relevant.
*Karyotype:* 2n = 40, NF = 68 [112].
*Additional information:* Ivlev et al. [25] and Lavrenchenko et al. [112] called this lineage *Acomys* sp. B. It is sympatric with *Cah8*, but the two lineages significantly differ by karyotypes, physiological and behavioural traits [112] and thus might represent different biological species.

*(23) Cah8*
*Distribution:* Alatish NP, Ethiopia.
*Available name:* None. Based on Lavrenchenko et al. [112], we use the name *Acomys* sp. A in Fig. 1.
Type locality: Not relevant.
*Karyotype:* 2n = 52, NF = 68 [112].
*Additional information:* Ivlev et al. [25] and Lavrenchenko et al. [112] called this lineage *Acomys* sp. A. It is also very abundant in the neighbouring Dinder NP in Sudan (J. Bryja et al., unpublished data). The taxonomic revision of *Cah7*, *Cah8*, *Cah9* and *Cah10* is necessary and more intensive sampling in Sudan and northern Ethiopia would be very helpful.

*(24) Cah9*
*Distribution:* Egypt, Greece (Crete), Cyprus, Turkey, Libya, northern Chad.
*Available name:* *Acomys cahirinus* (É. Geoffroy Saint-Hilaire, 1803).
*Type locality:* Cairo, Egypt.
*Karyotype:* 2n = 36–42, NF = 68 [54, 110].
*Additional information:* The species was described from Cairo (Egypt). It seems very likely that it colonized eastern Mediterranean area during Antiquity. Weak genetic differences revealed also by our multilocus analysis support the view that *A. cahirinus* should be synonymized with *A. minous* Bate, 1906 from Crete, *A. cilicicus* Spitzenberg, 1978 from Turkey and *A. nesiotes* Bate, 1903 from Cyprus (see [16, 54] and references there). The relationships with *A. seurati* (a distinct taxon from rocky areas in southern Algeria, differing by karyotype and dental morphology; [51]) should be investigated by using genetic data from the Algerian material.

*(25) Cah10*
*Distribution:* Sheraro, Ethiopia.
*Available name:* None.
*Type locality:* Not relevant.
*Karyotype:* Not known.
*Additional information:* *Cah10* is known only from one locality in North Ethiopia. It is a sister MOTU either to *Cah9* or *Cah11* and its conspecificity with *A. cahirinus* and/or *A. chudeaui* should be tested.

*(26) Cah11*
*Distribution:* Niger, Mauritania, Mali, Chad.
*Available name:* *Acomys chudeaui* Kollman, 1911.
*Type locality:* Atar, SW of Biskra, Mauritania.
*Karyotype:* 2n = 40–46, NFa = 66 [116].
*Additional information:* This taxon has been previously listed as a synonym of *A. cahirinus*, but most recent works consider it as a distinct species [36, 46, 116]. Nicolas et al. [43] synonymized *A. airensis* and *A. chudeaui* and provided a detailed phylogeographic analysis of this taxon.

## Conclusions

Using multilocus genetic data, comprehensive geographic sampling and multiple phylogenetic approaches, we revealed that the spiny mice (*Acomys*) are composed of five main species groups: *subspinosus, spinosissimus, russatus, wilsoni* and *cahirinus*. Three of them (*spinosissimus*, *wilsoni* and *cahirinus*) clearly represent species complexes. We delimited 26 genetic lineages as potential *Acomys* species, and their taxonomic status should now be assessed by multidisciplinary investigations. The origin of the genus is dated to the late Miocene in savannas of eastern Africa, when the first vicariance between "southern" and "northern" groups was probably caused by the development of the coast-to-coast forest belt. The evolutionary history of the genus in Plio-Pleistocene was influenced by global climatic transitions as well as by local geomorphological features (e.g. deserts, mountain blocks and/or large water bodies) and is characterized by repeated cycles of diversifications, especially in eastern Africa, and repeated dispersal events mainly to the North and West. The spiny mice can be thus used as very suitable model for testing specific hypotheses of the role of historical factors on the formation of current biodiversity of seasonally dry environments of Afro-Arabia.

## Methods

### Sampling

The genetic dataset is based on 700 individuals of spiny mice. We produced original genetic data from 421 individuals collected at more than one hundred localities, and complemented them by 279 georeferenced mitochondrial sequences from GenBank. This material covers large part of the distribution of the genus as predicted by the IUCN [122] (see Fig. 2a). All individuals were DNA-barcoded at mitochondrial markers to get as precise distributional maps of genetic clades as possible, but part of sequences was removed as redundant from subsequent phylogenetic analyses (see Additional file 5). All fieldwork performed in the frame of this study complied with legal regulations in particular countries and sampling was in accordance with local legislation (see more details in Ethics approval section). Rodents were trapped in Sherman live traps (H.B. Sherman Traps Inc., Tallahassee, USA) and snap traps baited with a mixture of peanut butter, maize flour and dried fish. Mice caught in live traps were euthanized by cervical dislocation or an overdose of Isoflurane prior to dissection (Directive 2010/63/EU). When present, the spiny mice are generally the most abundant component of the small mammal communities and are not listed as endangered. Each individual was identified to the genus by the external features and the tissue sample (tail, toe, spleen, etc.) was stored in 96% ethanol until DNA extraction. GPS coordinates of each locality were recorded. For more details on particular specimens, localities and collectors, see Additional file 5.

### DNA extraction, amplification and sequencing

DNA from 96% ethanol-preserved tissue samples was extracted using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For phylogenetic analysis we selected four genetic markers; two mitochondrial fragments, cytochrome *b* (*CYTB*) and control region (*D-loop*) and two nuclear exons, Interphotoreceptor Binding Protein gen (*IRBP*) and Recombination activating gene 1 (*RAG1*). Individual markers were amplified by the polymerase chain reaction (PCR) using following combination of primers: L14723 and H15915 [123] for *CYTB*; 'primers 1–4' [124] for *D-loop*; IRBP217 and IRBP1531 [125] for *IRBP* and RAG1F1705 and RAG1R2951 [126] for *RAG1*. Each locus was amplified using a final concentration of 3 mM of $MgCl_2$ (for *IRBP* only 2 mM), 0.2 mM of each dNTP, 0.4 μM of each primer, 1 x Taq buffer (Thermo Fisher Scientific, Waltham, USA), 0.2 μl of Taq polymerase (5 U/μl, Thermo Fisher Scientific), 30 ng/μl of genomic DNA, and $ddH_2O$ to a total volume of 15 μl. PCR products were purified with Calf Intestine Alkaline Phosphatase and Exonuclease I (New England Biolabs, Ipswich, USA), and Sanger-sequenced in both directions using the BigDye® Terminator chemistry (Thermo Fisher Scientific) either at the Institute of Vertebrate Biology CAS on an 'Applied Biosystems® 3130xl Genetic Analyzer' or commercially through the GATC Biotech company (Konstanz, Germany). All corresponding sequences were deposited in GenBank under accession numbers MH044731-MH045045 (see Additional file 5).

### Phylogenetic analysis

The final dataset for phylogenetic analyses consisted of 373 unique sequences of *CYTB*, 71 sequences of *IRBP*, 59 sequences of *RAG1* and 96 sequences of *D-loop*. The remaining mitochondrial sequences (usually identical and/or shorter sequences and/or from the same or neighbouring localities) were unambiguously assigned to particular mtDNA lineages by preliminary phylogenetic analysis and they were removed as redundant (see Additional file 5). These data were used only to increase the precision by which we mapped the geographical distribution of phylogenetic clades. Nuclear exons were sequenced only in the representative subset of 102 individuals, covering the geographic distribution and mitochondrial diversity as much as possible (see Additional files 5 and 6). As outgroups we used four taxa from subfamily Deomyinae, to which *Acomys* belong (*Deomys ferrugineus*, *Lophuromys flavopunctatus*, *Lophuromys sikapusi* and *Uranomys ruddi*; see Additional file 5). Sequences were aligned in MUSCLE [127] and the concatenated dataset with total length 4005 bp was created in Mesquite. For all three protein-coding genes (*CYTB*, *IRBP*, *RAG1*), we used Mesquite 3.03 [128] to check the coding frame for possible errors or stop codons.

Phylogenetic reconstructions were conducted using maximum likelihood (ML) and Bayesian inference (BI). For both phylogenetic approaches were carried out partitioned analyses to improve phylogenetic accuracy [129]. The molecular dataset was divided into ten partitions: we used three partitions for each of the protein-coding genes, and one partition for the control region (*D-loop*). The best partitioning scheme and substitution models were determined with PartitionFinder v1 [130] using a greedy heuristic algorithm with 'linked branch lengths' option. The Bayesian information criterion (BIC) was used to compare partitioning schemes and substitution models following the recommendation of Ripplinger and Sullivan ([131]; Table 2).

Maximum likelihood analyses were performed using RAxML v8.2.8 [132] for separate gene trees (*CYTB* and *D-loop* not shown same topology as Fig. 1, *IRBP* Additional file 2, *RAG1* Additional file 3) as well as for concatenate matrix. Based on the BIC results in PartitionFinder we used seven partitions for concatenate matrix, five partitions with GTR + I + G model and two

**Table 2** The substitution models used in particular phylogenetic analyses. They were selected by PartitionFinder using BIC model selection, greedy search, linked branch length

| Partitions | | RAxML | MrBayes | BEAST |
|---|---|---|---|---|
| 1 | *CYTB*_pos1 | GTR + I + G | GTR + I + G | GTR + I + G |
| 2 | *CYTB*_pos2 | GTR + I + G | HKY + I + G | HKY + I + G |
| 3 | *CYTB*_pos3 | GTR + I + G | GTR + I + G | GTR + I + G |
| 4 | *IRBP*_pos1, *RAG1*_pos2 | GTR + G | HKY + I | TrN + I |
| 5 | *IRBP*_pos2, *RAG1*_pos3 | GTR + G | HKY + G | TrN + G |
| 6 | *IRBP*_pos3, *RAG1*_pos1 | GTR + I + G | HKY + I + G | HKY + I + G |
| 7 | *D-loop* | GTR + I + G | GTR + I + G | GTR + I + G |

with GTR + G substitution model (Table 2). The ML tree was obtained using heuristic searches with 100 random addition replicates and the clade support was then assessed using a non-parametric bootstrap procedure with 1000 replicates. Following Hillis and Bull [133], nodes supported by bootstrap values (BP) ≥ 70 were considered strongly supported.

Bayesian inference analyses were carried out using MrBayes v3.2.6 [134] with seven partitions (Table 2). Two independent runs with four MCMC (one cold and three incrementally heated) were conducted: they ran for 50 million generations, with trees sampled every 1000 generations. A conservative 25% burn-in was applied after checking for stability on the log-likelihood curves and the split-frequencies of the runs. Support of nodes for MrBayes analyses was provided by clade posterior probabilities (PP) as directly estimated from the majority-rule consensus topology. Following Erixon et al. [135], nodes supported by PP ≥ 0.95 were considered strongly supported.

### Estimates of species richness

For estimation of *Acomys* species richness we applied multiple species delimitation methods (as suggested by Carstens et al. [136]): (1) "by-eye" analysis of genetic structure (based primarily on *CYTB* barcodes) and geographical distribution of genetic lineages; (2) species discovery approach to assign individuals to putative groups based on the variability of *CYTB* sequences [137, 138]; (3) species delimitation based on multi-locus data and multispecies coalescent methods [139].

In the first simplest approach, we produced ML tree based only on *CYTB* sequences. We then compared the revealed clades with the species names used in previous studies (e.g. [31, 32, 41, 43, 45]), and the distribution of particular clades with positions of type localities of nominal species. By using this approach we newly identified several highly supported phylogenetic clades with parapatric distribution to previously analysed and named species, which might represent new species and are worth of further taxonomic studies. Genetic distances (within species and to the genetically nearest lineage) from BI tree were additionally also computed using the species delimitation package [140] implemented in Geneious v9.1.8 [141].

Second, we performed two analyses of species discovery based on diversity of *CYTB* marker. The Automatic Barcode Gap Discovery approach (ABGD; [137]) was used to identify barcode gap between intraspecific and interspecific genetic distances. An alternative Poisson Tree process (PTP) approach models intra- and interspecies processes by directly using the number of substitutions [138]. We used a recently improved algorithm based on PTP, the so-called multi-rate PTP (mPTP; [142]), which works better for phylogenies that have different rates of speciation-coalescence and allows to account for the different rates of branching events within each delimited species [142]. Both analyses (ABGD and mPTP) were performed using the ultrametric *CYTB* phylogeny produced by Bayesian method with strict clock in BEAST v2.4.7 [143].

The last used species delimitation approach, Species Tree And Classification Estimation, Yarely (STACEY; [139]), is a Bayesian method based on the multispecies coalescent model and estimates the probability of distinct species delimitation hypotheses given multilocus data. By utilizing multi-species coalescent theory and phylogenetic inference under a full probability Bayesian network, STACEY simultaneously estimates gene trees, the species tree and species delimitations under the assumption that all individuals that are affected by the same coalescent process, also belong to the same species/clade. We assumed conspecificity of individuals bearing mtDNA of the same lineage, identified by the first arbitrary approach described above. All but one lineages were represented at least by two individuals genotyped minimally at 3–4 markers (see Additional file 5). To relax the prior assumptions about species delimitation, we estimated a species tree using the birth-death-collapse model [144] as implemented in STACEY for BEAST 2 [145]. STACEY does not require guide tree, therefore errors resulting from a priori phylogenetic assumptions are avoided. Parameters and priors for the analysis were set according to the recommendations of STACEY manual [139]. Sequence alignments were imported into BEAUTI where they were assigned separate and unlinked substitution, clock and tree models. For mitochondrial markers (*CYTB* + *D-loop*) the ploidy was set of 0.5, for nuclear genes (*IRBP, RAG1*) 2.0. For the species tree prior the collapse height was set to $10^{-3}$. Three independent MCMC chains were run for $10^{-7}$ generations and log every 5000 generations. The burn-in was 25%, and the outputs from three runs were combined in LogCombiner 2.4.7 [146]. The similarity matrix was created using SpeciesDelimationAnalyser version 1.8.9 [147] with 25% burn-in and

collapse height of $10^{-3}$. Species tree was visualised as a cloudogram using DensiTree [148].

### Divergence dating

To calibrate a molecular clock, we compiled the set of usable fossils for the genus *Acomys* and its ancestors: (1) an extinct genus †*Preacomys* with three described species: †*P. griffini* Mein et al., 2004 and †*P. karsticus* Mein et al., 2004 from Harasib, Namibia (9 Ma; [83, 149]) and †*P. kikiae* Geraads, 2001 from Chorora, Ethiopia (8.5 Ma; [150, 151]). Because the position of these three fossils on phylogenetic tree is not unequivocally clear, we used a minimum age 8.5 Ma as a root for the genus *Acomys*. (2) *Acomys* from Lemudong'o locality in Kenya (6.08–6.12 Ma; [152, 153]) is the oldest *Acomys* and we considered it as the most recent common ancestor (MRCA) for taxa living currently in the northern part of eastern Africa (with the centre of their distribution in Somali-Masai savanna) and in Arabia, i.e. the clade encompassing *cahirinus* + *wilsoni* + *russatus* groups. (3) The oldest fossil of the *spinosissimus* group (sensu Verheyen et al. [45]) was discovered in Transvaal, South Africa (3 Ma; [154]), and we used it as MRCA for this group. Bayesian analyses of divergence dating were conducted on a species tree in *BEAST v2.4.7 [143]. The species were defined based on STACEY results. The mitochondrial (*CYTB* + *D-loop*) and nuclear genes (*IRBP*, *RAG1*) were imported in BEAUTI where they were assigned separate and unlinked substitution, clock and tree models. Bayesian analysis run with uncorrelated lognormal relaxed clocks [155], birth-death tree prior [156] and selected fossil constraints were defined by using lognormal statistical distributions (see Table 3 for more details). Two independent runs were carried out for $10^7$ generations with sampling every 1000 generations in BEAST. We discarded first 25% as burn-in and the resulting parameter and tree files were examined for convergence and effective sample sizes (> 200) in Tracer 1.6 [157]. The two runs were combined in LogCombiner and the species tree was visualized in TreeAnotator.

### Biogeographical reconstructions

The BioGeoBEARS approach [158] was used to reconstruct the ancestral distributions and diversification patterns. Six major biogeographic regions with *Acomys* occurrence were defined on the basis of Holt et al. [159] and Linder et al. [3]: South Africa region (S – South Africa), Zambezian region (Z – Zambezian region), Somali region (E – East Africa), Sudanian region (W – West Africa), Sahara region (N – North Africa) and the Arabian region (A – Arabian region). Dispersal rate between adjacent areas (S-Z, Z-E, E-W, E-N, N-A) was fixed to 1, whereas the dispersal of 0.5 (S-E, Z-W, Z-N, E-A) was defined for long distance dispersal (i.e. biogeographical areas separated by another region) or whenever a geographical barrier had to be crossed (e.g. multiple water bodies). Dispersal was disallowed between geographical areas separated by two or more areas (S-W, S-A, S-N, Z-N, Z-A, W-A). Biogeographic reconstruction relied on the Dispersal-Extinction Cladogenesis model (DEC) of range evolution [160]. DEC model estimates geographical range evolution using a phylogenetic tree with branch lengths scaled to time, geographical (habitat) areas for all tips, and an adjacent matrix of plausibly connected areas [85]. Because of concerns with its statistical validity [161] we did not use the +J model of Matzke [158] in our analyses.

### Species distribution modelling

Assuming phylogenetic niche conservatism [162] and generally similar ecological requirements for all taxa of spiny mice, we modelled the present and past distribution of suitable climatic conditions for the genus *Acomys* by the maximum entropy approach [163]. We used 282 presence records (unique localities, Additional file 5, Fig. 2) as the input data to train the model. We modelled the suitable conditions in the recent, but we also produced paleoclimatic projections for the last glacial maximum (21 ka; MIROC resolution 2.5 min [164]) and for the last interglacial (120–140 ka; resolution 30 s [165]) using 19 bioclimatic variables from the WorldClim database [166]. The background was restricted to whole Africa and Arabia (Figs. 2 and 3). The species distribution modelling (SDM) analysis was performed using MaxEnt v3.3.3 k [167]. We used 10 replicates and the importance of environmental variables was tested using jackknife option, and for the regularization multiplier we used the default value of 1. The SDM results were converted in a map using QGIS with a maximized sum threshold [168, 169].

**Table 3** List of fossils associated with the genus *Acomys* used in the divergence dating. The offset and mean represent the specification of lognormal priors used for the calibration of molecular clock. All fossil constrains were used as a crown

| Fossil | MRCA | Locality | Author | Age (Ma) | Offset | Mean |
|---|---|---|---|---|---|---|
| †*Preacomys kikiae* | *Acomys* | Chorora, Ethiopia | Geraads (2001, 2002); Suwa et al., (2015) | 8.5 | 8.5 | 1.0 |
| †*Acomys* I. Geoffroy | *cahirinus* + *wilsoni* + *russatus* | Lemudong'o, Kenya | Manthi (2007); Manthi and Ambrose (2007) | 6.08–6.12 | 6.08 | 1.0 |
| †*Acomys spinosissimus* Peters | *spinosissimus* | Transvaal, South Africa | Denys (1999) | 3 | 3 | 1.0 |

## Additional files

**Additional file 1:** Taxonomic classifications of the genus *Acomys*. Bold italic names represent species reported in particular lists, standard italics represent subspecies (in Ellerman [34]; Setzer [38]; Denys et al. [16]). (XLSX 41 kb)

**Additional file 2:** ML phylogenetic tree based on *IRBP* sequences (nexus file). (NEXUS 6 kb)

**Additional file 3:** ML phylogenetic tree based on *RAG1* sequences (nexus file). (NEXUS 5 kb)

**Additional file 4:** Maximum clade credibility tree from STACEY with PP support (nexus file). (NEXUS 24 kb)

**Additional file 5:** Complete list of individuals used in this study, with details on localities and genetic data. (XLSX 97 kb)

**Additional file 6:** Alignment of 369 ingroup and 4 outgroup concatenated sequences of *CYTB*, *IRBP*, *RAG1* and *D-loop*. (NEXUS 1470 kb)

### Abbreviations

2n: Diploid number of chromosomes; a.s.l.: Above sea level; ABGD: Automatic Barcode Gap Discovery; BI: Bayesian Inference; BIC: Bayesian information criterion; bp: Base pairs; BP: Bootstrap value; *CYTB*: Cytochrome b gene; DEC: Dispersal-Extinction Cladogenesis model; *D-loop*: Control region; EAM: Eastern Arc Mountains; EARS: The East African Rift System; GRV: The Great Rift Valley; HPD: Highest posterior density; iNHG: The intensification of Northern Hemisphere glaciation; *IRBP*: Interphotoreceptor Binding Protein gen; ka: Thousand years; LGM: Last Glacial Maximum; LIG: Last Interglacial; Ma: Million of years ago; MaxEnt: Maximum entropy; MCMC: Markov chain Monte Carlo; ML: Maximum likelihood; MOTU(s): Molecular operational taxonomic unit(s); mPTP: Multi-rate Poisson Tree process; MRCA: The most recent common ancestor; MSC: Messinian salinity crisis; Mt. (s): Mountain(s); mtDNA: Mitochondrial DNA; NF: Fundamental number of chromosome arms; NFa: Fundamental number of autosomal chromosome arms; NP: National park; PCR: The polymerase chain reaction; PP: Posterior probability; PTP: Poisson Tree process; *RAG1*: Recombination activating gene 1; SDM: Species distribution modelling; SRM: Southern Rift Mountains; STACEY: Species Tree And Classification Estimation, Yarely

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### Availability of data and materials

All corresponding sequences were deposited in GenBank under accession numbers MH044731-MH045045 (see Additional file 5). The final alignment of concatenated sequences used in phylogenetic analyses is in Additional file 6.

### Authors' contributions

DF, RS and JB conceived and designed the study; TA, DF, RS, LL, YM, JS, JV, JM, DM, JB collected important part of samples; TA and KP performed laboratory analysis; TA analysed data; TA, JB, KP drafted the first version of the manuscript. All authors made substantial contribution on acquisition of data, revised the draft, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

### Ethics approval and consent to participate

We are indebted to many local authorities for providing permits to carry out the research (including the ethics approval, when applicable), especially the Zambia Wildlife Authority (ZAWA), the National Directorate for Protected Areas (DINAC – Mozambique), the National Research Council and Forestry Department in Malawi, Sokoine University of Agriculture in Morogoro (Tanzania), the Kenyan Forest Service and the Kenyan Wildlife Service (Kenya), the Ethiopian Wildlife Conservation Authority (EWCA), Government of Ethiopia and the Oromia Forest and Wildlife Enterprise (OFWE) in Ethiopia. Furthermore, Josef Bryja is holder of the certificate of competency on Protection of Animals against Cruelty (reg. no. V/1/2005/05) and all manipulations with animals, i.e. when captured in life traps, followed the specific rules for working with alive animals as specified by the Central Commission for Animal Welfare of Ministry of Agriculture of the Czech Republic, and the general protocols were approved by the Committee for Animal Welfare of the Czech Academy of Sciences. Newly analysed material from other countries has been collected in last decades within other research projects and we are indebted to museum curators for allowing us to study the tissue samples in their care.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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### References

1. Mayaux P, Bartholomé E, Fritz S, Belward A. A new land-cover map of Africa for the year 2000. J Biogeogr. 2004;31:861–77.
2. Sayre R, Comer P, Hak J, Josse C, Bow J, Warner H, Larwanou M, Kelbessa E, Bekele T, Kehl H, Amena R, Andriamasimanana R, Ba T, Benson L, Boucher T, Brown M, Cress J, Dassering O, Friesen B, Gachathi F, Houcine S, Keita M, Khamala E, Marangu D, Mokua F, Morou B, Mucina L, Mugisha S, Mwavu E, Rutherford M, Sanou P, Syampungani S, Tomor B, Vall A, Weghe JV, Wangui E, Waruingi L. A new map of standardized terrestrial ecosystems of Africa. Washington DC: Association of American Geographers; 2013.

3. Linder HP, de Klerk HM, Born J, Burgess ND, Fjeldså J, Rahbek C. The partitioning of Africa: statistically defined biogeographical regions in sub-Saharan Africa. J Biogeogr. 2012;39:1189–205.
4. Maslin MA, Trauth MH. Plio-Pleistocene east African pulsed climate variability and its influence on early human evolution. In: Grine FE, Leakey RE, Fleagle JG, editors. The first humans - origins of the genus homo. New York: Springer Verlag; 2009. p. 151–8.
5. Kingston JD, Deino AL, Edgar RK, Hill A. Astronomically forced climate change in the Kenyan Rift Valley 2.7–2.55 Ma: implications for the evolution of early hominin ecosystems. J Hum Evol. 2007;53:487–503.
6. Maslin MA, Christensen B. Tectonics, orbital forcing, global climate change, and human evolution in Africa: introduction to the African paleoclimate special volume. J Hum Evol. 2007;53:443–64.
7. Pickford M. Uplift of the roof of Africa and its bearing on the evolution of mankind. Hum Evol. 1990;5:1–20.
8. Prömmel K, Cubasch U, Kaspar F. A regional climate model study of the impact of tectonic and orbital forcing on African precipitation and vegetation. Palaeogeogr Palaeoclimatol Palaeoecol. 2013;369:154–62.
9. Cerling TE, Harris JM, Macfadden BJ, Leakey MG, Quadek J, Eisenmann V, Ehleringer JR. Global vegetation change through the Miocene/Pliocene boundary. Nature. 1997;389:153–8.
10. Cerling TE, Ehleringer JR, Harris JM. Carbon dioxide starvation, the development of C4 ecosystems, and mammalian evolution. Philos Trans R Soc B Biol Sci. 1998;353:159–71.
11. Huang W, Takebayashi N, Qi Y, Hickerson MJ. MTML-msBayes: approximate Bayesian comparative phylogeographic inference from multiple taxa and multiple loci with rate heterogeneity. BMC Bioinf. 2011;12:1.
12. Le Houérou HN. Outline of the biological history of the Sahara. J Arid Environ. 1992;22:3–30.
13. Swezey CS. Cenozoic stratigraphy of the Sahara. Northern Africa J African Earth Sci. 2009;53:89121.
14. Brito JC, Godinho R, Martínez-Freiría F, Pleguezuelos JM, Rebelo H, Santos X, Vale CG, Velo-Antón G, Boratyński Z, Carvalho SB, Ferreira S, Gonçalves DV, Silva TL, Tarroso P, Campos JC, Leite JV, Nogueira J, Alvares F, Sillero N, Sow AS, Fahd S, Crochet P-A, Carranza S. Unravelling biodiversity, evolution and threats to conservation in the Sahara-Sahel. Biol Rev Camb Philos Soc. 2014; 89:215–31.
15. Gonçalves DV, Martínez-Freiría F, Crochet PA, Geniez P, Carranza S, Brito JC. The role of climatic cycles and trans-Saharan migration corridors in species diversification: biogeography of *Psammophis schokari* group in North Africa. Mol Phylogenet Evol. 2018;118:64–74.
16. Denys C, Taylor PJ, Aplin KP. Family Muridae. In: Wilson DE, Lacher Jr TE, Mittermeier RA, editors. Hadbook of the mammals of the world, volume 7: rodents II. Barcelona: Lynx Edicions; 2017.
17. Carere C, Casetti R, De Acetis L, Perretta G, Cirulli F, Alleva E. Behavioural and nociceptive response in male and female spiny mice (*Acomys cahirinus*) upon exposure to snake odour. Behav Process. 1999;47:1–10.
18. Frynta D, Fraňková M, Čížková B, Skarlandtová H, Galeštoková K, Průšová K, Šmilauer P, Šumbera R. Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*). Acta Theriol (Warsz). 2011;56:289–95.
19. Gutman R, Dayan T. Temporal partitioning: an experiment with two species of spiny mice. Ecol. 2005;86:164–73.
20. Haim A, Yedidia I, Haim D, Zisapel N. Photoperiodicity in daily rhythms of body temperature, food and energy intake of the golden spiny mouse (*Acomys russatus*). Isr J Zool. 1994;40:145–50.
21. Kronfeld-Schor N, Shargal E, Haim A, Dayan T, Zisapel N, Heldmaier G. Temporal partitioning among diurnally and nocturnally active desert spiny mice: energy and water turnover costs. J Therm Biol. 2001;26:139–42.
22. Seifert AW, Kiama SG, Seifert MG, Goheen JR, Palmer TM, Maden M. Skin shedding and tissue regeneration in African spiny mice (*Acomys*). Nature. 2012;489:561–5.
23. Ehrhardt N, Heldmaier G, Exner C. Adaptive mechanisms during food restriction in *Acomys russatus*: the use of torpor for desert survival. J Comp Physiol B. 2005;175:193–200.
24. Horowitz M, Borut A. The spiny mous (*Acomys cahirinus*) - a rodent prototype for studying plasma volume regulation during thermal dehydration. Isr J Zool. 1994;40:117–25.
25. Ivlev YF, Lavrenchenko LA, OF Chernova, Bekele A. Response to overheating in spiny mice (the genus *Acomys*) from arid regions in northwestern Ethiopia. Dokl Biol Sci. 2011;440:335–9.
26. Weissenberg S, Shkolnik A. Metabolic rate and water economy in the desert and Mediterranean populations of the common spiny mouse (*Acomys cahirinus*) in Israel. Isr J Zool. 1994;40:135–43.
27. Dieterlen F. Vergleichende Untersuchungen zur Ontogenese von Stachelmaus (*Acomys*) und Wanderratte (*Rattus norvegicus*). Beiträge zum Nesthocker-Nestflüchter-Prolem bei Negetieren. Z Säugetierkd. 1963;28:193–227.
28. Hadid Y, Pavlicek T, Beiles A, Ianovici R, Raz S, Nevo E. Sympatric incipient speciation of spiny mice *Acomys* at "Evolution Canyon," Israel. Proc Natl Acad Sci. 2014;111:1043–8.
29. Tučková V, Šumbera R, Čížková B. Alloparental behaviour in Sinai spiny mice *Acomys dimidiatus*: a case of misdirected parental care? Behav Ecol Sociobiol. 2016;70:437–47.
30. Barome PO, Monnerot M, Gautun JC. Intrageneric phylogeny of *Acomys* (Rodentia, Muridae) using mitochondrial gene cytochrome b. Mol Phylogenet Evol. 1998;9:560–6.
31. Barome PO, Monnerot M, Gautun JC. Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome *b* mitochondrial gene: implications on taxonomy and phylogeography. Mammalia. 2000;64:423–38.
32. Barome PO, Lymberakis P, Monnerot M, Gautun JC. Cytochrome b sequences reveal *Acomys minous* (Rodentia, Muridae) paraphyly and answer the question about the ancestral karyotype of *Acomys dimidiatus*. Mol Phylogenet Evol. 2001a;18:37–46.
33. Frynta D, Palupčiková K, Bellinvia E, Benda P, Skarlantová H, Schwarzová L, Modrý D. Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia : Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula. Zootaxa. 2010;2660:46–56.
34. Ellerman JR. The Families and Genera of Living Rodents. In: Family muridae, volume II. London: British Museum (Natural History); 1941.
35. Honacki JH, Kinman KE, Koeppl JW. Mammal species of the world: a taxonomic and geographic reference. Kansas: Allen Press Inc. and The Association of Systematics Collections; 1982.
36. Monadjem A, Taylor P, Denys C, Cotterill FPD. Rodents of sub-Saharan Africa. A biogeographic and taxonomic synthesis. Berlin, Boston: De Gruyter; 2015.
37. Musser GG, Carleton MD. Superfamily Muroidea. In: Wilson DE, DAM R, editors. Mammal Species of the World. A Taxonomic and Geographic Reference. Baltimore: The Johns Hopkins University Press; 2005. p. 894–1531.
38. Setzer HW. Genus *Acomys*. In: Meester J, Setzer HW, editors. The mammals of Africa: an identification manual. Washington: Smithsonian Institution Press; 1975. p. 1–2.
39. Chevret P, Denys C, Jaeger JJ, Michaux J, Catzeflis FM. Molecular evidence that the spiny mouse (*Acomys*) is more closely related to gerbils (Gerbillinae) than to true mice (Murinae). Proc Natl Acad Sci U S A. 1993;90: 3433–6.
40. Matthey R. Cytogenetique et taxonomie du genre *Acomys A. percivali* Dollman et *A. wilsoni* Thomas, especes D'Abyssinie. Mammalia. 1968;32:621–7.
41. Barome PO, Volobouev V, Monnerot M, Mfune JK, Chitaukali W, Gautun JC, Denys C. Phylogeny of *Acomys spinosissimus* (Rodentia, Muridae) from North Malawi and Tanzania: evidence from morphological and molecular analysis. Biol J Linn Soc. 2001b;73:321–40.
42. Mgode GF. Application of a multidisciplinary approach to the systematics of *Acomys* (Rodentia: Muridae) from northern Tanzania. PhD thesis: University of Pretoria, Zoology and Entomology; 2006. http://www.suaire.sua.ac.tz:8080/xmlui/bitstream/handle/123456789/1433/Mgode18.pdf?sequence=1&isAllowed=y. Accessed 8 Feb 2019.
43. Nicolas V, Granjon L, Duplantier JM, Cruaud C, Dobigny G. Phylogeography of spiny mice (genus *Acomys*, Rodentia: Muridae) from the south-western margin of the sahara with taxonomic implications. Biol J Linn Soc. 2009;98: 29–46.
44. Petružela J, Šumbera R, Aghová T, Bryjová A, Katakweba AS, Sabuni CA, Chitaukali WN, Bryja J. Spiny mice of the Zambezian bioregion–phylogeny, biogeography and ecological differentiation within the *Acomys spinosissimus* complex. Mamm Biol. 2018;91:79–90.
45. Verheyen W, Hulselmans J, Wendelen W, Leirs H, Corti M, Backeljau T, Verheyen E. Contribution to the systematics and zoogeography of the east-African *Acomys spinosissimus* Peters 1852 species complex and the description of two new species (Rodentia: Muridae). Zootaxa. 2011;3059:1–35.
46. Alhajeri BH, Hunt OJ, Steppan SJ. Molecular systematics of gerbils and deomyines (Rodentia: Gerbillinae, Deomyinae) and a test of desert adaptation in the tympanic bulla. J Zool Syst Evol Res. 2015;53:312–30.

47. Bryja J, Kostin D, Meheretu Y, Šumbera R, Bryjová A, Kasso M, Mikula O, Lavrenchenko LA. Reticulate Pleistocene evolution of Ethiopian rodent genus along remarkable altitudinal gradient. Mol Phylogenet Evol. 2018;118: 75–87.
48. Correa C, Vásquez D, Castro-Carrasco C, Zúñiga-Reinoso Á, Ortiz JC, Palma RE. Species delimitation in frogs from south American temperate forests: the case of *Eupsophus*, a taxonomically complex genus with high phenotypic variation. PLoS One. 2017;12:e0181026.
49. Sukumaran J, Knowles LL. Multispecies coalescent delimits structure, not species. Proc Natl Acad Sci U S A. 2017;114:1607–12.
50. Haughton CL, Gawriluk TR, Seifert AW. The biology and husbandry of the African spiny mouse (*Acomys cahirinus*) and the research uses of a laboratory Colony. J Am Assoc Lab Anim Sci. 2016;55:9–17.
51. Denys C, Gautun J-C, Tranier M, Volobouev V. Evolution of the genus *Acomys* (Rodentia, Muridae) from dental and chromosomal patterns. Isr J Zool. 1994;40:215–46.
52. Bray TC, Bennett NC, Mohammed OB, Alagaili AN. On the genetic diversity of spiny mice (genus *Acomys*) and gerbils (genus *Gerbillus*) in the Arabian peninsula. Zool Middle East. 2013;59:283–8.
53. Fernandes CA, Rohling EJ, Siddall M. Absence of post-Miocene Red Sea land bridges: biogeographic implications. J Biogeogr. 2006;33:961–6.
54. Giagia-Athanasopoulou EB, Rovatsos MTH, Mitsainas GP, Martimianakis S, Lymberakis P, Angelou LXD, Marchal JA, Sánchez A. New data on the evolution of the Cretan spiny mouse, *Acomys minous* (Rodentia: Murinae), shed light on the phylogenetic relationships in the cahirinus group. Biol J Linn Soc. 2011;102:498–509.
55. Brouat C, Tatard C, Bâ K, Cosson J-F, Dobigny G, Fichet-Calvet E, Granjon L, Lecompte E, Loiseau A, Mouline K, Piry S, Duplantier J-M. Phylogeography of the Guinea multimammate mouse (*Mastomys erythroleucus*): a case study for Sahelian species in West Africa. J Biogeogr. 2009;36:2237–50.
56. Dobigny G, Tatard C, Gauthier P, Ba K, Duplantier J, Granjon L, Kergoat GJ. Mitochondrial and Nuclear Genes-Based Phylogeography of *Arvicanthis niloticus* (Murinae) and Sub-Saharan Open Habitats Pleistocene History. PLoS One. 2013; doi.org/10.1371/journal.pone.0077815.
57. Granjon L, Colangelo P, Tatard C, Colyn M, Dobigny G, Nicolas V. Intrageneric relationships within *Gerbilliscus* (Rodentia, Muridae, Gerbillinae), with characterization of an additional west African species. Zootaxa. 2012; 25:1–25.
58. Kingdon J. The Kingdon field guide to African mammals. 2nd ed. London: Bloomsbury Publishing Plc; 2015.
59. Douady CJ, Catzeflis F, Raman J, Springer MS, Stanhope MJ. The Sahara as a vicariant agent, and the role of Miocene climatic events, in the diversification of the mammalian order Macroscelidea (elephant shrews). Proc Natl Acad Sci U S A. 2003;100:8325–30.
60. Franck P, Garnery L, Loiseau A, Oldroyd BP, Hepburn HR, Solignac M, Cornuet JM. Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. Heredity. 2001;86:420–30.
61. Guillaumet A, Crochet PA, Pons JM. Climate-driven diversification in two widespread Galerida larks. BMC Evol Biol. 2008;8:32.
62. Rosevear D. The rodents of West Africa. London: Trustees of the British Museum Natural History; 1969.
63. Sicard B, Tranier M. Caractères et répartition de trois phénotypes d'*Acomys* (Rodentia, Muridae) au Burkina Faso. Mammalia. 1996;60:53–68.
64. Diamond JM, Hamilton AC. The distribution of forest passerine birds and quaternary climatic change in tropical Africa. J Zool. 1980;191:379–402.
65. Kingdon J. Where have the colonists come from? A zoogeographical examination of some mammalian isolates in eastern Africa. Afr J Ecol. 1981; 19:115–24.
66. Jesus FF, Wilkins JF, Solferini VN, Wakeley J. Expected coalescence times and segregating sites in a model of glacial cycles. Genet Mol Res. 2006;5:466–74.
67. Colangelo P, Verheyen E, Leirs H, Tatard C, Denys C, Dobigny G, Duplantier JM, Brouat C, Granjon L, Lecompte E. A mitochondrial phylogeographic scenario for the most widespread African rodent, *Mastomys natalensis*. Biol J Linnean Soc. 2013;108:901–16.
68. Bryja J, Mikula O, Šumbera R, Meheretu Y, Aghová T, Lavrenchenko LA, Mazoch V, Oguge N, Mbau JS, Welegerima K, Amundala N, Colyn M, Leirs H, Verheyen E. Pan-African phylogeny of *Mus* (subgenus *Nannomys*) reveals one of the most successful mammal radiations in Africa. BMC Evol Biol. 2014;14:256–76.
69. Bryja J, Konvičková H, Bryjová A, Mikula O, Makundi R, Chitaukali WN, Šumbera R. Differentiation underground: range-wide multilocus genetic structure of the silvery mole-rat does not support current taxonomy based on mitochondrial sequences. Mamm Biol. 2018;93:82–92.
70. Mazoch V, Mikula O, Bryja J, Konvičková H, Russo IR, Verheyen E, Šumbera R. Phylogeography of a widespread sub-Saharan murid rodent *Aethomys chrysophilus*: the role of geographic barriers and paleoclimate in the Zambezian bioregion. Mammalia. 2018;82:373–87.
71. Mikula O, Šumbera R, Aghova T, Mbau JS, Katakweba AS, Sabuni CA, Bryja J. Evolutionary history and species diversity of African pouched mice (Rodentia: Nesomyidae: *Saccostomus*). Zool Scr. 2016;45:595–617.
72. Aghová T, Šumbera R, Piálek L, Mikula O, Mcdonough MM, Lavrenchenko LA, Meheretu Y, Mbau JS, Bryja J. Multilocus phylogeny of east African gerbils (Rodentia, *Gerbilliscus*) illuminates the history of the Somali-Masai savanna. J Biogeogr. 2017;44:2295–307.
73. Trauth MH, Maslin MA, Deino AL, Junginger A, Lesoloyia M, Odada EO, Olago DO, Olaka LA, Strecker MR, Tiedemann R. Human evolution in a variable environment: the amplifier lakes of eastern Africa. Quat Sci Rev. 2010;29:2981–8.
74. Bartáková V, Reichard M, Blažek R, Polačik M, Bryja J. Terrestrial fishes: rivers are barriers to gene flow in annual fishes from the African savanna. J Biogeogr. 2015;42:1832–44.
75. McDonough MM, Šumbera R, Mazoch V, Ferguson AW, Phillips CD, Bryja J. Multilocus phylogeography of a widespread savanna-woodland-adapted rodent reveals the influence of Pleistocene geomorphology and climate change in Africa's Zambezi region. Mol Ecol. 2015;24:5248–66.
76. Keller C, Roos C, Groeneveld LF, Fischer J, Zinner D. Introgressive hybridization in southern African baboons shapes patterns of mtDNA variation. Am J Phys Anthropol. 2010;142:125–36.
77. Cotterill FPD. A biogeographic review of tsessebe antelopes *Damaliscus lunatus* (Bovidae: *Alcelaphini*) in south-Central Africa. Durban Mus Novit. 2003;28:45–55.
78. Bryja J, Granjon L, Dobigny G, Patzenhauerová H, Konečný A, Duplantier J-M, Gauthier P, Colyn M, Dumez L, Lalis A, Nicolas V. Plio-Pleistocene history of west African Sudanian savanna and the phylogeography of the *Praomys daltoni* complex (Rodentia): the environment/geography/genetic interplay. Mol Ecol. 2010;19:4783–99.
79. Jacquet F, Denys C, Verheyen E, Bryja J, Hutterer R, Peterhans JCK, Stanley WT, Goodman SM, Couloux A, Colyn M, Nicolas V. Phylogeography and evolutionary history of the *Crocidura olivieri* complex (Mammalia, Soricomorpha): from a forest origin to broad ecological expansion across Africa. BMC Evol Biol. 2015;15:1–15.
80. Aghová T, Kimura Y, Bryja J, Dobigny G, Granjon L, Kergoat GJ. Fossils know it best: using a new set of fossil calibrations to improve the temporal phylogenetic framework of murid rodents (Rodentia: Muridae). Mol Phylogenet Evol. 2018;128:98–111.
81. Steppan SJ, Schenk JJ. Muroid rodent phylogenetics: 900-species tree reveals increasing diversification rates. PLoS One. 2017;12:e0183070. https://doi.org/10.1371/journal.pone.0183070.
82. Ellerman JR, TCS M-S. A Checklist of Palaeartic and Indian Mammals, 1758-1946. London: Trustees of the British museum (natural history); 1951.
83. Pickford M. Age of supergene ore bodies at berg Aukas and Harasib 3a, Namibia. Commun Geol Surv Namibia. 1992;8:147–50.
84. Geraads D. Rongeurs du Miocene superieur de Chorora (Ethiopie): Dendromuridae, Muridae et conclusions. Palaeovertebrata. 2001;30:89–109.
85. Bryja J, Šumbera R, Peterhans K, Julian C, Aghová T, Bryjová A, Mikula O, Nicolas V, Denys C, Verheyen E. Evolutionary history of the thicket rats (genus *Grammomys*) mirrors the evolution of African forests since late Miocene. J Biogeogr. 2017;44:182–94.
86. Couvreur TL, Chatrou LW, Sosef MS, Richardson JE. Molecular phylogenetics reveal multiple tertiary vicariance origins of the African rain forest trees. BMC Biol. 2008;6:54.
87. Lorenzen ED, Heller R, Siegismund HR. Comparative phylogeography of African savannah ungulates. Mol Ecol. 2012;21:3656–70.
88. Randi E, D'Huart JP, Lucchini V, Aman R. Evidence of two genetically deeply divergent species of warhthog, *Phacochoerus africanus* and *P. aethiopicus* (Artiodactyla: Suiformes) in eastern Africa. Mamm Biol. 2002;67:91–6.
89. Brown DM, Brenneman RA, Koepfli K-P, Pollinger JP, Milá B, Georgiadis NJ, Louis EE, Grether GF, Jacobs DK, Wayne RK. Extensive population genetic structure in the giraffe. BMC Biol. 2007;5:57.
90. Fennessy J, Bidon T, Reuss F, Kumar V, Elkan P, Nilsson MA, Vamberger M, Fritz U, Janke A. Multi-locus analyses reveal four giraffe species instead of one. Curr Biol. 2016;26:1–7.

91. Hsü KJ, Giovanoli F. Messinian event in the black sea. Palaeogeogr Palaeoclimatol Palaeoecol. 1979;29:75–93.
92. Hodell DA, Curtis JH, Sierro FJ, Raymo ME. Correlation of Late Miocene to early Pliocene sequences between the Mediterranean and North Atlantic. Paleoceanography. 2001;16:164–78.
93. Maslin MA, Haug GH, Sarnthein M, Tiedemann R, Erlenkeuser H, Stax R. 21. Northwest pacific site 882: The initiation of Northern Hemisphere Glaciation. In: Rea DK, Basov IA, Scholl DW, Allan JF, editors. Proceedings of the Ocean Drilling Program Vol 145. Scientific Results; 1995. p. 179–94.
94. Maslin MA, Li XS, Loutre MF, Berger A. The contribution of orbital forcing to the progressive intensification of northern hemisphere glaciation. Quat Sci Rev. 1998;17:411–26.
95. Ravelo AC, Andreasen DH, Lyle M, Lyle AO, Wara MW. Regional climate shifts caused by gradual global cooling in the Pliocene epoch. Nature. 2004; 429:263–70.
96. Berger WH, Jansen E. Mid-Pleistocene climate shift: the Nansen connection. Geophys Monogr Ser. 1994;85:295–311.
97. Potts R. Hominin evolution in setings of strong environmental variability. Quat Sci Rev. 2013;73:1–13.
98. Kebede F, Rosenbom S, Khalatbari L, Moehlman PD, Beja-Pereira A, Bekele A. Genetic diversity of the Ethiopian Grevy's zebra (*Equus grevyi*) populations that includes a unique population of the Alledeghi plain. Mitochondrial DNA A DNA Mapp Seq Anal. 2016;27:397–400.
99. Baker RJ, Bradley RD. Speciation in mammals and the genetic species concept. J Mammal. 2006;87:643–62.
100. Groves CP. The nature of species: a rejoinder to Zachos et al. Mamm Biol. 2013;78:7–9.
101. Dippenaar NJ, Rautenbach IL. Morphometrics and karyology of the southern African species of the genus *Acomys* I. Geoffroy saint-Hilaire, 1838 (Rodentia: Muridae). Ann Transvaal Museum. 1986;34:129–83.
102. Matthey R. Etudes de cytogenetique Sur des Murinae Africains appartenant aux genres *Arvicanthis, Acomys* et *Mastomys* (Rodentia). Mammalia. 1965;29: 228–49.
103. Janecek LL, Schlitter DA, Rautenbach IL. A genetic comparison of spiny mice, genus *Acomys*. Am Soc Mammal. 1991;72:542–52.
104. Musser G, Carleton M. Family Muridae. In: Wilson DE, Reeder DM, editors. Mammal species of the world. Washington DC: Smithsonian Institution Press; 1993.
105. Nevo E. Natural selection of body size differentiation in spiny mice, *Acomys*. Z Säugetierkd. 1989;54:81–99.
106. Corti M, Castiglia R, Colangelo P, Capanna E, Beolchini F, Bekele A, Oguge N, Makundi RH, Sichilima AM, Leirs H, Verheyen W, Vergagen R. Cytotaxonomy of rodent species from Ethiopia, Kenya, Tanzania and Zambia. Belgian J Zool. 2005;135:197–216.
107. Sokolov VE, Orlov VN, Baskevich MI, Bekele A, Mebrate A. A karyological study of the spiny mouse *Acomys* Geoffroy 1838 (Rodentia, Muridae) along the Ethiopian Rift Valley. Trop Zool. 1993;6:227–35.
108. Petter F. Eléments d'une révision des *Acomys* africains. Un sous-genre nouveau, *Peracomys* Petter and Roche, 1981 (Rongeurs, Muridés). Ann Mus R Afr Cent Sci Zool. 1983;237:109–19.
109. Volobouev VT, Gautun JC, Tranier M. Chromosome evolution in the genus *Acomys* (Rodentia, Muridae): chromosome banding analysis of *Acomys cahirinus*. Mammalia. 1996;60:217–22.
110. Volobouev V, Auffray JC, Debat V, Denys C, Gautun JC. Species delimitation in the *Acomys cahirinus – dimidiatus* complex (Rodentia, Muridae) inferred from chromosomal. Biol J Linn Soc. 2007;91:203–14.
111. Lavrenchenko LA, Kruskop SV, Bekele A, Belay G, Morozov PN, Ivlev YF, Warshavsky AA. Mammals of the Babille elephant sanctuary (eastern Ethiopia). Russ J Theriol. 2010;9:47–60.
112. Lavrenchenko LA, Nadjafova RS, Bulatova NS. Three new karyotypes extend a Robertsonian fan in Ethiopian spiny mice of the genus *Acomys* I. Geoffroy, 1838 (Mammalia, Rodentia). Comp Cytogenet. 2011;5:423–31.
113. Hollister N. East African mammals in the United States National Museum. II. Rodentia, Lagomorpha and Tubulidentata. Bull U S Natn Mus. 1919;99:1–184.
114. Kunze B, Dieterlen F, Traut W, Winking H. Karyotype relationship among four species of spiny mice (*Acomys*, Rodentia). Z Säugetierkd. 1999;64:220–9.
115. Happold DCD, editor. Volume 3: Rodents, Hares and Rabbits. Mammals of Africa. London: Bloomsbury Publishing; 2013. p. 789.
116. Granjon L, Duplantier JM. Les Rongeurs de l'Afrique sahélo-soudanienne. Marseille: Editions de l'IRD (Collection Faune et Flore tropicales); 2009.
117. Gautun JC, Sankhon I, Tranier M. Nouvelle contribution à la connaissance des rongeurs du massif guinéen des monts Nimba (Afrique occidentale). Systématique et aperçu quantitatif. Mammalia. 1986;50:205–17.
118. Morrison-Scott TCS. The identity of the *Acomys megalotis* (Lichtenstein), described from Arabia. Ann Mag Nat Hist. 1939;11:238–40.
119. Setzer HW. The spiny mice (*Acomys*) of Egypt. J Egypt Public Health Assoc. 1959;34:93–101.
120. Ellerman JR, Morrison-Scott TCS, Hayman RW. Southern African mammals. London: British Museum; 1953.
121. Yalden DW, Largen MJ, Kock D. Catalogue of the mammals of Ethiopia. Ital J Zool. 1976;1:1–118.
122. IUCN The IUCN red list of threatened species. Version 2017–3. 2017. http://www.iucnredlist.org. Accessed 17 Dec 2017.
123. Ducroz JF, Volobouev V, Granjon L. A molecular perspective on the systematics and evolution of the genus *Arvicanthis* (Rodentia, Muridae): inferences from complete cytochrome *b* gene sequences. Mol Phylogenet Evol. 1998;10:104–17.
124. Bellinvia E. A phylogenetic study of the genus *Apodemus* by sequencing the mitochondrial DNA control region. J Zool Syst Evol Res. 2004;42:289–97.
125. Stanhope MJ, Czelusniak J, Si JS, Nickerson J, Goodman M. A molecular perspective on mammalian evolution from the gene encoding interphotoreceptor retinoid binding protein, with convincing evidence for bat monophyly. Mol Phylogenet Evol. 1992;1:148–60.
126. Teeling EC, Scally M, Kao DJ, Romagnoli ML, Springer MS, Stanhope MJ. Molecular evidence regarding the origin of echolocation and flight in bats. Nature. 2000;403:188–92.
127. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–7.
128. Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis. Version 3.31. 2017. http://mesquiteproject.org.
129. Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL. Bayesian phylogenetic analysis of combined data. Syst Biol. 2004;53:47–67.
130. Lanfear R, Calcott B, Ho SY, Guindon S. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol. 2012;29:1695–701.
131. Ripplinger J, Sullivan J. Does choice in model selection affect maximum likelihood analysis? Syst Biol. 2008;57:76–85.
132. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3.
133. Hillis DM, Bull JJ. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol. 1993;42:182–92.
134. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–42.
135. Erixon P, Svennblad B, Britton T, Oxelman B. Reliability of Bayesian posterior probabilities and bootstrap frequencies in phylogenetics. Syst Biol. 2003;52: 665–73.
136. Carstens BC, Pelletier TA, Reid NM, Satler JD. How to fail at species delimitation. Mol Ecol. 2013;22:4369–83.
137. Puillandre N, Lambert A, Brouillet S, Achaz G. ABGD, automatic barcode gap discovery for primary species delimitation. Mol Ecol. 2012;21:1864–77.
138. Zhang J, Kapli P, Pavlidis P, Stamatakis A. A general species delimitation method with applications to phylogenetic placements. Bioinformatics. 2013;29:2869–76.
139. Jones G. Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. J Mat Biol. 2017;74:447–67.
140. Masters BC, Fan V, Ross HA. Species delimitation – a geneious plugin for the exploration of species boundaries. Mol Ecol Resourses. 2011;11:154–7.
141. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9.
142. Kapli P, Lutteropp S, Zhang J, Kobert K, Pavlidis P, Stamatakis A, Flouri T. Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. Bioinformatics. 2017;33:1630–8.
143. Drummond AJ, Bouckaert RR. Bayesian evolutionary analysis with BEAST. Cambridge: Cambridge University Press; 2015.
144. Jones G, Aydin Z, Oxelman B. DISSECT: an assignment-free Bayesian discovery method for species delimitation under the multispecies coalescent. Bioinformatics. 2015;31:991–8.

145. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, Suchard MA, Rambaut A, Drummond AJ. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput Biol. 2014;10:e1003537.
146. Rambaut A, Drummond AJ. LogCombiner v2.4.7; 2017.
147. Jones G. Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. J Math Biol. 2017;74:447–67.
148. Bouckaert RR. DensiTree: making sense of sets of phylogenetic trees. Bioinformatics. 2010;26:1372–3.
149. Senut B, Pickford M, Mein P, Conroy G, Van Couvering J. Discovery of 12 new Late Cainozoic fossiliferous sites in palaeokarsts of the Otavi Mountains, Namibia. Comptes rendus de l'Académie des sciences. Série 2, Mécanique, Physique, Chimie, Sciences de l'univers. Sci Terre. 1992;314:727–33.
150. Geraads D. Plio-pleistocene mammalian biostratigraphy of Atlantic Morocco. Quaternaire. 2002;13:43–53.
151. Suwa G, Beyene Y, Nakaya H, Bemor RL, Boisserie JR, Bibi F, Ambrose SH, Sano K, Katoh S, Asfaw B. Newly discovered cercopithecid, equid and other mammalian fossils from the Chorora formation, Ethiopia. Anthropol Sci. 2015;123:19–39.
152. Manthi FK. A preliminary review of the rodent fauna from Lemudong'o, southwestern Kenya, and its implication to the late Miocene paleoenvironments. Kirtlandia. 2007;56:92–105.
153. Deino AL, Ambrose SH. 40Ar/39Ar dating of the Lemudong'o late Miocene fossil assemblages, southern Kenya rift. Kirtlandia. 2007;56:65–71.
154. Denys C. Of mice and men. Evolution in East and South Africa during Plio-Pleistocene times. In: Bromage GT, Schrenk F, editors. African Biogeography, Climate Change and Human Evolution. New York: Oxford University Press; 1999. p. 226–52.
155. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. PLoS Biol. 2006;4:e88.
156. Gernhard T. The conditioned reconstructed process. J Theor Biol. 2008;253:769–78.
157. Rambaut A, Suchard MA, Xie D, Drummond AJ. Tracer v1.6; 2014.
158. Matzke NJ. Model selection in historical biogeography reveals that founder-event speciation is a crucial process in island clades. Syst Biol. 2014;63:951–70.
159. Holt BG, Lessard JP, Borregaard MK, Fritz SA, Araújo MB, Dimitrov D, Fabre PH, Graham CH, Graves GR, Jønsson KA, Nogués-Bravo D, Wang Z, Whittaker RJ, Fjeldså J, Rahbek C. An update of Wallace's zoogeographic regions of the world. Science. 2013;339:74–8.
160. Ree RH, Smith SA. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. Syst Biol. 2008;57:4–14.
161. Ree RH, Sanmartín I. Conceptual and statistical problems with the DEC+J model of founder-event speciation and its comparison with DEC via model selection. J Biogeogr. 2018;45:741–9.
162. Wiens JJ, Donoghue MJ. Historical biogeography, ecology, and species richness. Trends Ecol Evol. 2004;19:639–44.
163. Phillips SJ, Anderson RP, Schapire RE. Maximum entropy modeling of species geographic distributions. Ecol Model. 2006;190:231–59.
164. Braconnot P, Otto-Bliesner B, Harrison S, Joussaume S, Peterchmitt JY, Abe-Ouchi A, Crucifix M, Driesschaert E, Fichefet T, Hewitt CD, Kageyama M, Kitoh A, Laîné A, Loutre MF, Marti O, Merkel U, Ramstein G, Valdes P, Weber SL, Yu Y, Zhao Y. Results of PMIP2 coupled simulations of the mid-Holocene and last glacial maximum - part 1: experiments and large-scale features. Clim Past. 2007;3:261–77.
165. Otto-Bliesner BL. Simulating arctic climate warmth and icefield retreat in the last interglaciation. Science. 2006;311:1751–3.
166. Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. Very high resolution interpolated climate surfaces for global land areas. Int J Climatol. 2005;25:1965–78.
167. Phillips SJ, Dudík M, Schapire RE. A maximum entropy approach to species distribution modeling, Proceedings of the 21st International Conference on Machine Learning. Canada: Banff; 2004. p. 655–62.
168. Jiménez-Valverde A, Lobo JM. Threshold criteria for conversion of probability of species presence to either-or presence-absence. Acta Oecol. 2007;31:361–9.
169. Manel S, Williams HC, Ormerod SJ. Evaluating presence absence models in ecology; the need to count for prevalence. J Appied Ecol. 2001;38:921–31.

## *6.3* The first report of spiny mouse belonging to *Acomys ignitus* group in Somaliland: Phylogenetic affinities of a new distinct mitochondrial lineage

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**The first report of spiny mouse belonging to *Acomys ignitus* group in Somaliland: Phylogenetic affinities of a new distinct mitochondrial lineage**

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## Abstract

We report here a first record of spiny mouse of the genus *Acomys* belonging to *ignitus* clade from Somaliland. This clade is distributed in Southern Kenya, Northernmost Tanzania and Southernmost Ethiopia. Our finding extends the distribution of this clade considerably to the East. The locality is situated in a dry savanna with red sand semi-desert elements, which is quite different from the typical rocky habitats of the other *Acomys* species in the Horn of Africa.

Molecular phylogenetic analyses placed our specimen to the *cahirinus* group of the genus *Acomys*. It represents a well-supported new lineage within *ignitus* clade that we called *Ign5*. Although the inner relationships have remained resolved only partially, it is clearly more related to *A. ignitus* than to *A. kempi*. Besides phylogenetic affinities, we briefly discuss basic morphology and habitat requirements.

Keywords: Acomys; ignitus; Horn of Africa, phylogeny, Somaliland, Somalia-Masai bushland

## Introduction

Taxonomy of spiny mice (*Acomys* Geoffroy Saint-Hilaire, 1838) within the *ignitus* clade has been the subject of many contradictory revisions during the last century (Dollman 1914; Hollister 1919; Ellerman 1941; Matthey 1965). Fiery spiny mouse (*A. ignitus* Dollman, 1910) was recognized as a valid species by Hollister (1919) and lately confirmed by Ellerman (1941). Chromosomal (Matthey 1965), electrophoretic (Janecek et al. 1991) and cytochrome c (Barome et al. 2000) studies revealed similar results. Janecek et al. (1991) regard *A. ignitus* as closely related to *A. cahirinus*. Kemp's spiny mouse (*A. kempi* Dollman, 1911) was traditionally considered as a subspecies of *A. ignitus* (Dollman 1914; Hollister 1919; Ellerman 1941) but was later proved as a valid species according to the analyses of electrophoretic data by Janecek et al. (1991). Later, Lavrenchenko et al. (2010) described genetically and cytogenetically divergent lineage of *Acomys* sp. C in Babile Elephant Sanctuary in Eastern Ethiopia. This possibly new species was later identified as *Ign1* (Aghová et al. 2019) and placed as a sister taxon of *A. kempi* (*Ign4* in Aghová et al. 2019). Moreover, in South-Eastern Ethiopia was firstly described new lineage called *Ign2*, which cluster with *A. ignitus* (*Ign3* in Aghová et al. 2019). To conclude, the most recent multi-locus phylogeny of spiny mice based on three genetic markers suggest delimitation of *ignitus* clade to four molecular operational taxonomic units (*Ign1, Ign2, Ign3 and Ign4*), which potentially corresponds to separate species and support the inclusion of this clade inside the *cahirinus* group (Aghová et al. 2019).

The distribution of *A. ignitus* is in Southern Kenya and Northernmost Tanzania, while *A. kempi* is common in Kenya and Southernmost Ethiopia. The published molecular samples of *ignitus* were from Ethiopia and Kenya (Aghová et al. 2019) but are completely missing from the rest of the Horn of Africa (HOA). *Ign1* with the northernmost known locality of *ignitus* forms the northern boundary of its distribution. Lavrenchenko et al. (2010) mentioned that this spiny mouse was very common and abundant in Babile. *A. kempi* and *A. ignitus* were mentioned to be distributed in South Somalia (Petter 1983; Happold 2013) but the proper localities remained unknown. These findings suggest that the distribution of *ignitus* clade can be larger than originally expected. Nevertheless, the region of HOA (especially Ogaden, Somalia and Somaliland) are not well studied due to the political instability. The new samples from this biodiversity hotspot (Brook et al. 2001; Burges et al. 2004) will be worth of interest.

In this work, we report the first record of *ignitus* from South of Somaliland - an independent state internationally recognized as an autonomous region of Somalia. We describe

morphology and ecology of this specimen and the phylogenetic affinity of this specimen to the rest of the *ignitus* clade.

## Material and Methods

### Sampling

The survey was carried out in Somaliland. The reported specimen was caught near the watering-place close to Shanshacade village (Fig. 1). The mouse was trapped in snap trap baited with peanut butter. The specimen was identified to the genus by the external characters, measured with digital calliper to the nearest 0.01 mm, weighed by digital balance to the nearest 0.01 g, photographed and a small piece of tissue sample (spleen) was collected and stored in 96% ethanol until DNA extraction. The specimen is deposited at zoological collection of Charles University (CUP/MAMM/SOMALILAND/170). GPS coordinates of the locality were recorded. We measured five standard characters including head and body length (HB), tail length (T), hindfoot from the 'ankle bone' to the tip of the longest digit without including the claw (HF), length of external (outer) ear measured from tip of ear to the posterior point of the ear conch (E) and body weight (W). Skulls were purified and magnified under an Olympus SZX 12 stereomicroscope and the detailed photos of the skull, lower jaw, upper and lower molars were taken with an Olympus DP70 camera. Moreover, we visualised lower jaw by μCT (Bruker SkyScan 1275 micro-CT) and processed videos in CT Vox software (version 3.1.1 r1191 Brucker). We employed QGIS to prepare a map of localities (QGIS Development Team 2019).

All fieldwork in this study complied with legal Somaliland regulations and sampling was in accordance with local legislation (export permit Ref. MOERD/M/I/251/2017).

### DNA extraction, amplification and sequencing

Genomic DNA was extracted from ethanol-preserved tissue samples using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. We amplified and sequenced two mitochondrial fragments, cytochrome b (CYTB) and control region (D-*loop*) and one nuclear exon Interphotoreceptor Binding Protein (IRBP). For amplification and sequencing methods see Methods in Aghová et al. (2019). New sequences were deposited in GenBank under accession numbers MN547495 (CYTB), MN547497 (D-loop), and MN547496 (IRBP).

## Phylogenetic analysis

Sequences were aligned and manually checked using Chromas Lite 2.01 (http://www.technelysium.com.au/chromas_lite.html), BioEdit (Hall 1999) and Clustal X 1.81 (Thompson et al. 1997). The final dataset for phylogenetic analyses contains sequences of CYTB, D-loop and IRBP with total length 2665 bp (for the list of specimens see Table 1). As outgroups were used clades (KE734, K4_210, SIN1, IRA2, ABC-006, CAIR, CHAD, E12-S37, VV1998-087, ETH0548, ETH0610, ET168, ETH0033, 601476, 602643, LAV2211, LAV2229, ABC-008, RUS2, ETH0323, KE697, KE030, KE042, ETH1057, KE628) from Aghová et al. (2019). Bayesian inference analysis was performed using MrBayes v3.2.6 (Ronquist et al. 2012) and the multiple alignment was selected into seven partitions (see Aghová et al. 2019). Two independent runs with four MCMC were conducted. It ran for 40 million generations, with trees sampled every 1000 generations. A conservative 25% burn-in was applied after checking for stability on the log-likelihood curves and the split-frequencies of the runs. Support of nodes for MrBayes analysis was provided by clade posterior probabilities as directly estimated from the majority-rule consensus topology.

Maximum likelihood (ML) analysis was performed using RAxML v8.2.8 (Stamatakis 2014) with the GTR + G model substitution model. The ML tree was obtained using heuristic searches with 100 random addition replicates and the clade support was then assessed using a non-parametric bootstrap procedure with 1000 replicates.

Maximum parsimony (MP) analysis was performed using Mega X (Kumar et al. 2018). We conducted heuristic search analyses with 1000 random replicates of taxa additions using tree-bisection and reconnection (TBR) branch swapping. The branch support was evaluated using 1000 bootstrap pseudo replicates (Felsenstein 1985). All characters were equally weighted and unordered.

# Results

The specimen of spiny-mouse of the genus *Acomys* was caught during the field survey in Somaliland near the watering-place close to Shanshacade village (8.658 latitude and 45.956 longitude, Fig. 1). The habitat was dry savanna with red sand semi-desert elements (Fig. 2a). It became the easternmost specimen belonging to *ignitus* clade (see below) ever reported. Thus, this finding extends the distribution range of *ignitus* clade further East.

The specimen was an adult female with HB = 99.27 mm, T = 97.40 mm, HF = 15.74 mm, E = 15.71 mm and W = 25.92 g. The dorsal pelage was spiny from shoulders to base of the tail. The colour of dorsal pelage was greyish brown with darker spines towards the tail. Ventral pelage was pure white and soft (Fig. 2b). Uterus duplex contained one placental scar on the left and one embryo on the right horn of the uterus. We cannot conclude any species-specific morphological characters due to low sample size. Nevertheless, we include detailed photos of lower jaw and molars for further examination (Fig. 3, SI1,2).

Both Bayesian inference (BI) and Maximum Likelihood (ML) analyses of concatenated multi-locus data provided similar phylogenetic relationships and supports (see Fig. 4 for BA tree). The molecular characterization of the specimen from Shanshacade revealed that it is a member of *ignitus* clade ($BA_{PP}$ = 1, $ML_{bootstrap}$ =100) belonging to the *cahirinus* group of the genus *Acomys*. The specimen represents a new lineage within *ignitus* clade that we hereafter call *Ign5*. Thus, the *ignitus* clade is currently composed of five deep branches. It further splits into two well-supported sister clades ($BA_{PP}$ = 1, $ML_{bootstrap}$ =100). There is a distinct clade consisting of *Ign5*, *Ign2* and *Ign3* (= *A. ignitus* s.str.) with unresolved inner relationships. The second clade includes both remaining MOTU's, *Ign1* and *Ign4 (= A. kempi).* Divergences among MOTU's of the *ignitus* clade are deep, P-distances range from 6.1 to 7.8% (Table 2).

## Discussion

Spiny mice of the genus *Acomys* belonging to the *ignitus* clade of *cahirinus* group are rodents distributed in Somalia-Masai Bushland biotic zone of east Africa. Nevertheless, the area of Somalia located in the Horn of Africa is not well studied. In basic monographs devoted to the systematics of rodents in Africa (Happold 2013; Monadjem et al. 2015), there is just scarce information (lacking specific coordinates) about the distribution of *A. ignitus* and *A. kempi* in Somalia. In this paper, we report the first record of *ignitus* in Somaliland with proper GPS locality, morphological and molecular characterization. Our finding extends the expected boundary of *ignitus* clade and is the easternmost locality ever reported.

Our results revealed that the *ignitus* clade occurs not only in eastern part of Ethiopia, northernmost Tanzania and southern Kenya, but the distribution covers also Somaliland (Fig. 1). It is possible that the *ignitus* clade is more specious and cover the whole Horn of Africa, which is famous for its high level of endemism (Agnelli et al. 1990; Gippoliti 2006; Varshavsky et al. 2007; Lewin et al. 2016).

Phylogenetic analysis revealed clear evidence for the existence of new *ignitus* lineage (*Ign5*), which belongs close to the *A. ignitus* (*Ign3*) and MOTU *Ign2*. The cluster of these three lineages (*Ign2, Ign3 and Ign5*) is well supported, nevertheless, the inner relationships remained unresolved. Our analysis supported *A. kempi* (*Ign4*) and *Ign1* as sister taxa representing a sister clade to the group of *Ign2, Ign3 and Ign5.* Genetic distances calculated from BI phylogenetic tree by the species delimitation algorithm is rather high, which suggest that our five MOTU's are potentially separate species. Nevertheless, more samples are needed for final consideration of these lineages as distinct species.

Generalized morphology of the genus *Acomys* encourages to use molecular methods for taxonomic identification (Barome et al. 1998, 2000, 2001a, 2001b; Nicolas et al. 2009; Verheyen et al. 2011; Alhajeri et al. 2015; Petružela et al. 2018), even though morphological characters (Ellerman 1941; Chevret et al. 1993) and chromosomes (Matthey 1968; Setzer 1975) are also employed. Utilization of morphological characters can distinguish across consecutive groups of spiny mice (Petter and Roche 1981; Petter 1983; Denys et al. 1994), nevertheless, the discrimination inside the lineages is much less reliable. First morphological characters delimitating *A. ignitus* from other spiny mice are from Petter (1983), who studied teeth and skull morphology. The teeth morphology of this species is characterized by: "the existence of a crest between t4 and t8, by very longitudinal t1 and t4, as well as by differentiated t3 and t6. The t8 is well separated from t9 and is more anterior." (Denys et al. 1994). Later Janecek et al. (1994) confirmed that this species has a long and narrow skull with rounded rather than V-shaped fronto-parietal suture. Unfortunately, the skull of our specimen was slightly damaged during trapping and several upper molars were missing. We can confirm rounded shaped fronto-parietal suture (Fig. 3a). Nevertheless, the comparison of molars is problematic due to low sample size. Moreover, the detailed molar description of separate *ignitus* lineages is missing. The tail of our specimen is slightly shorter than head and body length, which is consistent with Denys et al. 1994. More specimens of *Ign5* are needed for proper morphological characterization. Thus, we only report the body size measurements and the photos of whole body and skull elements.

The habitat of *cahirinus* group is reported to be rocky dry savanna and semi-desert. Our specimen was caught near the watering-place close to Shanshacade village. The habitat was more desert-like with predominant red sand and shrubs (Fig. 2a). This type of habitat is completely different from other Somaliland localities where spiny mice (*Acomys louisae* and *Acomys mullah*, Frynta et al. in prep.) were common.

In conclusion, we report the first record of *Acomys* belonging to *ignitus* clade from the territory of Somaliland. This finding extends the east border of *ignitus* distribution considerably. We refer this specimen as MOTU *Ign5.* The multi-locus phylogeny classifies this new lineage into a group including also *Ign2* and *Ign3*. Thus, it is more related to *A. ignitus* (= *Ign3*) rather than to *A. kempi* (= *Ign4*) and *Ign1*.

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A.I.A., wrote the paper K.P., D.F., P.F., all co-authors approved the final version of MS.

## References


Aghová T, Palupčíková K, Šumbera R, Frynta D, Lavrenchenko LA, Meheretu Y, Sádlová J, Votýpka J, Mbau JS, Modrý D, Bryja J. 2019. Multiple radiations of spiny mice (Rodentia: *Acomys*) in dry open habitats of Afro-Arabia: evidence from a multi-locus phylogeny. *BMC Evolutionary Biology* 19:1-22.

Agnelli P, Azzaroli ML, Simonetta AM. 1990. Some remarks on the mammals of Somalia. *Biogeographia–The Journal of Integrative Biogeography* 14:499-513.

Alhajeri BH, Hunt OJ, Steppan SJ. 2015. Molecular systematics of gerbils and deomyines (Rodentia: Gerbillinae, Deomyinae) and a test of desert adaptation in the tympanic bulla. *Journal of Zoological Systematics and Evolutionary Research* 53:312-330.

Barome PO, Monnerot M, Gautun JC. 1998. Intrageneric Phylogeny of *Acomys* (Rodentia, Muridae) Using Mitochondrial Gene Cytochrome b. *Molecular Phylogenetics and Evolution* 9:560-566.

Barome PO, Monnerot M, Gautun JC. 2000. Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. *Mammalia* 64:423-438.

Barome PO, Lymberakis P, Monnerot M, Gautun JC. 2001a. Cytochrome b sequences reveal *Acomys minous* (Rodentia, Muridae) paraphyly and answer the question about the ancestral karyotype of *Acomys dimidiatus*. *Molecular phylogenetics and evolution* 18:37-46.

Barome PO, Volobouev V, Monnerot M, Mfune JK, Chitaukali W, Gautun JC, Denys C. 2001b. Phylogeny of *Acomys spinosissimus* (Rodentia, Muridae) from north Malawi and Tanzania: evidence from morphological and molecular analysis. *Biological Journal of the Linnean Society* 73:321-340.



Barome PO, Monnerot M, Gautun JC. 2000. Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. *Mammalia* 64:423-438.

Brooks T, Balmford A, Burgess N, Fjeldså JON, Hansen LA, Moore J, Rahbek C, Williams P. 2001. Toward a Blueprint for Conservation in Africa: A new database on the distribution of vertebrate species in a tropical continent allows new insights into priorities for conservation across Africa. *AIBS Bulletin* 51:613–624.

Burgess N, Hales JA, Underwood E, Dinerstein E, Olson D, Itoua I, Schipper J, Ricketts T, Newman K. 2004. *Terrestrial ecoregions of Africa and Madagascar: a conservation assessment*. Washington: Island Press.

Chevret P, Denys C, Jaeger JJ, Michaux J, Catzeflis FM. 1993. Molecular evidence that the spiny mouse (*Acomys*) is more closely related to gerbils (Gerbillinae) than to true mice (Murinae). *Proceedings of National Academy of Science USA* 90: 3433–3436.

Denys C, Gautun JC, Tranie M, Volobouev V. 1994. Evolution of the genus *Acomys* (Rodentia, Muridae) from dental and chromosomal patterns. *Israel Journal of Ecology and Evolution* 40:215-246.

Dollman G. 1914. On the spiny mice of British East Africa, with a description of a new species from Magadi. *Annals and Magazine of Natural History* 14:485-488.

Ellerman JR. 1941. The Families and Genera of Living Rodents. Family muridae, volume II. In: Hayman RW, Holt GWC (eds), *The Families and Genera of Living Rodents. With a List of Named Forms (1758-1936)*. London: British Museum (Natural History).

Felsenstein J. 1985. Confidence limits on phylogeny: an approach using the bootstrap. *Evolution* 39:783–789.

Gippoliti S. 2006. Zammarano's monkey *Cercopithecus mitis zammaranoi* de Beaux 1923: The forgotten monkey of Somalia. *African Primates* 6:26–32.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41:95–98.

Happold DC. 2013. *Mammals of Africa: Rodents, Hares and Rabbits*. Bloomsbury.

Hollister N. 1919. East African Mammals in the United States National Museum. Part 2, Rodentia, Lagomorpha, and Tubulidentata. *Bulletin of the United States National Museum* 99:1-184.

Janecek LL, Schlitter DA, Rautenbach IL. 1991. A genic comparison of spiny mice, genus *Acomys*. *Journal of mammalogy* 72:542-552.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution* 35:1547-1549.

Lavrenchenko LA, Kruskop SV, Bekele A, Belay G, Morozov PN, Ivlev YF, Warshavsky AA. 2010. Mammals of the Babille elephant sanctuary (eastern Ethiopia). *Russian Journal of Theriology* 9:47-60.

Lewin A, Feldman A, Bauer AM, Belmaker J, Broadley DG, Chirio L, Itescu I, LeBreton M, Maza E, Meirte D, Nagy ZT, Novosolov M, Roll U, Tallowin O, Traep JF, Vidan E, Meiri S. 2016. Patterns of species richness, endemism and environmental gradients of African reptiles. *Journal of Biogeography* 43:2380-2390.

Matthey R. 1965. Etudes de cytogènètique sur des Murinae Africains appartenant aux genres *Arvicanthis, Praomys, Acomys* et *Mastomys* (Rodentia). *Mammalia* 29:228-249.

Matthey R. 1968. Cytogénétique et taxonomie du genre *Acomys A. percivali* Dollman et *A. wilsoni* Thomas, espéces d'Abyssinie. *Mammalia* 32:621-627.

Monadjem A, Taylor P, Denys C, Cotterill FPD. 2015. *Rodents of sub-Saharan Africa. A biogeographic and taxonomic synthesis*. Berlin, Boston: De Gruyter.

Nicolas V, Granjon L, Duplantier JM, Cruaud C, Dobigny G. 2009. Phylogeography of spiny mice (genus *Acomys*, Rodentia: Muridae) from the south-western margin of the Sahara with taxonomic implications. *Biological Journal of the Linnean Society* 98:29-46.

Petružela J, Šumbera R, Aghová T, Bryjová A, Katakweba AS, Sabuni CA, Chitaukali WN, Bryja J. 2018. Spiny mice of the Zambezian bioregion–phylogeny, biogeography and ecological differentiation within the Acomys *spinosissimus* complex. *Mammalian Biology* 91:79-90.

Petter F. 1983. Eléments d'une revision des *Acomys* africains. Un sous-genre nouveau, *Peracomys* Petter et Roche 1981. *Annales du Musée Royal de l'Afrique Centrale, Sciences Zoologiques* 237:109-119.

Petter F, Roche J. 1981. Remarques préliminaires sur la systematique des *Acomys* (Rongeurs, Muridae). *Peracomys*, sous-genre nouveau. *Mammalia* 45:381-383.

QGIS Development Team 2019. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org.

Setzer HW. 1975. Genus *Acomys*. In: Meester J, Setzer HW (eds), *The mammals of Africa: an identification manual*. Washington: Smithsonian Institution Press. pp 1–2.

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology* 61:539-542.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312-1313.

Thompson DJ, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tool. *Nucleic Acids Research* 24:4876–4882.

Varshavsky AA, Karimova TY, Neronov VM. 2007. Specific features of the rodent fauna formation in Somali Peninsula. *Zoologichesky Zhurnal* 86:1498–1506

Verheyen W, Hulselmans J, Wendelen W, Leirs H, Corti M, Backeljau T, Verheyen E. 2011. Contribution to the systematics and zoogeography of the east-African *Acomys spinosissimus* Peters 1852 species complex and the description of two new species (Rodentia: Muridae). *Zootaxa* 3059:1–35.

**Table 1** List of specimens included in the molecular analysis.

| ID | Species | Lineage | Country | Locality | Latitude | Longitude | Publication |
|---|---|---|---|---|---|---|---|
| ETH0054 | sp. C | Ign1 | Ethiopia | Babile Elephant Sanctuary | 9.12 | 42.257 | Aghová et al. (2019) |
| ETH0055 | sp. C | Ign1 | Ethiopia | Babile Elephant Sanctuary | 9.12 | 42.257 | Aghová et al. (2019) |
| ETH1020 | sp. Ign2 | Ign2 | Ethiopia | Sof Omar caves | 6.906 | 40.849 | Aghová et al. (2019) |
| ETH1058 | sp. Ign2 | Ign2 | Ethiopia | Imi | 6.431 | 42.132 | Aghová et al. (2019) |
| KE519 | ignitus | Ign3 | Kenya | Tsavo West NP | -2.747 | 38.133 | Aghová et al. (2019) |
| KE625 | ignitus | Ign3 | Kenya | Gede | -3.309 | 40.018 | Aghová et al. (2019) |
| ETH0332 | kempi | Ign4 | Ethiopia | Turmi | 4.933 | 36.569 | Aghová et al. (2019) |
| KE824 | kempi | Ign4 | Kenya | Marigat, Egerton University Field Station | 0.489 | 35.921 | Aghová et al. (2019) |
| 170 | | Ign5 | Somaliland | Shanshacade | 8.658 | 45.956 | this study |

**Table 2** Genetic distances calculated from multiple alignments in Geneious. Interspecific distances from the nearest lineage in percents (%).

| Lineage | Nearest lineage | Inter Dist |
|---|---|---|
| 170 | Ign3 | 6.1 |
| 170 | Ign2 | 6.9 |
| 170 | Ign4 | 7.4 |
| 170 | Ign1 | 7.8 |

**Figure 1.** Map of Somaliland and adjoining parts of Ethiopia and Somalia forming the Horn of Africa with the locality of *Ign5* (star) extending the border of *ignitus* clade considerably to the East. This specimen was caught in close proximity of Shanshacade village. Remaining symbols represent previously published localities of spiny mice belonging to *ignitus* clade.

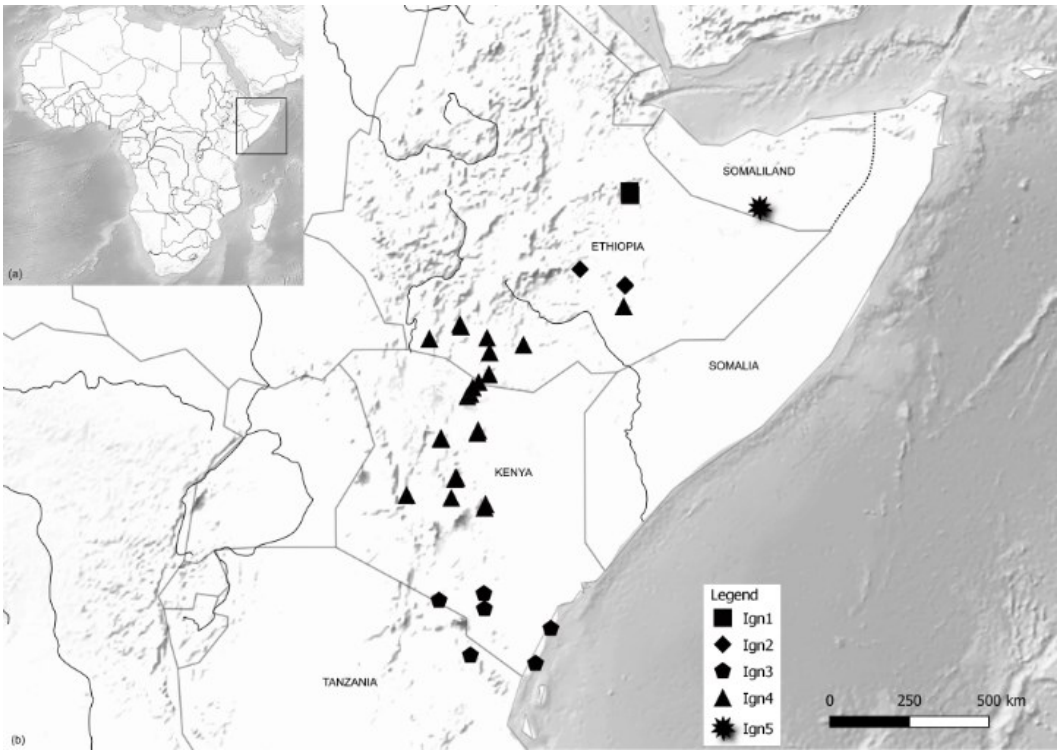


**Figure 2.** Semi-desert habitat in Shanshacade (Somaliland), where the specimen *Ign5* was caught (a); general body habitus (b).

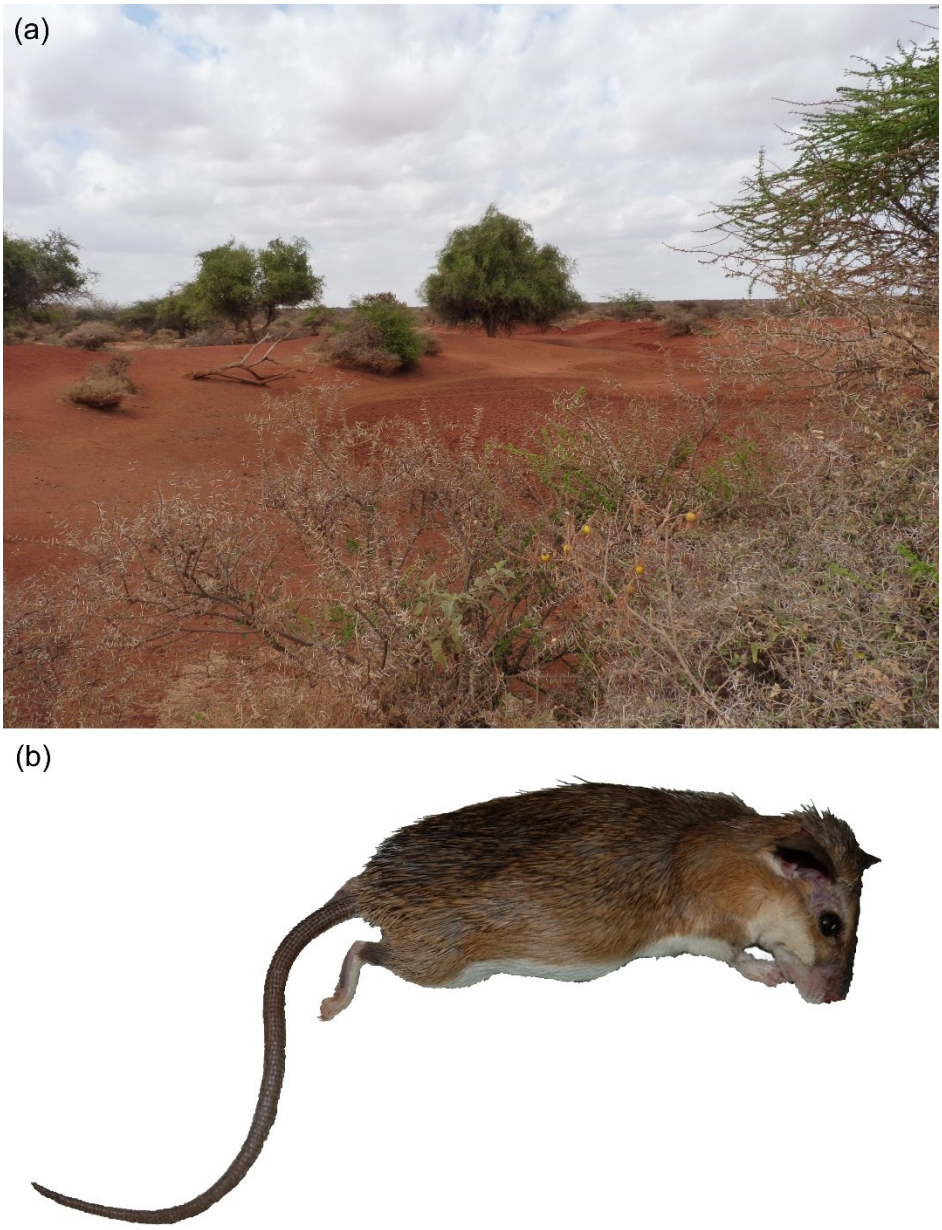


**Figure 3.** Skull morphology with the detail of the dorsal (a) and ventral (b) part of the skull; lower jaw from the lateral (c) and medial (d) view; detail of lower molars (e) and upper $M^2$ (f).

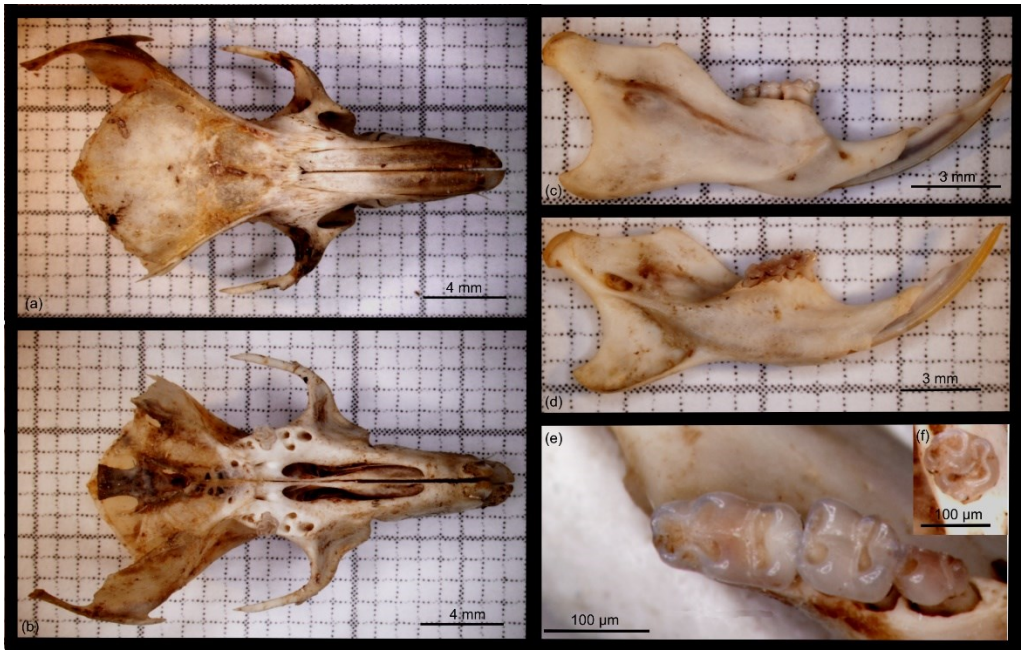
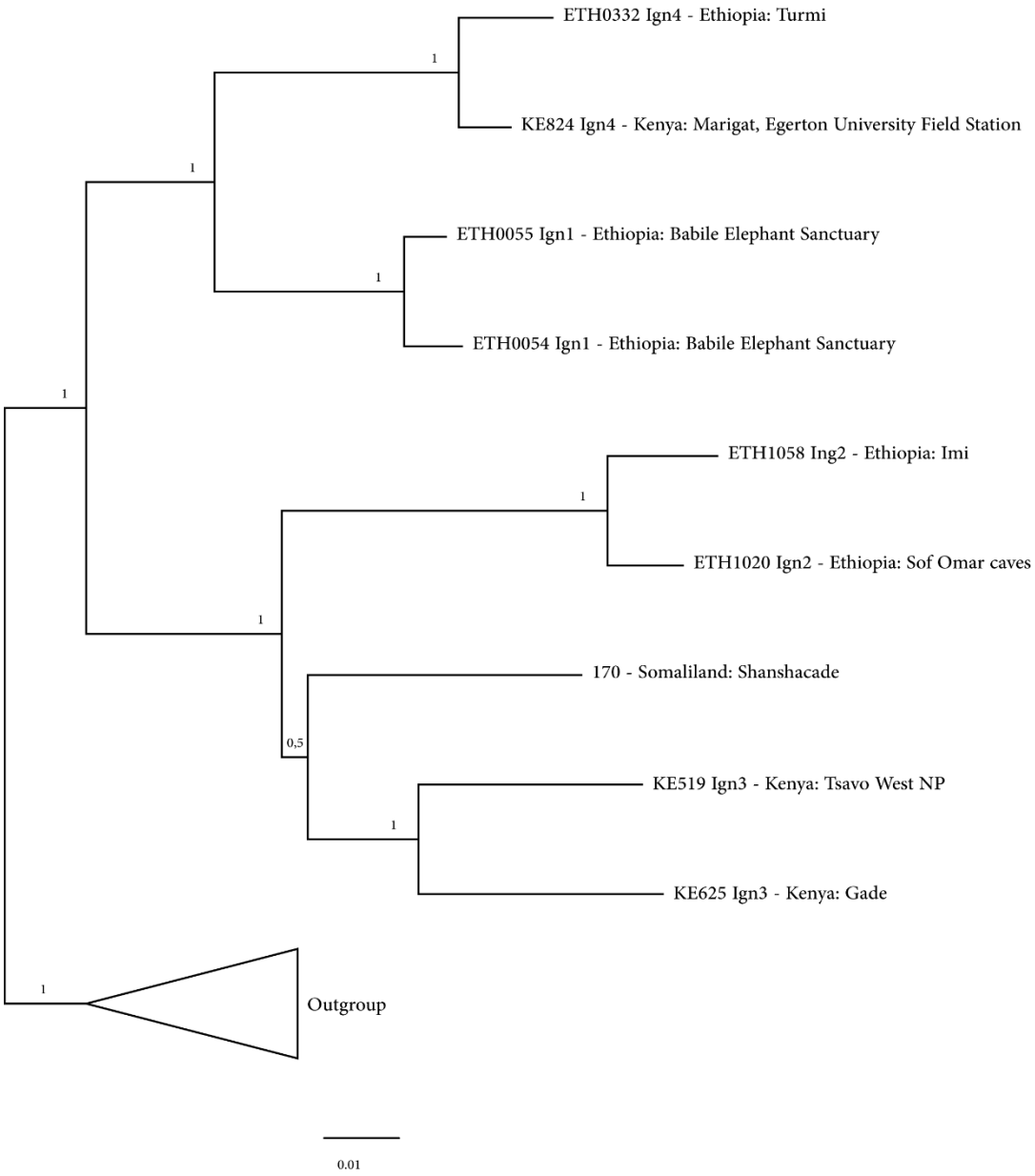


**Figure 4.** Multi-locus phylogeny of the *ignitus* clade. Bayesian phylogeny of concatenated multi-locus matrix calculated in MrBayes with posterior probabilities.



## 6.4 Molecular characterization of *Acomys louisae* from Somaliland: A deep divergence and contrasting genetic patterns in a rift zone

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**Příspěvek autora:**

Prohlašuji, že Klára Palupčíková přispěla k publikaci:

- KP provedla laboratorní práce následně se podílela na analýze dat, interpretaci výsledků a sepisování rukopisu.

V Praze dne

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**Molecular characterization of *Acomys louisae* from Somaliland: A deep divergence and contrasting genetic patterns in a rift zone**

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Authors declare no competing interests.

**Abstract**

Phylogeographic patterns in the Horn of Africa have recently attracted researchers searching for hidden diversity and explaining the evolutionary history of this region. In this paper, we focus on an endemic spiny mouse *Acomys louisae.* We examined 88 samples from 13 localities across Somaliland and sequenced CYTB, control region and IRBP genes. Phylogenetic analysis confirmed clear distinctness of *A. louisae* from the other clades of *Acomys*, but it also revealed deep splits within *A. louisae* clade. Samples from Central and Eastern Somaliland, including those from the type locality, form a clearly distinct Somaliland clade while remaining ones from the very NW of Somaliland and 5 previously published sequences from Djibouti and E Ethiopia form a Djibouti group. At two localities in the contact zone, we detected sympatric occurrence of both. The clades exhibit sharply contrasting patterns of variability, the Somaliland clade is characterized by a sufficient mitochondrial haplotype diversity, but low sequence divergence. The population parameters and haplotype networks suggest that the populations belonging to the Somaliland clade probably underwent a recent expansion of its range and population size. It may be explained by a repopulation after the interglacial period providing poor environmental conditions for spiny mice in E and C Somaliland. In contrast, the Djibouti group shows extremely high nucleotide diversity besides that of haplotype one. This suggests a long-term persistence of large and/or structured populations. It may be attributed to a specific history of the Ethiopian Rift and Afar. The results emphasize importance of this area for generating species diversity in the Horn of Africa.

Keywords: Spiny mice; Somalia; Genetic diversity; Population expansion

## Introduction

Spiny mice of the genus *Acomys* evolved several unique characters as menstrual cycles (Bellofiore et al., 2017) and extreme ability of tissue regeneration (Seifert et al., 2012) which recently attracted the attention of laboratory researchers (Haughton et al., 2016). Spiny mice also exhibit sympatric speciation (Hadid et al., 2014), giving birth to precocial neonates (Dieterlen, 1963, Dewsbury and Hodges, 1987, Dempster et al., 1992), nutritionally induced diabetes (Shafrir, 2000, Shafrir et al., 2006), individual recognition (Porter and Wyrick, 1979, Porter, 1988), social stress (Nováková et al., 2008, Frynta et al., 2010a), male parental investment (Dieterlen, 1962, Makin and Porter, 1984), physiological adaptations to arid environment (Horowitz and Borut, 1994, Weissenberg and Shkolnik, 1994, Ehrhardt et al., 2005), shifts from nocturnal to diurnal way of life (Haim et al., 1994, Kronfeld-Schor et al., 2001, Gutman and Dayan, 2005), specific antipredator (Carere et al., 1999) and reproductive strategies (Nováková et al., 2010, Frynta et al. 2011). Moreover, they are epidemiologically important as a reservoir species for *Leishmania* infection (Kassahun et al. 2015). Therefore, they belong to handful rodent taxa that have been extensively studied for decades and become a model in multiple fields of ecology, physiology, and evolution. Nevertheless, vast majority of the above studies were performed in *A.cahirinus*, *A.dimidiatus* and *A.russatus*, i.e., those species inhabiting Egypt, Israel, Jordan, Mediterranean Islands and neighbouring areas of the Near East and representing just terminal offshoots of the phylogenetic tree of the genus. For proper evolutionary interpretation of the results of the above-mentioned studies, distribution of the respective characters on species tree is urgently needed. Nevertheless, many Sub-Saharan species of this genus have remained nearly neglected. This is especially applicable to species inhabiting the area of the Horn of Africa.

Recently, Aghová et al. (2019) published a comprehensive multilocus phylogenetic tree of the genus *Acomys*. A representative sampling of taxa and zoogeographic regions enabled them to uncover 26 molecular operational taxonomic units (MOTUs), putatively corresponding to distinct species. Aghová et al. (2019) supported phylogenetic hypotheses revealed by previous molecular studies (Barome et al., 1998, 2000, Frynta et al., 2010b, Alhajeri et al., 2015, Steppan and Schenk, 2017; Petružela et al. 2018). There are five major phylogenetic groups referred to as cahirinus, wilsoni, russatus, subspinosus and spinosissimus (Aghová et al., 2019). The cahirinus superclade, which originally diversified in E Africa is the most speciose and, moreover, contains principal model species as *A.cahirinus*, *A.dimidiatus* and *A.ignitus*. It further splits into three major clades, cahirinus-dimidiatus, ignitus and Djibuti,

consisting of 11, 4 and 1 MOTUs, respectively (Aghová et al., 2019). We can reasonably expect that this list of potential species is still incomplete due to limited sampling in some areas. The principal role of E Africa in generating genetic and species diversity was repeatedly demonstrated in other taxa including ungulates (Lorenzen et al., 2012), baboons (Zinner et al., 2011), ostriches (Miller et al., 2011), warthogs (D'Huart and Grubb 2001, Randi et al., 2002) and rodents (Bryja et al., 2014, 2017, 2019, Mazoch et al., 2018, Krásová et al., 2019). A thorough phylogenetic study of the genus *Gerbilliscus*, rodents with partially similar requirements as those of spiny mice, revealed extensive speciation in the region of Somali-Masai savanna (Aghová et al., 2017). Importance of this region, in particular the territory of Somaliland, as a biodiversity hotspot was currently demonstrated in other animal taxa like lizards (Šmíd et al., 2013, Wagner et al., 2013a,b), snakes (Mazuch et al., 2018) and invertebrates (Kovařík et al., 2013, 2016a,b, 2017, 2018, 2019, Král et al., 2019).

Timing of the first splits of the cahirinus superclade estimated to 5.47 and 4.74 mye (for cahirinus-dimidiatus from ignitus-Djibuti and ignitus from Djibuti, respectively; see Aghová et al., 2018, 2019) clearly corresponds to a climate aridification period following Messinian salinity crisis at the end of the Miocene. This resulted in a reduction of originally forested areas in W and E Africa (Duggen et al., 2003, Schuster et al., 2006, Jacobs et al., 2010) and its replacement by savannas and deserts (Bonnefille, 2010) including emerging Sahara desert (7 - 2.5 Mye; Schuster et al., 2006, Swezey, 2009). This was accompanied by slight warming of the climate during the first half of the Pliocene (Poore & Sloan, 1996, Maslin et al., 1998). Thus the preferred habitats of spiny mice (Aghová et al., 2019 and references herein) expanded at this time. Simultaneously, Himalaya uplift resulted in regular monsoons providing seasonal rains in E Africa (Partridge et al., 1995). This supported persistence of forested areas representing dispersal barriers for savanna species in NE Africa (e.g., in Kenya until 3.7 Mye; Hill et al., 2002, Sepulcher et al., 2006). In the following period, tectonic processes associated with formation of the Rift Valley (in Kenya from ca 3.2 mye, Veldkamp et al., 2007) became a key factor determining the climate, vegetation and barriers for dispersal and gene flow. In the Pleistocene, these were furthermore affected by glacial cycles (Cowling et al., 2008).

In this paper, we focus on *A. louisae* Thomas, 1896. This species was originally described from former British Somalia, Henweina Plain, 65 km S of Berbera (at present this area belongs to Somaliland Republic). According to its unique dental characters, Petter and Roché, (1981) placed this species into a separate subgenus *Peracomys* (see also Petter, 1983, Denys et al., 1994). Its karyotype (2n = 68, NF = 68) was described by Sokolov et al. (1993).

Nevertheless, molecular data were limited, to just five specimens from Djibouti and Ethiopia that were examined by Aghová et al. (2019) and unpublished record from Dire Dawa mentioned therein. These samples form a clearly distinct Djibouti clade within the cahirinus superclade. It is deeply separated from the nearest ignitus clade by 9.6% sequence divergence (Aghová et al., 2019).

The aim of this paper was a molecular characterization and searching for a hidden phylogeographic diversity within *A. louisae* sensu lato. For these purposes we (1) collected samples across the territory of Somaliland including vicinity of the type locality of *A. louisae*; (2) performed a phylogenetic analysis of concatenated fragments of CYTB, D-loop and IRBP genes; (3) analyzed phylogenetic relationships among haplotypes of mitochondrial cytochrome b (CYTB) gene; (4) analysed a genetic variability and constructed a haplotype network; (5) inferred a demographic history of studied populations; (6) interpreted the results in the context of climatic history of the Horn of Africa during the Pleistocene.

## Material and methods

*Sampling*

We analyzed 88 new samples of spiny mice from 13 localities across the territory of Somaliland (see Table 1, Fig. 1).

*DNA Extractions and Sequencing*

We sequenced up to two mitochondrial genes and one nuclear exon, combined the new sequences with our previously published data (Aghová et al., 2019), and supplemented them with sequences from GenBank. The three genes included 864 base pairs (bp) of cytochrome *b* (CYTB), 949 bp of the control region (D-loop) and 823 bp of exon 1 of the Interphotoreceptor Retinoid Binding Protein (IRBP) gene. These genes were chosen on the basis of their phylogenetic information content in previous studies with the same taxonomic scope, appropriate rates of evolution in muroids, and availability of sequences. Nuclear exon IRBP and D-loop were sequenced only in the representative subset (see Table 1). Genomic DNA was extracted from 96% ethanol-preserved tissue samples by a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Individual markers were amplified by the polymerase chain reaction (PCR) using the same combination of primers like in previous study (Aghová et al., 2019) for chosen genes as well as subsequent steps to obtain clear sequences.

*Phylogenetic analysis*

The final dataset for phylogenetic analyses consisted of 134 unique sequences of CYTB, 44 sequences of D-loo*p* and IRB*P* (for additional *Acomys* sequences and outgroups included into the final alignments see Appendix 1 and 2). As outgroups, we used three taxa from subfamily Deomyinae like Aghová et al. (2019).

Phylogenetic reconstructions were conducted using Bayesian analysis (BA), maximum likelihood (ML) and maximum parsimony (MP) for separate gene trees (CYTB, D-loop, IRBP and CYTB*)*. Bayesian analysis (BA) of multiple alignment (CYTB, D-loop, IRBP) was performed using MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001, Ronquist et al., 2012) with seven partitions (three partitions for each of the protein-coding genes and one partition for the D-loop see Aghová et al., 2019). Two independent runs of BA were conducted with a random starting tree and run for 40,000,000 generations, with trees sampled every 1,000 generations and with 25% burn-in. BA of the CYTB data were performed under the GTR+I+G for pos1, HKY+I+G for pos2, GTR+I+G for pos3 for 50,000,000 generations, with 25% burn-in.

Maximum likelihood analyses were performed using RAxML v8.2.8. (Stamatakis, 2014). For ML analysis was used the heuristic search with 100 random replicates of taxa additions. Support for the ML tree topology was assessed by bootstrap analysis with 1,000 replicates.

Maximum parsimony analyses were performed using Mega7 (Kumar et al., 2016). For MP, we conducted heuristic search analyses with 1,000 random replicates of taxa additions using tree-bisection and reconnection (TBR) branch swapping. The branch support was evaluated using 10,000 bootstrap pseudoreplicates (Felsenstein, 1985). All characters were equally weighted and unordered.

These methods produced phylogenetic trees with very similar topologies and thus we present only BA trees. Besides BA posterior probabilities, we provide bootstrap supports for principal nodes as revealed by ML and MP.

Relationships between haplotypes of *A. louisae* sensu lato were also represented by using the TCS statistical parsimony network approach (Clement et al., 2000) in the program Population Analysis with Reticulate Trees (PopART; Leigh and Bryant, 2015).

To test the hypothesis that the observed pattern of geographic variation can be explained by isolation by distance, we employed Mantel tests (Mantel, 1967). We compared the matrix of

geographic distances (based on decimal degree coordinates) with standard genetic distances using Mantel matrix correlations implemented in GENALEX (Peakall and Smouse, 2012). Significance was evaluated based on 9,999 permutations.

*Demographic inferences*

Polymorphism for populations was worked out by the statistic software DnaSP v5 5.10.01 (Librado and Rozas, 2009) which estimated the following: haplotype diversity (*h*), segregating site (S), nucleotide diversity ($\pi$) and Tajima's D, Fu & Li's $F^*$, Fu & Li's $D^*$ and Fu's FS tests. According to Russell et al. (2005), high values of *h* and $\pi$ indicate a constant large size of a population. However, a low value of $\pi$ and high value of h signify a recent expansion. To estimate population dynamics through time, we have run Markov chain Monte Carlo simulations with 30 million iterations and 10 million burn-ins using the GTR model and molecular clock with setting a rate 0.05 per million years. We have summarized the results and displayed them as Bayesian skyline plots in Tracer v1.7.1.

**Results**

*Phylogenetic analyses*

Analyses of multiple alignment (CYTB, D-loop and IRBP genes; for BA tree see Fig. 2) support a clear genetic distinctness of samples belonging to *A. louisae* sensu lato. These form a deep clade within the cahirinus superclade of *Acomys* species ($BA_{posterior\ probability}$ = 1.0, $ML_{bootstrap}$ = 100, $MP_{bootstrap}$ = 100). We further referred to this clade as Djibouti-Somaliland. It further splits into two sister clades that we further referred to as Somaliland and Djibouti ($BA_{posterior\ probability}$ = 1.0, $ML_{bootstrap}$ = 100, $MP_{bootstrap}$ = 100 for both).

Phylogenetic analyses of mitochondrial CYTB alignment (for BA tree see Fig. 3) revealed that all examined haplotypes of A. louisae sensu lato belong to a deep and clearly distinct Djibouti-Somaliland clade ($BA_{posterior\ probability}$ = 1.0, $ML_{bootstrap}$ = 98, $MP_{bootstrap}$ = 97). Within this clade, there was a well-supported Somaliland clade ($BA_{posterior\ probability}$ = 1.0, $ML_{bootstrap}$ = 100, $MP_{bootstrap}$ = 99) containing 58 previously unknown haplotypes from the Central and Eastern Somaliland. The remaining 21 haplotypes of the Djibouti-Somaliland clade form a "Djibouti" group. It includes 16 new haplotypes from the very NW of Somaliland and 5 already published sequences from Djibouti and Ethiopia (Dero Park; Aghová et al., 2019). In this separate analysis of CYTB alignment, the Djibouti group is paraphyletic with respect to the

Somaliland clade. This contrasts to the above analyses of multiple alignment supporting its monophyly.

Although, BA of CYTB alignment supported a sister relationship between the cahirinus-dimidiatus and ignitus clades ($BA_{posterior\ probability} = 1.0$), neither of the other above-mentioned phylogenetic analyses resolved relationships among the Djibouti-Somaliland, cahirinus-dimidiatus and ignitus clades.

*Genetic diversity and its phylogeographic structure*

Median-joining haplotype network based on cytochrome b data showed a clearly distinct Somaliland haplogroup (Fig. 4). It includes 71 sequences (58 haplotypes) coming from ten localities of the Central and Eastern Somaliland. Its sequence divergence was low but still followed a geographic pattern. A test for isolation by distance was significant ($r = 0.216$, $R^2 = 0.0466$, $P << 0.0001$).

The rest of the network we call Djibouti group. It contains 22 sequences (21 haplotypes) from eight localities from NW Somaliland (Quljeet, Dacar Budhuq, Jidha, Ruqi and Cavi Haid), Djibouti and Ethiopia (Fig. 4). It is characterized by an extraordinarily high sequence divergence with a chain structure resulting in an unclear delimitation of partial haplogroups. At the locality Quljeet, we detected the simultaneous occurrence of extremely distant haplotypes of the Djibouti group (Fig. 4). In spite of this, there was a significant congruence of geographic and genetic distances ($r = 0.165$, $R^2 = 0.0272$, $P < 0.0001$) supporting isolation by distance within Djibouti group.

At two localities situated in a contact zone, i.e., Ruqi and Cavi Haid, we detected co-occurrence of haplotypes belonging to Somaliland and Djibouti groups.

*Demographic history*

Population parameters clearly demonstrated a sharp difference between a demographic history of the Djibouti and Somaliland haplogroups (Table 2). Haplotype diversity was high in both examined haplogroups ($h = 0.996$ and 0.949, respectively), while nucleotide diversity was seven times higher in the former ($\pi = 0.0622$) than in the latter one ($\pi = 0.00879$). Fu's Fs was high and significant, especially in the case of Somaliland haplogroup ($Fs = -16.61$, $P < 0.001$). Taken together with non-significant D and F it supports a population expansion of this population.

The Bayesian skyline plot (BSP) analyses of sequence variability (CYTB) showed that within both examined haplogroups, population size was fairly stable during the time covered by the plot. Nevertheless, an effective population size of Djibouti group was higher and consequently the coalescent time was many times longer than in the case of the Somaliland clade (Figs 5 and 6).

**Discussion**

In a recent extensive phylogenetic analysis of the genus *Acomys* performed by Aghová et al. (2019), *A. louisae* sensu lato was represented by only a handful of samples from Djibouti and Ethiopia. Moreover, these samples contained mutually closely similar haplotypes belonging to a single terminal offshoot of the Djibouti group (Fig. 4). Our extensive sampling across the territory of the Somaliland Republic uncovered a surprisingly high sequence divergence within this taxon. In spite of this, our phylogenetic analyses revealed that all these mice form a clearly distinct Djibouti-Somaliland clade. They also clearly confirmed that this clade belongs to cahirinus group of the genus *Acomys*, but as with previous analysis (Aghová et al., 2019), failed to resolve phylogenetic relationships within this group, i.e., among the Djibouti-Somaliland, cahirinus-dimidiatus and ignitus clades. These clades were separated at the Miocene/Pliocene boundary (Aghová et al., 2018, 2019). Thus, the Pliocene is the period of further splits within the Djibouti-Somaliland clade.

Pliocene climate was more humid and slightly cooler than nowadays (WoldeGabriel, 2009). Findings of fossil hominids like *Ardipithecus* and *Australopithecus* (3.5 – 2.2 mye), enhanced paleoecological research in ERV and Afar area, in particular in Hadar (Campisano and Feibel, 2007, King and Bailey, 2006). The analyses of vegetation types suggest that this area was covered by fairly heterogeneous habitats (mainly consisting of steppe, tropical xerophytic woods/scrub and temperate xerophytic woods/scrub) providing opportunities for a wide range of animal species (Bonnefille et al., 2004). Taken together, these findings made the hypothesis that ancestors of *A. louisae* evolved somewhere in this part of ERV plausible.

We confirmed that *A. louisae* sensu lato is a dominant species of the genus *Acomys* in the territory of Somaliland. We found this species at almost all examined localities providing suitable rocks and stones. This makes *A. louisae* sensu lato a proper model for evaluation of phylogeographic, climatic and evolutionary scenarios in a neglected biodiversity hotspot in the Horn of Africa.

We report here that the geographic range of *A. louisae* sensu lato splits into two parts following highly contrasting patterns of a genetic variation. Haplotypes from the Central and Eastern part of Somaliland forming Somaliland clade were poorly diversified but exhibited a tendency to form localized clusters on the haplotype network (Fig. 4). In contrast, the remaining haplotypes exhibited an extremely high sequence diversity which considerably exceeds those previously reported in the other MOTUs of spiny mice (Aghová et al., 2019). These haplotypes here referred to as Djibouti group come exclusively from the very NW of Somaliland and adjacent parts of the Ethiopian Rift Valley (ERV) in Djibouti and Ethiopia. Although isolation by distance was significant, the sequence diversity cannot be fully explained by the current geographic structure and barriers. Long-term persistence of highly diversified haplotypes of the Djibouti group can be explained by extremely large population numbers and/or complex speciation/metapopulation dynamics during the Pleistocene. In contrast, the estimated population size was one order lower in the case of the Somaliland group. Moreover, our sequence data for the Somaliland group support a recent population expansion prior the resolution of the Bayesian skyline plots.

The geographic ranges of the Somaliland and Djibouti groups are parapatric with no clear ecological boundary. This suggests that these seemingly similar landscapes underwent sharply different evolutionary past. MaxEnt modelling of the bioclimatic niches performed by Aghová et al. (2019) provides a possible explanation for this observed phylogeographic pattern. The models for both present and last glacial maximum predict an occurrence of *Acomys* in C and E Somaliland. Nevertheless, a model for the last interglacial (120-140 mye) predicts a low probability of suitable conditions in areas corresponding to a current range of the Somaliland haplotype group. This is consistent with a scenario suggesting a partial or complete extinction of spiny mice in C and E Somaliland during the last interglacial and a subsequent recolonization from a local refugium or the ERV area. Rapid population expansion of the Somaliland clade inferred from genetic variation may be explained as an inevitable consequence of such a colonization/recolonization event. The above-mentioned models also supported the view that the ERV provided large areas of suitable habitats throughout the glacial cycles of the Pleistocene (Aghová et al., 2019). It is consistent with our estimates of effective population size and its dynamics inferred from sequence data of the Djibouti group. These results suggest maintenance of extremely large population numbers for at least the second half of the Pleistocene period.

Our findings in NE Somaliland suggest that the geographic range of the Djibouti group corresponds well to Afar Depression. This extension of the ERV separated from the Red Sea by Ali-Sabieh and Danakil Blocks, had a fairly complex geological history (Keir et al., 2013). The Danakil block was originally a part of the landbridge between the Nubian plate and Arabian Peninsula which shifted and rotated to its present-day position (Redfield et al., 2003, Beyene and Abdelsalam, 2005). Afar Depression is well-bounded by marginal escarpments and seashore (Beyene and Abdelsalam, 2005). These provided barriers promote speciation processes (Redfield et al., 2003). The shape of Afar Depression is triangular, covering an area of 200,000 $km^2$. Nowadays, Afar Depression is covered mostly by Pliocene–Pleistocene volcanic rocks (Redfield et al., 2003) providing suitable shelters for spiny mice. Thus, the territory is probably large enough to support large populations. This is consistent with our estimates of effective population sizes.

*Taxonomic implications*

*A.louisae* was originally described from Henweina Plain, 65 km S of Berbera (Thomas 1896). Our locality Sheikh is ruins of a summer palace of the British Governor of Somalia. It is placed 50 km S of Berbera (strait distance), i.e., it should be close to the type locality. Both specimens captured there belong to the Somaliland clade. As this clade exhibits little divergence and its monophyly was supported by all phylogenetic analyses, it is reasonable to conclude that the Somaliland clade corresponds to *A.louisae* sensu stricto. The taxonomic status of the Djibouti clade is less obvious. On the one hand, its great genetic divergence from the Somaliland clade and almost parapatric range of these clades suggest that Djibuti group may represent at least one distinct unnamed species. On the other hand, a major contribution of mitochondrial genes to our results, a sympatric occurrence of the Somaliland and Djibuti clades at two localities in the contact zone, an extremely high genetic variability and a weaker support for monophyly of the Djibouti clade (it may be paraphyletic in regard to Somaliland clade) support the view that it would be premature to split *A.louisae.* Until a taxonomic decision can be issued, we have to await additional information regarding, e.g., morphology, karyotypes and ability to hybridize.

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**References**

Aghová, T., Šumbera, R., Piálek, L., Mikula, O., McDonough, M.M., Lavrenchenko, L.A., Meheretu, I., Mbau, J.S., Bryja, J., 2017. Multilocus phylogeny of East African gerbils (Rodentia, Gerbilliscus) illuminates the history of the Somali-Masai savanna. J. Biogeog. 44, 2295-2307.

Aghová, T., Kimura, Y., Bryja, J., Dobigny, G., Granjon, L., Kergoat, G.J., 2018. Fossils know it best: using a new set of fossil calibrations to improve the temporal phylogenetic framework of murid rodents (Rodentia: Muridae). Mol. Phylogenet. Evol. 128, 98-111.

Aghová, T., Palupčíková, K., Šumbera, R., Frynta, D., Lavrenchenko, L.A., Meheretu, Y., Sádlová, J., Votýpka, J., Mbau, J. S., Modrý, D., Bryja, J., 2019. Multiple radiations of spiny mice (Rodentia: Acomys) in dry open habitats of Afro-Arabia: evidence from a multi-locus phylogeny. BMC Evol. Biol. 19, 69.

Alhajeri, B.H., Hunt, O.J., Steppan, S.J., 2015. Molecular systematics of gerbils and deomyines (Rodentia: Gerbillinae, Deomyinae) and a test of desert adaptation in the tympanic bulla. J. Zool. Syst. Evol. Res. 53, 312–330.

Barome, P.O., Monnerot, M., Gautun, J.C., 1998. Intrageneric phylogeny of Acomys (Rodentia, Muridae) using mitochondrial gene cytochrome b. Mol. Phylogenet. Evol. 9, 560–6.

Barome, P.O., Monnerot, M., Gautun, J.C., 2000. Phylogeny of the genus Acomys (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. Mammalia. 64, 423–38.

Bellofiore, N., Ellery, S.J., Mamrot, J., Walker, D.W., Temple-Smith, P., Dickinson, H., 2017. First evidence of a menstruating rodent: the spiny mouse (Acomys cahirinus). Am. J. Obstet. Gynecol. 216, 40-e1.

Beyene, A., Abdelsalam, M. G., 2005. Tectonics of the Afar Depression: A review and synthesis. J. Afr. Earth Sci. 41(1-2), 41-59.

Bonnefille, R., 2010. Cenozoic vegetation, climate changes and hominid evolution in tropical Africa. Global Planet. Change. 72, 390-411.

Bonnefille, R., Potts, R., Chalié, F., Jolly, D., Peyron, O., 2004. High-resolution vegetation and climate change associated with Pliocene Australopithecus afarensis. Proc. Nat. Acad. Sci. 101(33), 12125-12129.

Bryja, J., Mikula, O., Šumbera, R., Meheretu, Y., Aghová, T., Lavrenchenko, L.A., Mazoch, V., Oguge, N., Mbau, J.S., Welegerima, K., Amundala, N., Colyn, M., Leirs, H., Verheyen, E., 2014. Pan-African phylogeny of Mus (subgenus Nannomys) reveals one of the most successful mammal radiations in Africa. BMC Evol. Biol. 14, 256.

Bryja, J., Šumbera, R., Kerbis Peterhans, J.C., Aghová, T., Bryjová, A., Mikula, O., Nicolas, V., Denys, C., Verheyen, E., 2017. Evolutionary history of the thicket rats (genus Grammomys) mirrors the evolution of African forests since late Miocene. J. Biogeogr. 44, 182-194.

Bryja, J., Meheretu, Y., Šumbera, R., Lavrenchenko, L.A., 2019. Annotated checklist, taxonomy and distribution of rodents in Ethiopia. Folia Zool. 68, 117-213.

Carere, C., Casetti, R., de Acetis, L., Parretta, G., Cirulli, F., Alleva, E., 1999. Behavioural and nociceptive response in male and female spiny mice (Acomys cahirinus) upon exposure to snake odour. Behav. Proc. 47, 1-10.

Campisano, C. J., Feibel, C. S., 2007. Connecting local environmental sequences to global climate patterns: evidence from the hominin-bearing Hadar Formation, Ethiopia. J. Hum. Evol. 53, 515-527.

Clement, M., Posada, D.C.K.A., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657-1659.

Cowling, S.A., Cox, P.M., Jones, C.D., Maslin, M.A., Peros, M., Spall, S.A., 2008. Simulated glacial and interglacial vegetation across Africa: implications for species phylogenies and trans-African migration of plants and animals. Glob. Change Biol. 14, 827-840.

Dempster, F.N., 1992. The rise and fall of the inhibitory mechanism: Toward a unified theory of cognitive development and aging. Develop. Rev. 12, 45-75.

Denys, C., Gautun, J.C., Tranie, M., Volobouev, V., 1994. Evolution of the genus Acomys (Rodentia, Muridae) from dental and chromosomal patterns. Israel J. Ecol. Evol. 40, 215-246.

Dewsbury, D.A., Hodges, A.W., 1987. Copulatory behavior and related phenomena in spiny mice (Acomys cahirinus) and hopping mice (Notomys alexis). J. Mammal. 68, 49-57.

Dieterlen, F., 1962. Geburt und Geburtshilfe bei der Stachelmaus, Acomys cahirinus. Ethology. 19, 191-222.

Dieterlen F., 1963. Vergleichende Untersuchungen zur Ontogenese vonStachelmaus (Acomys) und Wanderratte (Rattus norvegicus). Beiträgezum Nesthocker-Nestflüchter-Prolem bei Negetieren. Z. Säugetierkd. 28, 193–227.

Duggen, S., Hoernle, K., Van Den Bogaard, P., Rüpke, L., Morgan, J.P., 2003. Deep roots of the Messinian salinity crisis. Nature, 422, 602-606.

D'Huart, J. P., Grubb, P., 2001. Distribution of the common warthog (Phacochoerus africanus) and the desert warthog (Phacochoerus aethiopicus) in the Horn of Africa. African J. Ecol. 39, 156-169.

Ehrhardt N., Heldmaier G., Exner C., 2005. Adaptive mechanisms during food restriction in Acomys russatus: the use of torpor for desert survival. J. Comp. Psychol. 175, 193-200.

Felsenstein, J., 1985. Confidence limits on phylogeny: an approach using the bootstrap. Evolution. 39, 783–789.

Frynta, D., Volfová, R., Fraňková-Nováková, M., Stejskal, V., 2010a. Oestrous females investigate the unfamiliar male more than the familiar male in both commensal and non-commensal populations of house mice. Behav. Proc. 83, 54-60.

Frynta, D., Palupčíková, K., Bellinvia, E., Benda, P., Skarlantova, H., Schwarzova, L., Modrý, D., 2010b. Phylogenetic relationships within the cahirinus-dimidiatus group of the genus Acomys (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula. Zootaxa 2660, 46-56.

Frynta, D., Fraňková, M., Čížková, B., Skarlandtová, H., Galeštoková, K., Průšová, K., Šmilauer, P., Šumbera, R., 2011. Social and life history correlates of litter size in captive colonies of precocial spiny mice (Acomys). Acta Theriol. 56, 289-295.

Gutman, R., Dayan, T., 2005. Temporal partitioning: an experiment with two species of spiny mice. Ecology. 86, 164-173.

Hadid, Y., Pavlíček, T., Beiles, A., Ianovici, R., Raz, S., Nevo, E., 2014. Sympatric incipient speciation of spiny mice Acomys at “Evolution Canyon,” Israel. P. Natl. Acad. Sci. USA. 111, 1043-1048.

Haim A., Yedidia I., Haim D., Zisapel N., 1994. Photoperiodicity in daily rhythms of body temperature, food and energy intake of the golden spiny mouse (Acomys russatus). Isr. J. Zool. 40, 145-150.

Haughton, C.L., Gawriluk, T.R., Seifert, A.W., 2016. The biology and husbandry of the African spiny mouse (Acomys cahirinus) and the research uses of a laboratory colony. J. Am. Assoc. Lab. Anim. 55, 9-17.

Hill, A., Leakey, M., Kingston, J.D., Ward, S., 2002. New cercopithecoids and a hominoid from 12· 5Ma in the Tugen Hills succession, Kenya. J. Hum. Evol. 42, 75-93.

Horowitz, M., Borut, A., 1994. The spiny mouse (Acomys cahirinus) a rodent prototype for studying plasma volume regulation during thermal dehydration. Isr. J. Zool. 40, 117-125.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics. 17, 754–755.

Jacobs, B.F., Pan, A.D., Scotese, C.R., 2010. A review of the Cenozoic vegetation history of Africa, in: Werdelin, L., Sanders, W.J. (Eds.), Cenozoic Mammals of Africa, University of California Press, Berkeley, pp. 57-72.

Kassahun, A., Sadlova, J., Dvorak, V., Kostalova, T., Rohousova, I., Frynta, D., Aghová, T., Yasur-Landau, D., Lemma, W., Hailu, A., Baneth, G., Warburg, A., Wolf, P., Votýpka, J., 2015. Detection of Leishmania donovani and L. tropica in Ethiopian wild rodents. Acta Trop. 145, 39-44.

Keir, D., Bastow, I. D., Pagli, C., Chambers, E. L., 2013. The development of extension and magmatism in the Red Sea rift of Afar. Tectonophysics. 607, 98-114.

King, G., Bailey, G., 2006. Tectonics and human evolution. Antiquity. 80(308), 265-286.

Kovařík, F., Lowe, G., Plíšková, J., Šťáhlavský, F., 2013. A new scorpion genus, Gint gen. n., from the Horn of Africa (Scorpiones: Buthidae). Euscorpius. 173, 1-19.

Kovařík, F., Lowe, G., Plíšková, J., Šťáhlavský, F., 2016a. Scorpions of the Horn of Africa (Arachnida: Scorpiones). Part VII. Parabuthus Pocock, 1890 (Buthidae), with description of P. hamar sp. n. and P. kajibu sp. n. from Ethiopia. Euscorpius. 228, 1-58.

Kovařík, F., Lowe, G., Šťáhlavský, F., 2016b. Scorpions of the Horn of Africa (Arachnida: Scorpiones). Part IX. Lanzatus, Orthochirus, and Somalicharmus (Buthidae), with description of Lanzatus somalilandus sp. n. and Orthochirus afar sp. n. Euscorpius. 232, 1-38.

Kovařík, F., Lowe, G., Mazuch, T., Awale, A.I., Štundlová, J., Šťáhlavský, F., 2017. Scorpions of the Horn of Africa (Arachnida: Scorpiones). Part XII. Pandinurus hangarale sp. n.(Scorpionidae) from Somaliland and a review of type locality and true distribution of Pandinurus smithi (Pocock, 1897). Euscorpius. 253, 1-18.

Kovařík, F., Lowe, G., Just, P., Awale, A.I., Elmi, H.A., Šťáhlavský, F., 2018. Scorpions of the Horn of Africa (Arachnida: Scorpiones). Part XV. Review of the genus Gint Kovařík et al.,

2013, with description of three new species from Somaliland (Scorpiones, Buthidae). Euscorpius. 259, 1-41.

Kovařík, F., Lowe, G., Mazuch, T., 2019. Scorpions of the Horn of Africa (Arachnida: Scorpiones). Part XIX. Pandiborellius meidensis (Karsch, 1879) and Pandinurus fulvipes sp. n. (Scorpionidae) from Somaliland. Euscorpius. 275, 1.

Král, D., Hrůzová, L., Šípek, P., Awale, A.I., Hurre, A.A., Sommer, D., 2019. Pachnoda iskuulka (Coleoptera: Scarabaeidae: Cetoniinae), a new species from Somaliland, including description of its mature larva. Zootaxa. 4604, 482-496.

Krásová, J., Mikula, O., Mazoch, V., Bryja, J., Říčan, O., Šumbera, R., 2019. Evolution of the Grey-bellied pygmy mouse group: Highly structured molecular diversity with predictable geographic ranges but morphological crypsis. Mol. Phylogenet. Evol. 130, 143-155.

Kronfeld-Schor, N., Dayan, T., Elvert, R., Haim, A., Zisapel, N., Heldmaier, G., 2001. On the use of the time axis for ecological separation: diel rhythms as an evolutionary constraint. Am. Nat. 158, 451-457.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870-1874.

Leigh, J.W., Bryant, D., 2015. POPART: full-feature software for haplotype network construction. Methods Ecol. Evol. 6, 1110–1116.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25, 1451-1452.

Lorenzen, E.D., Heller, R., Siegismund, H.R., 2012. Comparative phylogeography of African savannah ungulates. Mol. Ecol. 21, 3656–70.

Makin, J.W., Porter, R.H., 1984. Paternal behavior in the spiny mouse (Acomys cahirinus). Behav. Neural. Biol. 41, 135-151.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209-220.

Maslin, M.A., Li, X.S., Loutre, M.F., Berger, A., 1998. The contribution of orbital forcing to the progressive intensification of Northern Hemisphere glaciation. Quaternary Sci. Rev. 17, 411-426.

Mazoch, V., Mikula, O., Bryja, J., Konvičková, H., Russo, I.R., Verheyen, E., Šumbera, R., 2018. Phylogeography of a widespread sub-Saharan murid rodent Aethomys chrysophilus: the role of geographic barriers and paleoclimate in the Zambezian bioregion. Mammalia. 82, 373-387.

Mazuch, T., Šmíd, J., Price, T., Frýdlová, P., Awale, A I., Elmi, H.A., Frynta, D., 2018. New records of one of the least known snakes, Telescopus pulcher (Squamata: Colubridae) from the Horn of Africa. Zootaxa. 4462, 483-496.

Miller, J. M., Hallager, S., Monfort, S. L., Newby, J., Bishop, K., Tidmus, S. A., Black, P., Houston, B., Matthee, C.A., Fleischer, R. C., 2011. Phylogeographic analysis of nuclear and mtDNA supports subspecies designations in the ostrich (Struthio camelus). Conserv. Genet. 12(2), 423-431.

Nováková, M., Palme, R., Kutalová, H., Janský, L., Frynta, D., 2008. The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (Acomys cahirinus). Physiol. Behav. 95, 187-193.

Nováková, M., Vašáková, B., Kutalová, H., Galeštoková, K., Průšová, K., Šmilauer, P., Šumbera, R., Frynta, D., 2010. Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: Acomys). Behav. Ecol. Sociobiol. 64, 371-379.

Partridge, T.C., Wood, B.A., DeMenocal, P.B., 1995. The influence of global climatic change and regional uplift on large-mammalian evolution in east and southern Africa, in: Vrba, E., Denton, G., Patridge, T., Buckle, L. (Eds), Paleoclimate and Evolution, with Emphasis on Human Origins. Yale University Press, New Haven, pp. 331–355.

Peakall, R., Smouse. P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics. 28, 2537–2539

Petružela, J., Šumbera, R., Aghová, T., Bryjová, A., Katakweba, A.S., Sabuni, C.A., Chitaukali, W.N., Bryja, J., 2018. Spiny mice of the Zambezian bioregion–phylogeny, biogeography and ecological differentiation within the Acomys spinosissimus complex. Mamm. Biol. 91, 79-90.

Petter, F., Roche, J., 1981. Remarques préliminaires sur la systematique des Acomys (Rongeurs, Muridae Peracomys, sous-genre nouveau. Mammalia. 45, 381-383.

Petter, F., 1983. Eléments d'une révision des Acomys africains. Un sous-genre nouveau, Peracomys Petter et Roche, 1981 (Rongeurs, Muridés). Ann. Mus. R. Afr. Cent. Sci. Zool. 237, 109-119.

Poore, R.Z., Sloan, L.C., 1996. Introduction climates and climate variability of the Pliocene. Mar. Micropaleontol. 27, 1-2.

Porter, R.H., 1988. The ontogeny of sibling recognition in rodents: superfamily Muroidea. Behav. Genetics. 18, 483-494.

Porter, R.H., Wyrick, M., 1979. Sibling recognition in spiny mice (Acomys cahirinus): influence of age and isolation. Anim. Behav. 27, 761-766.

Randi, E., Lucchini, V., Aman, R., 2002. Evidence of two genetically deeply divergent species of warthog, Phacochoerus africanus and P. aethiopicus (Artiodactyla: Suiformes) in East Africa. Mam. Biol. – Z. Säugetierk. 67(2), 91-96.

Redfield, T. F., Wheeler, W. H., Often, M. 2003. A kinematic model for the development of the Afar Depression and its paleogeographic implications. Earth Planet. Sci. Let. 216(3), 383-398.

Ronquist, F., Teslenko, M., van derMark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Russell, A.L., Medellín, R.A., McCracken, G.F., 2005. Genetic variation and migration in the Mexican free-tailed bat (Tadarida brasiliensis mexicana). Mol. Ecol. 14, 2207-2222.

Schuster, M., Duringer, P., Ghienne, J.F., Vignaud, P., Mackaye, H.T., Likius, A., Brunet, M., 2006. The age of the Sahara Desert. Science. 311, 821-821.

Seifert, A.W., Kiama, S.G., Seifert, M.G., Goheen, J.R., Palmer, T.M., Maden, M., 2012. Skin shedding and tissue regeneration in African spiny mice (Acomys). Nature. 489, 561.

Sepulchre, P., Ramstein, G., Fluteau, F., Schuster, M., Tiercelin, J. J., Brunet, M., 2006. Tectonic uplift and Eastern Africa aridification. Science. 313, 1419-1423.

Shafrir, E., 2000. Overnutrition in spiny mice (Acomys cahirinus): β-cell expansion leading to rupture and overt diabetes on fat-rich diet and protective energy-wasting elevation in thyroid hormone on sucrose-rich diet. Diabetes Metab. Res. 16, 94-105.

Shafrir, E., Ziv, E., Kalman, R., 2006. Nutritionally induced diabetes in desert rodents as models of type 2 diabetes: Acomys cahirinus (spiny mice) and Psammomys obesus (desert gerbil). ILAR J. 47, 212-224.

Sokolov, V.E., Orlov, V.N., Baskevich, M.I., Bekele, A., Mebrate, A., 1993. A karyological study of the spiny mouse Acomys Geoffroy 1838 (Rodentia Muridae) along the Ethiopian Rift Valley. Trop. Zool. 6, 227-235.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30, 1312-1313.

Steppan, S.J., Schenk, J.J., 2017. Muroid rodent phylogenetics: 900-species tree reveals increasing diversification rates. PLoS One. 12, e0183070.

Swezey, C.S., 2009. Cenozoic stratigraphy of the Sahara, northern Africa. J. Afr. Earth Sci. 53, 89-121.

Šmíd, J., Carranza, S., Kratochvíl, L., Gvoždík, V., Nasher, A.K., Moravec, J., 2013. Out of Arabia: A complex biogeographic history of multiple vicariance and dispersal events in the gecko genus Hemidactylus (Reptilia: Gekkonidae). PloS One. 8, e64018.

Thomas, O., 1896. Description of a new Acomys from Somaliland. Ann. Mag. Nat. Hist. 6(18), 269.

Veldkamp, A., Buis, E., Wijbrans, J.R., Olago, D.O., Boshoven, E.H., Marée, M., van Saparoea, R.V.D.B., 2007. Late Cenozoic fluvial dynamics of the River Tana, Kenya, an uplift dominated record. Quaternary Sci. Rev. 26, 2897-2912.

Wagner, P., Leaché, A., Mazuch, T., Böhme, W., 2013a. Additions to the lizard diversity of the Horn of Africa: Two new species in the Agama spinosa group. Amphibia-Reptilia. 34, 363-387.

Wagner, P., Mazuch, T., Bauer, A.M., 2013b. An extraordinary tail–integrative review of the agamid genus Xenagama. J. Zool. Syst. Evol. Res. 51, 144-164

Weissenberg, S., Shkolnik, A., 1994. Metabolic rate and water economy in the desert and mediterranean populatinos of the common spiny mouse (Acomys cahirinus) in Israel. Isr. J. Zool. 40, 135-143.

WoldeGabriel, G., Ambrose, S. H., Barboni, D., Bonnefille, R., Bremond, L., Currie, B., DeGusta, D., Hart. W. K., Murray. A. M., Renne, P. R., Jolly-Saad, M. C., Stewart, K. M., Jolly-Saad, M. C., 2009. The geological, isotopic, botanical, invertebrate, and lower vertebrate surroundings of Ardipithecus ramidus. Science. 326(5949), 65-65e5.

Zinner, D., Buba, U., Nash, S., Roos, C., 2011. Pan-African voyagers: the phylogeogrphy of baboons. In Primates of Gashaka (pp. 319-358). Springer, New York, NY

**Table 1.** List of specimens sampled in this study with localities, geographic coordinates and altitudes. Genes are scored as yes if sequenced.

| ID | Genus | Species | Locality | Latitude | Longitude | Altitude | Collector | CYTB | D-LOOP | IRBP |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 4 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 5 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | yes | yes |
| 6 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 7 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 9 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | yes | yes |
| 10 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 11 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 12 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 13 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 14 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 15 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 16 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 17 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 18 | *Acomys* | *louisae* | Somaliland: Las Geel | 9.781 | 44.443 | 1075 | Frynta D., Frýdlová P. | yes | | |
| 31 | *Acomys* | *louisae* | Somaliland: Sheikh | 9.944 | 45.183 | 1427 | Frynta D., Frýdlová P. | yes | yes | yes |

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|---|---|---|---|---|---|---|---|---|---|---|
| 35 | *Acomys* | *louisae* | Somaliland: Boorama | 9.947 | 43.221 | 1405 | Frynta D., Frýdlová P. | yes | | |
| 112 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 113 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 114 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 115 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 120 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 121 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 122 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 123 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 124 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 125 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 126 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 127 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 128 | *Acomys* | *louisae* | Somaliland: Mader Mage | 10.799 | 47.297 | 1403 | Frynta D., Frýdlová P. | yes | | |
| 129 | *Acomys* | *louisae* | Somaliland: Rugay | 10.845 | 47.311 | 482 | Frynta D., Frýdlová P. | yes | | |
| 130 | *Acomys* | *louisae* | Somaliland: Rugay | 10.845 | 47.311 | 482 | Frynta D., Frýdlová P. | yes | yes | yes |
| 131 | *Acomys* | *louisae* | Somaliland: Rugay | 10.845 | 47.311 | 482 | Frynta D., Frýdlová P. | yes | | |
| 132 | *Acomys* | *louisae* | Somaliland: Rugay | 10.845 | 47.311 | 482 | Frynta D., Frýdlová P. | yes | | |

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|---|---|---|---|---|---|---|---|---|
| 133 | *Acomys* | *louisae* | Somaliland: Rugay | 10.845 | 47.311 | 482 | Frynta D., Frýdlová P. | yes |
| 134 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 135 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 136 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 137 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 138 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 139 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 140 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 141 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 142 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 143 | *Acomys* | *louisae* | Somaliland: Erigavo university | 10.623 | 47.352 | 1805 | Frynta D., Frýdlová P. | yes |
| 144 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes |
| 146 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 147 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 148 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 149 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 150 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 151 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 152 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 153 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 154 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 155 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 159 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 160 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 161 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 162 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 163 | *Acomys* | *louisae* | Somaliland: Buq | 10.624 | 47.182 | 1726 | Frynta D., Frýdlová P. | yes | | | |
| 176 | *Acomys* | *louisae* | Somaliland: Sheikh | 9.944 | 45.183 | 1427 | Frynta D., Frýdlová P. | yes | yes | yes | |
| 177 | *Acomys* | *louisae* | Somaliland: Sheikh | 9.944 | 45.183 | 1427 | Frynta D., Frýdlová P. | yes | yes | yes | |
| 202 | *Acomys* | Dji | Somaliland: Dacar Budhuq | 10.240 | 43.051 | 1255 | Frynta D. | yes | | | |
| 203 | *Acomys* | Dji | Somaliland: Dacar Budhuq | 10.240 | 43.051 | 1255 | Frynta D. | yes | yes | yes | |

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|---|---|---|---|---|---|---|---|---|---|---|
| 214 | *Acomys* | Dji | Somaliland: Jidha | 10.621 | 43.069 | 462 | Frynta D. | yes | | |
| 216 | *Acomys* | Dji | Somaliland: Jidha | 10.621 | 43.069 | 462 | Frynta D. | yes | | |
| 217 | *Acomys* | Dji | Somaliland: Jidha | 10.621 | 43.069 | 462 | Frynta D. | yes | | |
| 224 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | | |
| 226 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | yes | yes |
| 227 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | | |
| 228 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | yes | yes |
| 229 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | | |
| 230 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | yes | yes |
| 232 | *Acomys* | Dji | Somaliland: Quljeet | 10.089 | 43.012 | 1575 | Frynta D. | yes | yes | yes |
| 235 | *Acomys* | Dji | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | | |
| 237 | *Acomys* | Dji | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | | |
| 238 | *Acomys* | *louisae* | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | | |
| 239 | *Acomys* | Dji | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | yes | yes |
| 240 | *Acomys* | Dji | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | | |
| 241 | *Acomys* | *louisae* | Somaliland: Ruqi | 9.967 | 43.427 | 1130 | Frynta D. | yes | | |
| 243 | *Acomys* | Dji | Somaliland: Cavi Haid | 10.047 | 43.786 | 1056 | Frynta D. | yes | | |
| 244 | *Acomys* | *louisae* | Somaliland: Cavi Haid | 10.047 | 43.786 | 1056 | Frynta D. | yes | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| 245 | *Acomys* | *louisae* | Somaliland: Agabar | 9.885 | 43.961 | 982 | Frynta D. | yes | | |
| 246 | *Acomys* | *louisae* | Somaliland: Agabar | 9.885 | 43.961 | 982 | Frynta D. | yes | | |
| 247 | *Acomys* | *louisae* | Somaliland: Agabar | 9.885 | 43.961 | 982 | Frynta D. | yes | | |
| 248 | *Acomys* | *louisae* | Somaliland: Agabar | 9.885 | 43.961 | 982 | Frynta D. | yes | | |
| 249 | *Acomys* | *louisae* | Somaliland: Agabar | 9.885 | 43.961 | 982 | Frynta D. | yes | | |
| 601474 | *Acomys* | *Dji* | Djibuti: SW Balbala | 11.519 | 43.098 | - | Peurach S. C. | yes | | |
| 601476 | *Acomys* | *Dji* | Djibuti: SW Balbala | 11.519 | 43.098 | - | Peurach S. C. | yes | | yes |
| 602643 | *Acomys* | *Dji* | Djibuti: Camp Lemonnier | 11.541 | 43.181 | - | McDonough M. | yes | | yes |
| LAV2211 | *Acomys* | *Dji* | Ethiopia: Dera Park | 8.339 | 39.328 | - | Lavrenchenko L. | yes | | yes |
| LAV2229 | *Acomys* | *Dji* | Ethiopia: Dera Park | 8.339 | 39.328 | - | Lavrenchenko L. | yes | | yes |

**Table 2.** Genetic diversity indices and demographic expansion in Djibouti, Somaliland and pooled groups of *Acomys louisae* sensu lato. The number of sequences (Ns), segregating sites (S), haplotype diversity (*h*), nucleotide diversity ($\pi$),Fu & Li's $F^*$, Fu & Li's $D^*$ and Fu's FS, Tajima's D tests and expansion coefficients (Exp) are provided.

| Group | Ns | S | *h* | $\pi$ | Fu & Li's F* | P | Fu & Li's D* | P | Fu's Fs | P | Tajima's D | P | Exp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Djibouti | 22 | 97 | 0.996 | 0.0622 | 0.24144 | > 0.10 | 0.03669 | > 0.10 | -3.82 | 0.019 | 0.58423 | > 0.10 | 2.998825 |
| Somaliland | 70 | 40 | 0.949 | 0.00879 | -2.30098 | > 0.05 | -2.18121 | > 0.05 | -16.61 | < 0.001 | -1.5061 | > 0.10 | 8.731718 |
| Djibouti-Somaliland | 92 | 118 | 0.97 | 0.03749 | -1.0937 | > 0.10 | -1.04327 | > 0.10 | -10.124 | < 0.000 | -0.7299 | > 0.10 | 6.053766 |

**Figure 1.** A map of Somaliland and adjacent parts of the Horn of Africa with localities where samples of *A. louisae* were collected. Open circles denote presence of haplotypes belonging to the Djibouti group while opened triangles those belonging to the Somaliland haplogroup.

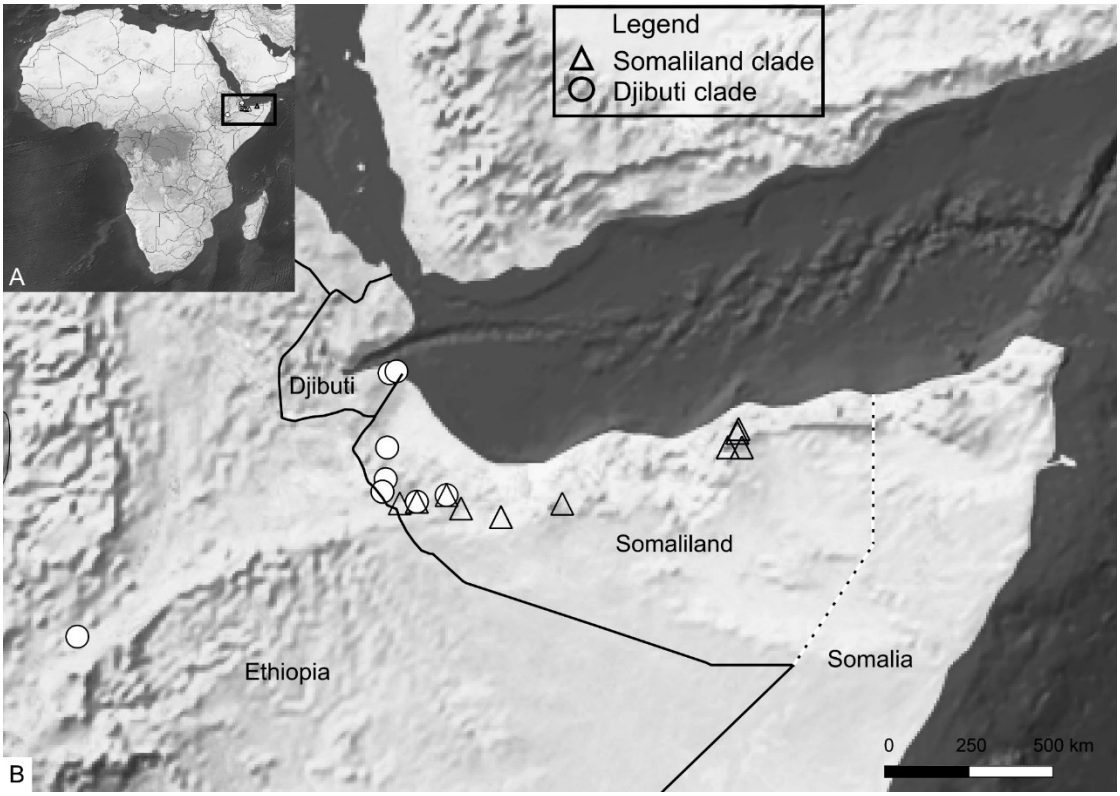


**Figure 2.** Bayesian tree computed from multiple alignment (CYTB, D-loop and IRBP genes). Statistical support of the nodes is expressed as a posterior probability.

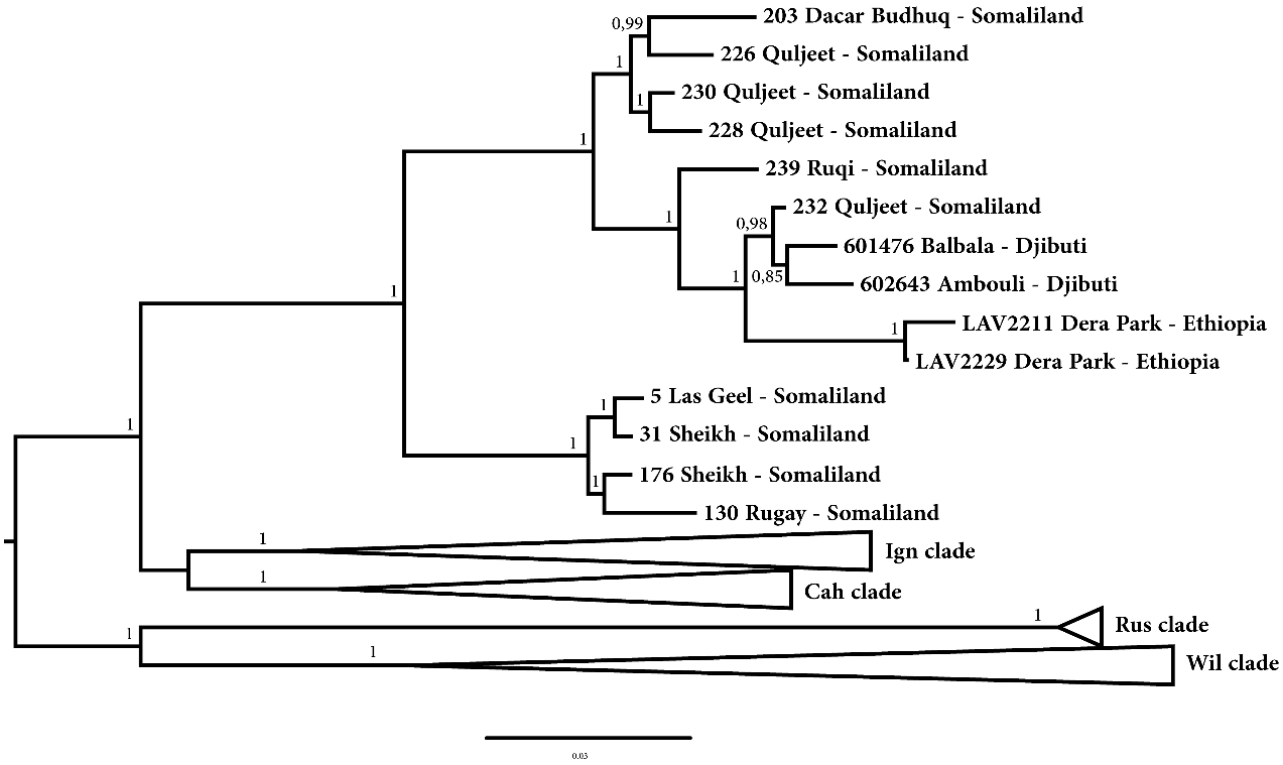


**Figure 3.** Bayesian tree computed from CYTB alignment. Statistical support of the nodes is expressed as a posterior probability.

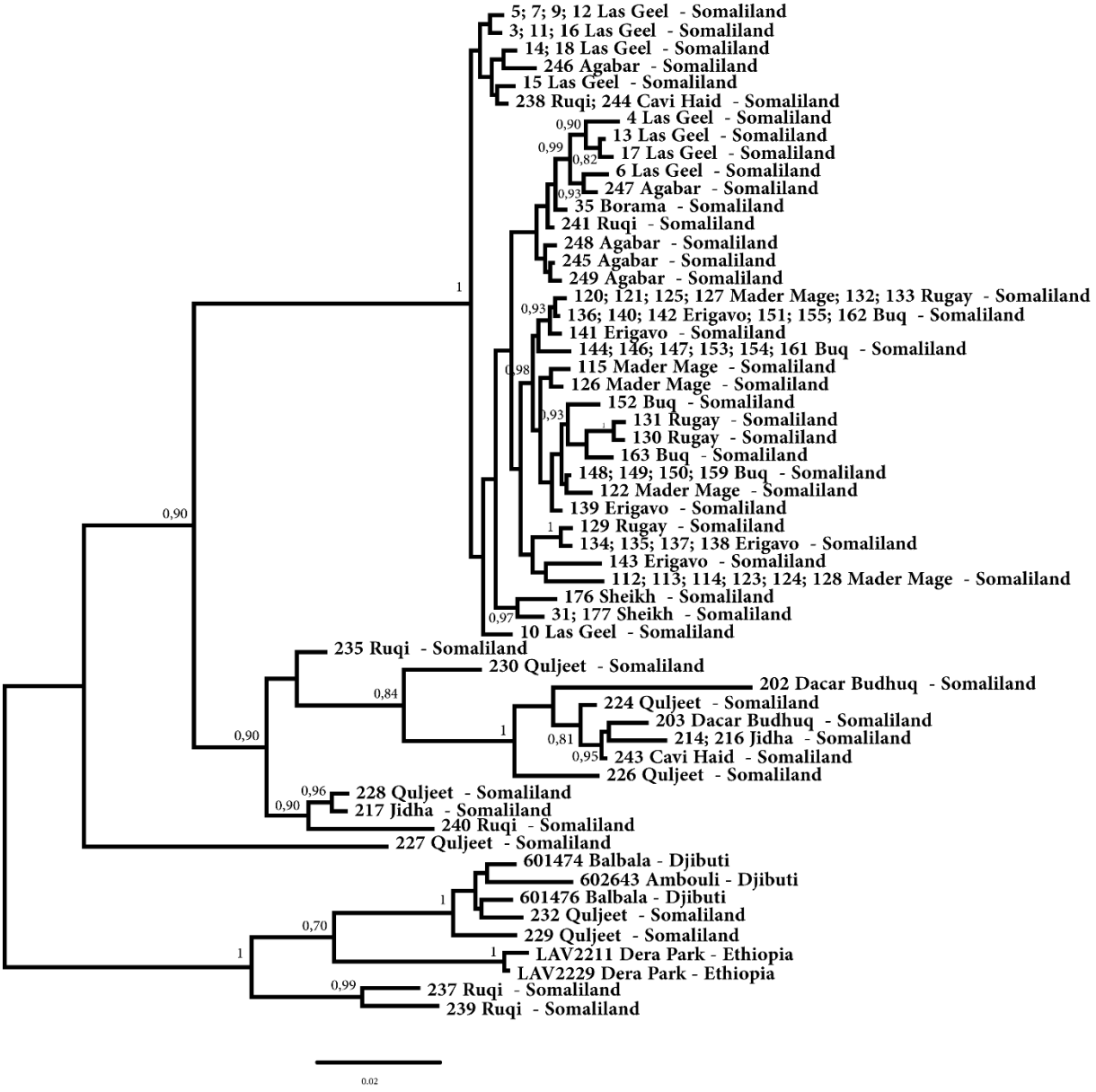


**Figure 4.** Median-joining haplotype network based on cytochrome b data in *Acomys louisae* sensu lato. The size of the circle is proportional to the frequency of haplotypes and the colours correspond to localities. The mutational steps are indicated on the branches.

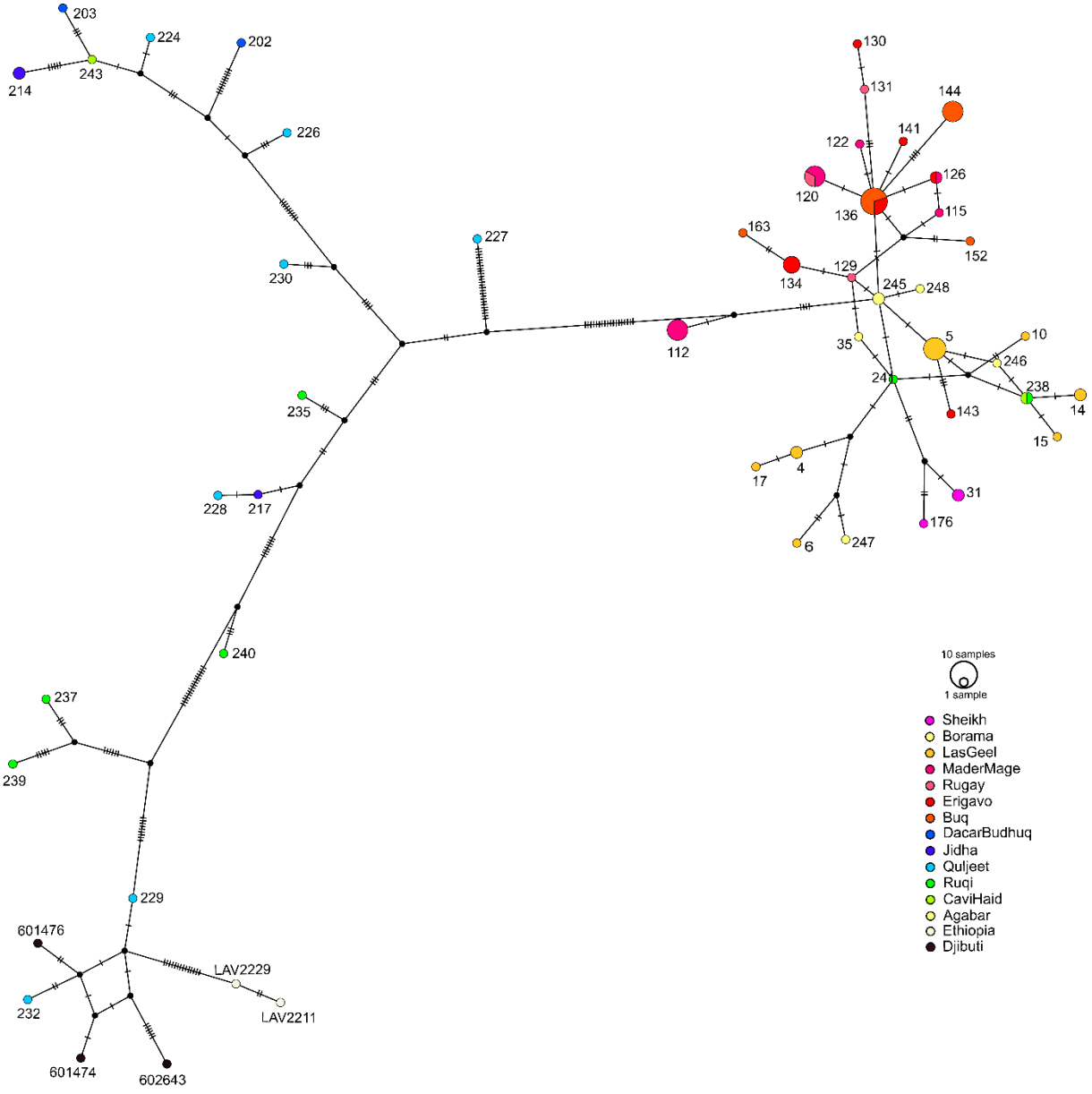


**Figure 5.** The Bayesian skyline plot (BSP) of sequence variability in CYTB for the Somaliland haplogroup.

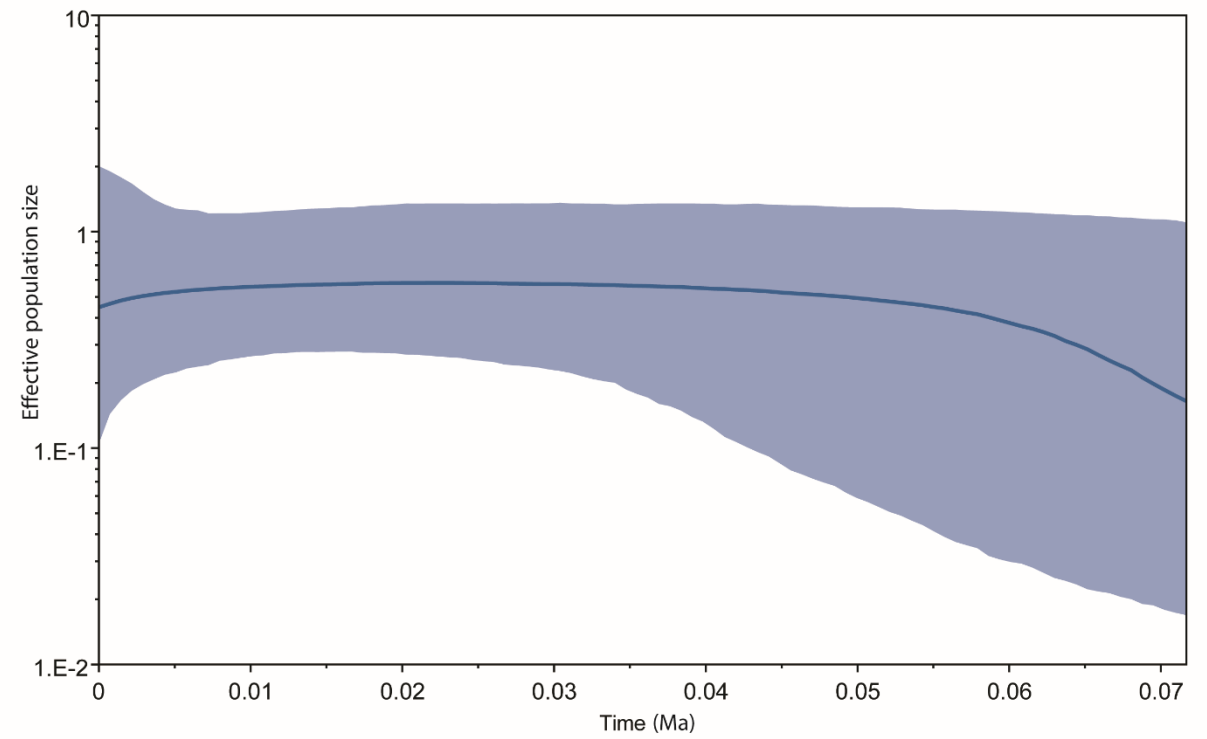
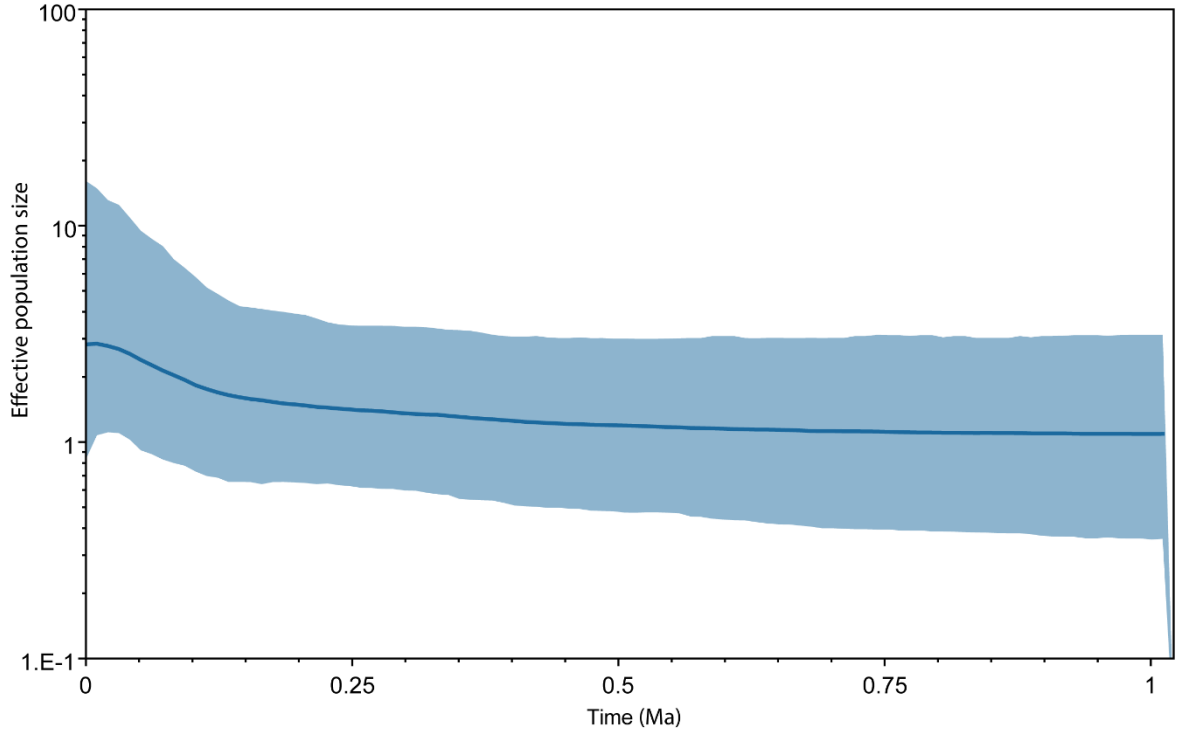


**Figure 6.** The Bayesian skyline plot (BSP) of sequence variability in CYTB for the Djibouti haplogroup.



**Appendix 1.** Additional *Acomys* sequences and outgroups included in the multiple alignments (CYTB, D-loop, IRBP).

| ID | Genus | Species | Lineage | Group | Country | Locality | Latitude | Longitude | Publication |
|---|---|---|---|---|---|---|---|---|---|
| KE734 | *Acomys* | *cineraceus* | *Cah1* | *cahirinus* | Kenya | Kosipirr | 2.895 | 34.990 | Aghová et al. 2019 |
| K4_210 | *Acomys* | *cineraceus* | *Cah1* | *cahirinus* | Kenya | North Horr | 3.317 | 37.050 | Aghová et al. 2019 |
| SIN1 | *Acomys* | *dimidiatus* | *Cah5* | *cahirinus* | Egypt | Wadi Gharandal, Sinai | 29.262 | 32.938 | Frynta et al. (2010) |
| IRA2 | *Acomys* | *dimidiatus* | *Cah5* | *cahirinus* | Iran | Zagros | 28.917 | 52.517 | Frynta et al. (2010) |
| ABC-006 | *Acomys* | *johannis* | *Cah4* | *cahirinus* | Chad | Zakouma National Park | 10.838 | 19.656 | Nicolas et al. (2009) |
| CAIR | *Acomys* | *cahirinus* | *Cah9* | *cahirinus* | Egypt | Cairo | 30.067 | 31.233 | Frynta et al. (2010) |
| CHAD | *Acomys* | *cahirinus* | *Cah9* | *cahirinus* | Chad | NE Chad, Tibesti Plateau, near Bardei-Zouar | 20.935 | 16.845 | Frynta et al. (2010) |
| E12-S37 | *Acomys* | sp. Cah10 | *Cah10* | *cahirinus* | Ethiopia | Geza Adura | 14.194 | 37.463 | Aghová et al. 2019 |
| VV1998-087 | *Acomys* | *chudeaui* | *Cah11* | *cahirinus* | Niger | Mont Baguezan | 17.670 | 8.798 | Aghová et al. 2019 |
| ETH0548 | *Acomys* | sp. B | *Cah7* | *cahirinus* | Ethiopia | Mai-Temen | 14.102 | 37.457 | Aghová et al. 2019 |
| ETH0610 | *Acomys* | sp. B | *Cah7* | *cahirinus* | Ethiopia | Alatish NP | 12.267 | 35.726 | Aghová et al. 2019 |
| ET168 | *Acomys* | *mullah* | *Cah6* | *cahirinus* | Ethiopia | Tuti, 15 km W of Metahara | 8.883 | 39.800 | Aghová et al. 2019 |
| ETH0033 | *Acomys* | *mullah* | *Cah6* | *cahirinus* | Ethiopia | Awash NP | 8.877 | 40.076 | Aghová et al. 2019 |
| ABC-008 | *Acomys* | *russatus* | *Rus* | *russatus* | Israel | | NA | NA | Nicolas et al. (2009) |
| RUS2 | *Acomys* | *russatus* | *Rus* | *russatus* | Jordan | Wadi Ramm | 29.600 | 35.400 | Frynta et al. (2010) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ETH0323 | *Acomys* | *percivali* | *Wil1* | *wilsoni* | Ethiopia | Turmi | 4.933 | 36.569 | Aghová et al. 2019 |
| KE697 | *Acomys* | *percivali* | *Wil1* | *wilsoni* | Kenya | Nassalot National Reserve | 1.831 | 35.402 | Aghová et al. 2019 |
| KE030 | *Acomys* | aff. *percivali* | *Wil3* | *wilsoni* | Kenya | Namanga | -2.530 | 36.785 | Aghová et al. 2019 |
| KE042 | *Acomys* | aff. *percivali* | *Wil3* | *wilsoni* | Kenya | Namanga | -2.530 | 36.785 | Aghová et al. 2019 |
| ETH1057 | *Acomys* | *wilsoni* | *Wil4* | *wilsoni* | Ethiopia | Gode | 5.919 | 43.554 | Aghová et al. 2019 |
| KE628 | *Acomys* | *wilsoni* | *Wil4* | *wilsoni* | Kenya | Shimoni | -4.642 | 39.380 | Aghová et al. 2019 |

**Appendix 2.** Additional *Acomys* sequences and outgroups included in the final alignments of CYTB.

| ID | Genus | Species | Lineage | Country | Locality | Latitude | Longitude | Publication |
|---|---|---|---|---|---|---|---|---|
| KE734 | *Acomys* | *cineraceus* | *Cah1* | Kenya | Kosipirr | 2.895 | 34.990 | Aghová et al. 2019 |
| K4_210 | *Acomys* | *cineraceus* | *Cah1* | Kenya | North Horr | 3.317 | 37.050 | Aghová et al. 2019 |
| KE738 | *Acomys* | *cineraceus* | *Cah1* | Kenya | Kosipirr | 2.895 | 34.990 | Aghová et al. 2019 |
| AJ012022 | *Acomys* | sp. 2 | *Cah2* | Burkina Faso | Oursi-BelDiaka | 14.600 | -0.483 | Barome et al. (1998) |
| JX292880.1 | *Acomys* | sp. 2 | *Cah2* | Mali | Doucombo | 14.355 | -3.565 | Schwan et al. (2012) |
| AJ010566 | *Acomys* | sp. 1 | *Cah3* | Burkina Faso | Toukabayal | 14.183 | -0.250 | Barome et al. (2000) |
| EMIR | *Acomys* | *dimidiatus* | *Cah5* | United Arab Emirates | Jabal Hafit | 24.067 | 55.783 | Frynta et al. (2010) |
| JOR1 | *Acomys* | *dimidiatus* | *Cah5* | Jordan | Wadi Ramm | 29.600 | 35.400 | Frynta et al. (2010) |
| YEM2 | *Acomys* | *dimidiatus* | *Cah5* | Yemen | Hawf | 16.650 | 53.050 | Aghová et al. 2019 |
| SIN1 | *Acomys* | *dimidiatus* | *Cah5* | Egypt | Wadi Gharandal, Sinai | 29.262 | 32.938 | Frynta et al. (2010) |
| IRA2 | *Acomys* | *dimidiatus* | *Cah5* | Iran | Zagros | 28.917 | 52.517 | Frynta et al. (2010) |
| AJ010565 | *Acomys* | *johannis* | *Cah4* | Cameroon | Mokolo | 10.817 | 13.900 | Barome et al. (2000) |
| ABC-006 | *Acomys* | *johannis* | *Cah4* | Chad | Zakouma National Park | 10.838 | 19.656 | Nicolas et al. (2009) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CAIR | *Acomys* | *cahirinus* | *Cah9* | Egypt | Cairo | 30.067 | 31.233 | Frynta et al. (2010) |
| CHAD | *Acomys* | *cahirinus* | *Cah9* | Chad | NE Chad, Tibesti Plateau, near Bardei-Zouar | 20.935 | 16.845 | Frynta et al. (2010) |
| CIL | *Acomys* | *cahirinus* | *Cah9* | Turkey | Silifke | 36.433 | 34.100 | Frynta et al. (2010) |
| LIB | *Acomys* | *cahirinus* | *Cah9* | Libya | Mts. Akakus | 25.889 | 12.192 | Frynta et al. (2010) |
| NES1 | *Acomys* | *cahirinus* | *Cah9* | Cyprus | Agirdag | 35.300 | 33.250 | Frynta et al. (2010) |
| MIN | *Acomys* | *cahirinus* | *Cah9* | Crete | | | | Frynta et al. (2010) |
| E12-S49 | *Acomys* | sp. Cah10 | *Cah10* | Ethiopia | Mentaptap | 14.260 | 37.442 | Aghová et al. 2019 |
| E12-S58 | *Acomys* | sp. Cah10 | *Cah10* | Ethiopia | Geza Adura | 14.194 | 37.463 | Aghová et al. 2019 |
| E12-S37 | *Acomys* | sp. Cah10 | *Cah10* | Ethiopia | Geza Adura | 14.194 | 37.463 | Aghová et al. 2019 |
| ABC-012 | *Acomys* | *chudeaui* | *Cah11* | Niger | Agadez, Indoudou | 17.150 | 9.150 | Nicolas et al. (2009) |
| VV1998-087 | *Acomys* | *chudeaui* | *Cah11* | Niger | Mont Baguezan | 17.670 | 8.798 | Aghová et al. 2019 |
| ETH0548 | *Acomys* | sp. B | *Cah7* | Ethiopia | Mai-Temen | 14.102 | 37.457 | Aghová et al. 2019 |
| ETH0610 | *Acomys* | sp. B | *Cah7* | Ethiopia | Alatish NP | 12.267 | 35.726 | Aghová et al. 2019 |
| ET168 | *Acomys* | *mullah* | *Cah6* | Ethiopia | Tuti, 15 km W of Metahara | 8.883 | 39.800 | Aghová et al. 2019 |
| ET169 | *Acomys* | *mullah* | *Cah6* | Ethiopia | Tuti, 15 km W of Metahara | 8.883 | 39.800 | Aghová et al. 2019 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ETH0033 | *Acomys* | *mullah* | *Cah6* | Ethiopia | Awash NP | 8.877 | 40.076 | Aghová et al. 2019 |
| ETH0595 | *Acomys* | sp. A | *Cah8* | Ethiopia | Alatish NP | 12.267 | 35.726 | Aghová et al. 2019 |
| LAV1795 | *Acomys* | sp. A | *Cah8* | Ethiopia | Alatish NP, Amjale | 12.109 | 34.993 | Aghová et al. 2019 |
| LAV1893 | *Acomys* | sp. A | *Cah8* | Ethiopia | Alatish NP, Megenagne | 12.109 | 34.993 | Aghová et al. 2019 |
| LAV1900 | *Acomys* | sp. A | *Cah8* | Ethiopia | Alatish NP, Megenagne | 12.109 | 34.993 | Aghová et al. 2019 |
| | | | | | | | | |
| ETH0054 | *Acomys* | sp. C | *Ign1* | Ethiopia | Babile Elephant Sanctuary | 9.120 | 42.257 | Aghová et al. 2019 |
| ETH0055 | *Acomys* | sp. C | *Ign1* | Ethiopia | Babile Elephant Sanctuary | 9.120 | 42.257 | Aghová et al. 2019 |
| ETH0056 | *Acomys* | sp. C | *Ign1* | Ethiopia | Babile Elephant Sanctuary | 9.114 | 42.260 | Aghová et al. 2019 |
| ETH1018 | *Acomys* | sp. Ign2 | *Ign2* | Ethiopia | Sof Omar caves | 6.906 | 40.849 | Aghová et al. 2019 |
| ETH1020 | *Acomys* | sp. Ign2 | *Ign2* | Ethiopia | Sof Omar caves | 6.906 | 40.849 | Aghová et al. 2019 |
| ETH1037 | *Acomys* | sp. Ign2 | *Ign2* | Ethiopia | Imi | 6.464 | 42.129 | Aghová et al. 2019 |
| KE013 | *Acomys* | *ignitus* | *Ign3* | Kenya | Namanga | -2.509 | 36.842 | Aghová et al. 2019 |
| KE519 | *Acomys* | *ignitus* | *Ign3* | Kenya | Tsavo West NP | -2.747 | 38.133 | Aghová et al. 2019 |
| KE625 | *Acomys* | *ignitus* | *Ign3* | Kenya | Gede | -3.309 | 40.018 | Aghová et al. 2019 |

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|---|---|---|---|---|---|---|---|---|
| ET104 | *Acomys* | *kempi* | *Ign4* | Ethiopia | Abaroba, 7 km SE of Konso | 5.283 | 37.466 | Aghová et al. 2019 |
| K4_139 | *Acomys* | *kempi* | *Ign4* | Kenya | Forole | 3.700 | 37.967 | Aghová et al. 2019 |
| K4_255 | *Acomys* | *kempi* | *Ign4* | Kenya | South Horr | 2.083 | 36.900 | Aghová et al. 2019 |
| KE824 | *Acomys* | *kempi* | *Ign4* | Kenya | Marigat, Egerton University Field Station | 0.489 | 35.921 | Aghová et al. 2019 |
| M8x0220 | *Acomys* | *muzei* | *Muz* | Malawi | Ntchisi | -13.381 | 34.003 | Petružela et al. (in press) |
| TA447 | *Acomys* | *muzei* | *Muz* | Tanzania | Chala (Ufipa Plateau) | -7.589 | 31.277 | Petružela et al. (in press) |
| TZ30606 | *Acomys* | *muzei* | *Muz* | Tanzania | Ikokoto | -7.651 | 36.135 | Petružela et al. (in press) |
| M8x0035 | *Acomys* | *ngurui* | *Ngu* | Malawi | Mulanje Mts FR | -15.849 | 35.705 | Petružela et al. (in press) |
| MOZ326 | *Acomys* | *ngurui* | *Ngu* | Mozambique | Gurue | -15.737 | 37.206 | Petružela et al. (in press) |
| TZ27680 | *Acomys* | *ngurui* | *Ngu* | Tanzania | Amboni caves | -5.071 | 39.050 | Petružela et al. (in press) |
| MOZ017 | *Acomys* | *spinosissimus* | *Spi* | Mozambique | Chimanimani | -19.699 | 33.022 | Petružela et al. (in press) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ABC-009 | *Acomys* | *subspinosus* | *Sup* | South Africa | Cape Peninsula National Park | -34.350 | 18.485 | Nicolas et al. (2009) |
| LEW | *Acomys* | *russatus* | *Rus* | Jordan | Al-Wisad-Heber | 31.833 | 38.133 | Frynta et al. (2010) |
| RUS2 | *Acomys* | *russatus* | *Rus* | Jordan | Wadi Ramm | 29.600 | 35.400 | Frynta et al. (2010) |
| ETH0323 | *Acomys* | *percivali* | *Wil1* | Ethiopia | Turmi | 4.933 | 36.569 | Aghová et al. 2019 |
| KE697 | *Acomys* | *percivali* | *Wil1* | Kenya | Nassalot National Reserve | 1.831 | 35.402 | Aghová et al. 2019 |
| E12-S28 | *Acomys* | *percivali* | *Wil1* | Ethiopia | Weyt'o | 5.131 | 37.021 | Aghová et al. 2019 |
| ET106 | *Acomys* | *percivali* | *Wil1* | Ethiopia | Gato, 22 km N of Konso | 5.533 | 37.417 | Aghová et al. 2019 |
| AJ010561 | *Acomys* | sp. 'Magadi' | *Wil2* | Kenya | Machakos district | -2.317 | 38.117 | Barome et al. (2000) |
| AJ012015 | *Acomys* | sp. 'Magadi' | *Wil2* | Kenya | Magadi | -1.883 | 36.300 | Barome et al. (2000) |
| TZ27881 | *Acomys* | aff. *percivali* | *Wil3* | Tanzania | Ikona WMA | -2.112 | 34.638 | Aghová et al. 2019 |
| KE030 | *Acomys* | aff. *percivali* | *Wil3* | Kenya | Namanga | -2.530 | 36.785 | Aghová et al. 2019 |
| KE042 | *Acomys* | aff. *percivali* | *Wil3* | Kenya | Namanga | -2.530 | 36.785 | Aghová et al. 2019 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ETH1057 | *Acomys* | *wilsoni* | *Wil4* | Ethiopia | Gode | 5.919 | 43.554 | Aghová et al. 2019 |
| KE628 | *Acomys* | *wilsoni* | *Wil4* | Kenya | Shimoni | -4.642 | 39.380 | Aghová et al. 2019 |
| E12-S26 | *Acomys* | *wilsoni* | *Wil4* | Ethiopia | Omorate | 4.802 | 36.054 | Aghová et al. 2019 |
| KE871 | *Acomys* | *wilsoni* | *Wil4* | Kenya | Meru NP | 0.231 | 38.165 | Aghová et al. 2019 |
| TZ27668 | *Acomys* | *wilsoni* | *Wil4* | Tanzania | Himo | -3.327 | 37.639 | Aghová et al. 2019 |
| TZ29964 | *Acomys* | *wilsoni* | *Wil4* | Tanzania | Mswaki | -5.460 | 37.784 | Aghová et al. 2019 |
| Lophuromys flavopunctatus | *Lophuromys* | *flavopunctatus* | | | | NA | NA | Rowe et al. (2008) |
| Lophuromys sikapusi | *Lophuromys* | *sikapusi* | | | | NA | NA | Barome et al. (1998) |
| Uranomys ruddi | *Uranomys* | *ruddi* | | | | NA | NA | Dobigny et al. (2011) |

# 7 Literatura


- Aghová T., Šumbera R., Piálek L., Mikula O., McDonough M. M., Lavrenchenko L. A., Bryja J. (2017). Multilocus phylogeny of East African gerbils (Rodentia, Gerbilliscus) illuminates the history of the Somali-Masai savanna. *Journal of biogeography*, *44*(10), 2295-2307.
- Alhajeri B. H., Hunt O. J., Steppan S. J. (2015). Molecular systematics of gerbils and deomyines (Rodentia: Gerbillinae, Deomyinae) and a test of desert adaptation in the tympanic bulla. *Journal of Zoological Systematics and Evolutionary Research*, *53*(4), 312-330.
- Barome P.-O., Lymberakis P., Monnerot M., Gautun J.-C. (2001b). Cytochrome b sequences reveal *Acomys minous* (Rodentia, Muridae) paraphyly and answer the question about the ancestral karyotype of *Acomys dimidiatus*. *Molecular Phylogenetics and Evolution*, 18(1): 37-46.
- Barome P.-O., Monnerot M., Gautun J.-C. (1998). Intrageneric phylogeny of Acomys (Rodentia, Muridae) using mitochondrial gene cytochrome b. *Molecular Phylogenetics and Evolution*, 9(3): 560-566.
- Barome P.-O., Monnerot M., Gautun J.-C. (2000). Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. *Mammalia,* 64(4): 423-438.
- Barome P.-O., Volobouev V., Monnerot M., Mfune J. K., Chitaukali W., Gautun J.-C., Denys Ch. (2001a). Phylogeny of *Acomys spinosissimus* (Rodentia, Muridae) from north Malawi and Tanzania: evidence from morphological and molecular analysis. *Biological Journal of the Linnean Society,* 73: 321-340.
- Bellofiore N., Ellery S. J., Mamrot J., Walker D. W., Temple-Smith P., Dickinson H. (2017). First evidence of a menstruating rodent: the spiny mouse (*Acomys cahirinus*). *American journal of obstetrics and gynecology*, 216(1), 40-e1.
- Benammi M., Calvo M., Prévot M., Jaeger J.J. (1996). Magnetostratigraphy and paleontology of Aït Kandoula basin (High Atlas, Morocco) and the African-European late Miocene terrestrial fauna exchanges. *Earth and Planetary Science Letters*, 145(1), 15-29.

- Bonnefille R. (2010). Cenozoic vegetation, climate changes and hominid evolution in tropical Africa. *Global and Planetary Change*, *72*(4), 390-411.
- Bosworth W., Huchon P., McClay K. (2005). The red sea and gulf of aden basins. *Journal of African Earth Sciences*, 43(1), 334-378.
- Bradshaw W.E., Holzapfel, C.M. (2007). Evolution of animal photoperiodism. Annu. 474 *Rev. Ecol. Evol. Syst.*, 38, 1-25.
- Brown J.H., Lomolino M.V. (1998). Biogeography, 2nd edn. Sinauer Associates, Sunderland, MA, USA.
- Broza M., Nevo E. (1994). Selective land snail predation by the spiny mouse, Acomys cahirinus, in Nahal Oren, Mt. Carmel, Israel. *Israel Journal of Zoology*, *40*(2), 173-176.
- Brunet M., Guy F., Pilbeam D., Lieberman D.E., Likius A., Mackaye H.T. Vignaud P. (2005). New material of the earliest hominid from the Upper Miocene of Chad. *Nature*, *434*(7034), 752-755.
- Brunjes P.C. (1990). The precocial mouse, *Acomys cahirinus. Psychobiology,* 18(3), 339-350.
- Bryja J., Mikula O., Šumbera R., Meheretu Y., Aghová T., Lavrenchenko L.A., Amundala N. (2014). Pan-African phylogeny of Mus (subgenus *Nannomys*) reveals one of the most successful mammal radiations in Africa. *BMC evolutionary biology*, 14(1), 256.
- Carere C., Casetti R., de Acetis L., Parretta G., Cirulli F., Alleva E. (1999). Behavioural and nociceptive response in male and female spiny mice (Acomys cahirinus) upon exposure to snake odour. *Behavioural Processes,* 47:1-10.
- Cerling T.E. (1992). Development of grasslands and savannas in East Africa during the Neogene. *Palaeogeography Palaeoclimatology Palaeoecology,* 97, 241–247.
- Cerling T.E., Harris J.M., MacFadden B.J., Leakey M.G., Quade J., Eisenmann, V., Ehleringer J.R. (1997). Global vegetation change through the Miocene/Pliocene boundary. *Nature*, *389*(6647), 153-158.
- Cerling T.E., Wang Y., Quade J. (1993). Expansion of C4 ecosystems as an indicator of global ecological change in the late Miocene. *Nature*, *361*(6410), 344-345.

- Chernova O.F., Kuznetsov, G.V. (2001). Structural features of spines in some rodents (Rodentia: Myomorpha, Hystricomorpha). *Biology Bulletin of the Russian Academy of Sciences*, *28*(4), 371-383.
- Chevret P., Catzefli, F., Michaux J. (2001). " Acomyinae": new molecular evidences for a muroid taxon (Rodentia: Muridae). *African small mammals*, 109-+.
- Chevret P., Denys C., Jaeger J.J., Michaux J., Catzeflis F.M. (1993). Molecular evidence that the spiny mouse (Acomys) is more closely related to gerbils (Gerbillinae) than to true mice (Murinae). *Proceedings of the National Academy of Sciences*, *90*(8), 3433-3436.
- Chevret P., Dobigny G. (2005). Systematics and evolution of the subfamily Gerbillinae (Mammalia, Rodentia, Muridae). *Molecular phylogenetics and evolution*, *35*(3), 674-688.
- Cosentino D., Buchwaldt R., Sampalmieri G., Iadanza A., Cipollari P., Schildgen T.F., Hinnov L.A., Ramezani J., Bowring S.A. (2013). Refining the Mediterranean “Messinian gap” with high-precision U-Pb zircon geochronology, central and northern Italy. *Geology*, 41(3), 323-326.
- Degen A.A. (1994). Field metabolic rates of *Acomys russatus* and *Acomys cahirinus*, and a comparison with other rodents. *Israel Journal of Ecology and Evolution*, 40(2), 127-134.
- Dempster E.R., Perrin M.R., Nuttall R.J. (1992). Postnatal development of three sympatric small mammal species of southern Africa. *Zeitschrift für Säugetierkunde*, 57: 103-111.
- Denys C. (1985). Paleoenvironmental and paleobiogeographical significance of the fossil rodent assemblages of Laetoli (Pliocene, Tanzania). *Palaeogeography, Palaeoclimatology and Palaeoecology*, 52: 77-97.
- Denys C., Taylor P.J., Aplin K.P. (2017). Family Muridae. In: Wilson et al. (Eds.) *Hadbook of the Mammals of the World*, Volume 7: Rodents II. Lynx Edicions. ISBN-13: 9788416728046
- Dewsbury D.A., Hodges A.W. (1987). Copulatory behavior and related phenomena in spiny mice (*Acomys cahirinus*) and hopping mice (*Notomys alexis*). *Journal of Mammalogy,* 68(1): 49-57.

- Dieterlen F. (1961). Beitrage zur Biologie der Stachelmaus, *Acomys cahirinus dimidiatus* Cretzschmar. *Z. Saugertierk26*, 1-13.
- Dieterlen F. (1962). Geburt und Geburtshilfe bei der Stachelmaus, *Acomys cahirinus. Ethology*, 19(2), 191-222.
- Dieterlen F. (1963). Vergleichende Untersuchungen zur Ontogenese von Stachelmaus (*Acomys*) und Wanderratte (Rattus norvegicus). *Z. Saugetierk*, 28(4).
- Duggen S., Hoernle K., Van Den Bogaard P., Rüpke L., Morgan J.P. (2003). Deep roots of the Messinian salinity crisis. *Nature*, *422*(6932), 602-606.
- Edut S., Eilam D. (2003). Rodents in open space adjust their behavioral response to the different risk levels during barn-owl attack. *BMC ecology*, 3(1), 10.
- Ehrhardt N., Heldmaier G., Exner C. (2005). Adaptive mechanisms during food restriction in *Acomys russatus*: the use of torpor for desert survival. *Journal of Comparative Psychology B*, 175: 193-200.
- Eilam D., Dayan T., Ben-Eliyahu S., Schulman I., Shefer G., Hendrie C.A. (1999). Differential behavioural and hormonal responses of voles and spiny mice to owl calls. *Animal Behaviour*, *58*(5), 1085-1093.
- Ellerman J.R. (1941). The families and genera of living rodents. Vol. 2. British Museum (Natural History), London.
- Elwood R.W. Broom D.M. (1978). The influence of litter size and parental behaviour on the development of Mongolian gerbil pups. *Animal Behaviour*, 26, 438-454.
- Fernandes C.A., Rohling E.J., Siddall M. (2006). Absence of post-Miocene Red Sea land bridges: Biogeographic implications. *Journal of Biogeography*, 33(6), 961-966.
- Frýdlová P., Palupčíková, K., Awale A.I., Frynta D. (2019). The first report of spiny mouse belonging to *Acomys ignitus* group in Somaliland: Phylogenetic affinities of a new distinct mitochondrial lineage. Manuscript submitted for publication.
- Frynta D., Fraňková M., Čížková B., Skarlandtová H., Galeštoková K., Průšová K., Šmilauer P., Šumbera R. (2011). Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*). *Acta Theriologica*, 56(3), 289-295.

- Frynta D., Palupčíková K., Bellinvia E., Benda P., Skarlandtová H., Schwarzova L., Modrý D. (2010). Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula. *Zootaxa*, *2660*, 46-56.
- Frynta D., Palupčíková K., Elmi H. S. A., Awale A.I., Frýdlová P. (2019). Molecular characterization of *Acomys louisae* from Somaliland: A deep divergence and contrasting genetic patterns in a rift zone. Manuscript sumbitted for publication.
- Frynta D., Sádlová J., (1998). Hybridisation experiments in spiny mice from the Eastern Mediterranean: Systematic position of *Acomys cilicicus* revisited. Z. Säugetierk. 63 (Suplementum) 18.
- Ghienne J.F., Schuster M., Bernard A., Duringer P., Brunet M. (2002). The Holocene giant Lake Chad revealed by digital elevation models. *Quaternary International*, 87(1), 81-85.
- Goudie A.S. (2005). The drainage of Africa since the Cretaceous. *Geomorphology*, 67(3), 437-456.
- Gutman R., Dayan T. (2005). Temporal partitioning: an experiment with two species of spiny mice. *Ecology* 86: 164-173.
- Hadid Y., Pavlíček T., Beiles A., Ianovici R., Raz S., Nevo E. (2014). Sympatric incipient speciation of spiny mice *Acomys* at "Evolution Canyon," Israel. *Proceedings of the National Academy of Sciences*, 111(3), 1043-1048.
- Haim A., Yedidia I., Haim D., Zisapel N. (1994). Photoperiodicity in daily rhythms of body temperature, food and energy intake of the golden spiny mouse (*Acomys russatus*). *Israel Journal of Zoology* 40: 145-150.
- Haq B.U., Hardenbol J., Vail P.R. (1987). Chronology of fluctuating sea levels since the Triassic. *Science*, 235(4793), 1156-1167.
- Harvey P H., Pagel M.D. (1991). The comparative method in evolutionary biology (Vol. 239). Oxford: *Oxford university press*.
- Haughton C.L., Gawriluk T.R., Seifert A.W. (2016). The biology and husbandry of the African spiny mouse (*Acomys cahirinus*) and the research uses of a laboratory colony. *Journal of the American Association for Laboratory Animal Science*, 55(1), 9-17.

- Hendrie C.A., Weiss S.M., Eilam D. (1998). Behavioural response of wild rodents to the calls of an owl: a comparative study. *Journal of Zoology*, 245(4), 439-446.
- Hill A., Leakey M., Kingston J.D., Ward S. (2002). New cercopithecoids and a hominoid from 12· 5Ma in the Tugen Hills succession, Kenya. *Journal of Human Evolution*, *42*(1-2), 75-93.
- Holbourn A., Kuhnt W., Schulz M., Erlenkeuser H. (2005). Impacts of orbital forcing and atmospheric carbon dioxide on Miocene ice-sheet expansion. *Nature*, *438*(7067), 483-487.
- Holt B.G., Lessard J.P., Borregaard M. K., Fritz S.A., Araújo M.B., Dimitrov D., Fabre P.H., Graham C.H., Graves G.R., Jønsson K.A., Nogués-Bravo D., Nogués-Bravo D., Wang Z., Whittaker R. J., Fjeldså J., Rahbek C. (2012). An update of Wallace's zoogeographic regions of the world. *Science*, 1228282.
- Horowitz M., Borut A. (1994). The spiny mouse (*Acomys cahirinus*) a rodent prototype for studying plasma volume regulation during thermal dehydration. *Israel Journal of Zoology,* 40: 117-125.
- Hsü K.J., Cita M.B., Ryan W.B.F. (1973). The origin of the Mediterranean evaporites. In: Ryan, W.B.F., Hsü, K.J., et al. (Eds.), Initial Rep. Deep Sea Drill. Prog., vol. 13. U.S. Govt. Printing Office, Washington, DC, pp. 1203–1231.
- Hsü K.J., Montadert L., Bernouilli D., Cita M.B., Erikson A., Garrison R.G., Kidd R.B., Mélières F., Müller C., Wright R. (1978). History of the Mediterranean salinity crisis. In: Hsü K.J., Montadert L. et al. (Eds.), Initial Rep. Deep Sea Drill. Proj., 42A. US Government Printing Office, Washington, DC, pp. 1053–1078.
- Huang Y.A., Street-Perrott F.A., Metcalfe S.E., Brenner M., Moreland M., Freeman K.H. (2001). Climate change as the dominant control on glacial-interglacial variations in C3 and C4 plant abundance. *Science*, *293*(5535), 1647-1651.
- Jansa S. A., Weksler M. (2004). Phylogeny of murid rodents: relationships within and among major lineages as determined by IRBP gene sequences. *Molecular Phylogenetics and Evolution*, 31: 256-276.
- Janus C. (1988). The development of responses to naturally occurring odours in spiny mice *Acomys cahirinus*. *Animal behaviour*, *36*(5), 1400-1406.

- Jiang T.X., Harn H.I.C., Ou K.L., Lei M., Chuong C.M. (2019). Comparative regenerative biology of spiny (*Acomys cahirinus*) and laboratory (Mus musculus) mouse skin. Experimental *dermatology*, *28*(4), 442-449.
- Jones M., Dayan T. (2000). Foraging behavior and microhabitat use by spiny mice, *Acomys cahirinus* and *A. russatus*, in the presence of Blanford's fox (Vulpes cana) odor. *Journal of Chemical Ecology*, 26(2), 455-469.
- Junginger A., Trauth, M.H. (2013). Hydrological constraints of paleo-Lake Suguta in the northern Kenya rift during the African humid period (15e5 ka BP). *Glob. Planet. Change,* 111, 174e188.
- Kam M.K., Decen A.A. (1993). Effect of dietary preformed water on energy and water budgets of two sympatric desert rodents, *Acomys russatus* and *Acomys calzivinus*. *Journal of Zoology*, 231(1), 51-59.
- Kingdon J., Happold D., Butynski T., Hoffmann M., Happold M., Kalina J. (eds) (2013). *Mammals of Africa* (Vol. 6). Bloomsbury Publishing, London.
- Kronfeld-Schor N., Dayan T. (1999). The dietary basis for temporal partitioning: food habits of coexisting Acomys species. *Oecologia*, *121*(1), 123-128.
- Kronfeld-Schor N., Dayan T., Elvert R., Haim A., Zisapel N., Heldmaier G. (2001). On the use of the time axis for ecological separation: diel rhythms as an evolutionary constraint. *The American Naturalist*, *158*(4), 451-457.
- Kryštufek B., Vohralík V., Janžekovič F. (2009). Mammals of Turkey and Cyprus: Rodentia II*: Cricetinae, Muridae, Spalacidae, Calomyscidae, Capromydae, Hystricidae, Castoridae*. Univerza na Primorskem, Znanstveno-raziskovalno središče, Založba Annales.
- Kunze B., Dieterlen F., Traut W., Winking H. (1999). Karyotype relationship among four species of spiny mice (*Acomys*, Rodentia). *Zeitschrift für Säugetierkunde*, 64: 220-229.
- Lambeck K. (1996). Shoreline reconstructions for the Persian Gulf since the last glacial maximum. *Earth and Planetary Science Letters*, *142*(1-2), 43-57.
- Linder H.P., de Klerk H.M., Born J., Burgess N.D., Fjeldså J., Rahbek C. (2012). The partitioning of Africa: statistically defined biogeographical regions in sub-Saharan Africa. *Journal of Biogeography*, *39*(7), 1189-1205.
- Lorenzen E.D., Heller R., Siegismund H.R. (2012). Comparative phylogeography of African savannah ungulates. *Molecular ecology*, *21*(15), 3656-3670.

- Macholán M., Zima J., Červená A., Červený, J. (1995). Karyotype of *Acomys cilicicus* Spitzenberger, 1978 (Rodentia, Muridae). *Mammalia*, 59(3), 397-402.
- Makin J. W., Porter R. H. (1984). Paternal behavior in the spiny mouse (*Acomys cahirinus*). *Behavioral and Neural Biology,* 41: 135-151.
- Mandelik Y., Jones M., Dayan T. (2000). The effect of moonlight on foraging behavior and habitat use of the common spiny mouse, *Acomys cahirinus*. Israel Journal of Zoology.
- Maslin M.A., Brierley C.M., Milner A.M., Shultz S., Trauth M.H., Wilson K.E. (2014). East African climate pulses and early human evolution. *Quaternary Science Reviews*, 101, 1-17.
- Maslin M.A., Li X.S., Loutre M.F., Berger A. (1998). The contribution of orbital forcing to the progressive intensification of Northern Hemisphere glaciation. *Quaternary Science Reviews*, *17*(4-5), 411-426.
- Matias Santos D., Rita A.M., Casanellas I., Brito Ova A., Araújo I.M., Power D., Tiscornia G. (2016). Ear wound regeneration in the African spiny mouse *Acomys cahirinus*. *Regeneration*, 3(1), 52-61.
- Matthey R. (1968). Cytogénétique et taxonomie du genre *Acomys A. percivali* Dollman et *A. wilsoni* Thomas, espéces d'Abyssinie. *Mammalia*, 32(4), 621-627.
- Mayr E. Co je evoluce. 1. vyd. Praha: *Academia*, 2009. 354 s.
- Medger K., Chimimba C.T., Bennett N.C. (2012). Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa. *Mammalian Biology-Zeitschrift für Säugetierkunde*, 77(3), 153-159.
- Mein P., Pickford M., Senut B. (2000). Late Miocene micromammals from the Harasib karst deposits, Namibia. Part 1- Large muroids and non-muroids rodents. Communications of the Geological Survey of Namibia 12: 375-390.
- Merkt J.R., Taylor C.R. (1994). " Metabolic switch" for desert survival. *Proceedings of the National Academy of Sciences*, 91(25), 12313-12316.
- Mescher A.L. (2017). Macrophages and fibroblasts during inflammation and tissue repair in models of organ regeneration. *Regeneration*, 4(2), 39-53.
- Monadjem A., Taylor P.J., Denys C., Cotterill F.P. (2015). *Rodents of sub-Saharan Africa: a biogeographic and taxonomic synthesis*. Walter de Gruyter GmbH and Co KG.

- Morley R.G. (2000). Geological Evolution of the Tropical Rain Forests. John Wiley and Sons, London.
- Neal B.R. (1984). Seasonal feeding habits of small mammals in Kenya. *Zeitschrift für Säugetierkunde*, 49(4), 226-234.
- Nicholson S.E. (1993). An overview of African rainfall fluctuations of the last decade. *Journal of climate*, 6(7), 1463-1466.
- Nováková M., Palme R., Kutalová H., Janský L., Frynta D. (2008). The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*). *Physiology and behavior*, 95(1), 187-193.
- Nováková M., Vašáková B., Kutalová H., Galeštoková K., Průšová K., Šmilauer P., Šumbera R., Frynta D. (2010). Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: *Acomys*). *Behavioral ecology and sociobiology*, 64(3), 371-379.
- Partridge T.C., Wood B.A., DeMenocal P.B. (1995). The influence of global climatic change and regional uplift on large-mammalian evolution in east and southern Africa. *Paleoclimate and evolution, with emphasis on human origins*, 331-355.
- Peitz B. (1981). The oestrous cycle of the spiny mouse (*Acomys cahirinus*). *Reproduction*, *61*(2), 453-459.
- Perrin M.R., Downs C.T. (1994). Comparative aspects of the thermal biology of the Cape spiny mouse, *Acomys subspinosus*, and the common spiny mouse, *A. spinosissimus*. *Israel Journal of Ecology and Evolution*, *40*(2), 151-160.
- Poore R.Z., Sloan L.C. (1996). Introduction climates and climate variability of the Pliocene. *Marine Micropaleontology*, *27*(1-4), 1-2.
- Porter R.H. (1986). Chemical Signals and Kin Recognition in Spiny Mice (*Acomys cahirinus*). *In Chemical Signals in Vertebrates 4* (pp. 397-411). Springer, Boston, MA.
- Porter R.H., Cavallaro S.A., Moore J.D. (1980). Developmental parameters of mother-offspring interactions in *Acomys cahirinus*. *Zeitschrift für Tierpsychologie*, 53(2), 153-170.
- Porter R.H., Ruttle K. (1975). The responses of one-day old *Acomys cabirinus* pups to naturally occurring chemical stimuli. *Zeitschrift fur Tierpsychologie*, 38(2), 154-162.

- Porter R.H., Wirick M. (1979). Sibling recognition in spiny mice (*Acomys cahirinus*): influence of age and isolation. *Animal Behaviour,* 27: 761-766.
- Prista G., Estevens M., Agostinho R., Cachão M. (2014). Euro-North African Sirenia biodiversity as a response to climate variations. Palaeogeography, Palaeoclimatology, *Palaeoecology*, 410, 372-379.
- Redfield T.F., Wheeler W.H., Often M. (2003). A kinematic model for the development of the Afar Depression and its paleogeographic implications. *Earth and Planetary Science Letters*, 216(3), 383-398.
- Rögl F. (1999). Mediterranean and Paratethys. Facts and hypotheses of an Oligocene to Miocene paleogeography (short overview). *Geologica carpathica*, 50(4), 339-349.
- Sarli J., Lutermann H., Alagaili A.N., Mohammed O.B., Bennett N.C. (2016). Seasonal reproduction in the Arabian spiny mouse, *Acomys dimidiatus* (Rodentia: Muridae) from Saudi Arabia: the role of rainfall and temperature. *Journal of arid environments*, 124, 352-359.
- Sayre R., Comer P., Hak J., et al. (2013). A new map of standardized terrestrial ecosystems of Africa. Washington, DC: Association of American Geographers. *African Geographical Review,* 24 pp.
- Schuster M., Duringer P., Ghienne J.F., Roquin C., Sepulchre P., Moussa A., Brunet M. (2009). Chad Basin: paleoenvironments of the Sahara since the Late Miocene. *Comptes Rendus Geoscience*, 341(8), 603-611.
- Schuster M., Duringer P., Ghienne J.F., Vignaud P., Mackaye H.T., Likius A., Brunet M. (2006). The age of the Sahara desert. *Science*, 311(5762), 821-821.
- Seifert A.W., Kiama S.G., Seifert M.G., Goheen J.R., Palmer T.M., Maden M. (2012). Skin shedding and tissue regeneration in African spiny mice (*Acomys*). *Nature*, 489(7417), 561-565.
- Seifert A.W., Maden M. (2014). New insights into vertebrate skin regeneration. In *International review of cell and molecular biology* (Vol. 310, pp. 129-169). Academic Press.
- Sepulchre P., Ramstein G., Fluteau F., Schuster M., Tiercelin J.J., Brunet M. (2006). Tectonic uplift and Eastern Africa aridification. *Science*, *313*(5792), 1419-1423.

- Shafrir E. (2000). Overnutrition in spiny mice (*Acomys cahirinus*): β-cell expansion leading to rupture and overt diabetes on fat-rich diet and protective energy-wasting elevation in thyroid hormone on sucrose-rich diet. *Diabetes/metabolism research and reviews*, 16(2), 94-105.
- Shafrir E., Ziv E., Kalman R. (2006). Nutritionally induced diabetes in desert rodents as models of type 2 diabetes: *Acomys cahirinus* (spiny mice) and *Psammomys obesus* (desert gerbil). *ILAR journal*, 47(3), 212-224.
- Shargal E., Rath-Wolfson L., Kronfeld N., Dayan T. (1999). Ecological and histological aspects of tail loss in spiny mice (Rodentia: Muridae, *Acomys*) with a review of its occurrence in rodents. *Journal of Zoology*, *249*(2), 187-193.
- Simkin J., Gawriluk T.R., Gensel J.C., Seifert A.W. (2017). Macrophages are necessary for epimorphic regeneration in African spiny mice. *Elife*, 6, e24623.
- Simon B., Guillocheau F., Robin C., Dauteuil O., Nalpas T., Pickford M., et al. (2017). Deformation and sedimentary evolution of the Lake Albert Rift (Uganda, East African Rift System). *Marine and Petroleum Geology*, 86, 17-37.
- Steckler M.S., Berthelot F., Lyberis N., Le Pichon X. (1988). Subsidence in the Gulf of Suez: implications for rifting and plate kinematics. *Tectonophysics*, 153(1-4), 249-270.
- Steppan S.J., Schenk J.J. (2017). Muroid rodent phylogenetics: 900-species tree reveals increasing diversification rates. *PloS one*, *12*(8), e0183070.
- Swezey C.S. (2009). Cenozoic stratigraphy of the Sahara, northern Africa. *Journal of African Earth Sciences*, *53*(3), 89-121.
- Szijarto K., Coffee R.J., Boyle C., Bailey D., Mulé M., Iacovone D., Deni R. (1985). Postpartum social interactions in families of spiny mice (*Acomys cahirinus*) observed in a laboratory environment. *Bulletin of the Psychonomic Society*, 23(3), 253-255.
- Tieszen L.L., Senyimba M.M., Imbamba S.K., Troughton J.H. (1979). The distribution of C3 and C4 grasses and carbon isotope discrimination along an altitudinal and moisture gradient in Kenya. *Oecologia*, 37(3), 337-350.
- Trauth M.H., Maslin M.A., Deino A.L., Junginger A., Lesoloyia M., Odada E.O., Olago D.O., Olaka L.A., Strecker M.R., Tiedemann R. (2010). Human evolution in a variable environment: the amplifier lakes of Eastern Africa. *Quaternary Science Reviews*, 29(23), 2981-2988.

- Uchupi E., Swift S.A., Ross D.A. (1999). Late Quaternary stratigraphy, paleoclimate and neotectonism of the Persian (Arabian) Gulf region. *Marine Geology*, *160*(1-2), 1-23.
- Van Damme D., Van Bocxlaer B. (2009). Freshwater molluscs of the Nile Basin, past and present. In *The Nile* (pp. 585-629). Springer Netherlands.
- Veldkamp A., Buis E., Wijbrans J.R., Olago D.O., Boshoven E.H., Marée M., van Saparoea R.V.D.B. (2007). Late Cenozoic fluvial dynamics of the River Tana, Kenya, an uplift dominated record. *Quaternary Science Reviews*, *26*(22), 2897-2912.
- Verheyen W., Hulselmans J., Wendelen W., Leirs H., Corti M., Backeljau T. Verheyen E. (2011). Contribution to the systematics and zoogeography of the East–African *Acomys spinosissimus* Peters 1852 species complex and the description of two new species (Rodentia: Muridae). *Zootaxa*, *3059*, 1-35.
- Volobouev V., Auffray J.C., Debat V., Denys C., Gautun J.C., Tranier M. (2007). Species delimitation in the *Acomys cahirinus-dimidiatus* complex (Rodentia, Muridae) inferred from chromosomal and morphological analyses. *Biological Journal of the Linnean Society,* 91: 203-214.
- Weissenberg S., Shkolnik A. (1994). Metabolic rate and water economy in the desert and mediterranean populatinos of the common spiny mouse (*Acomys cahirinus*) in Israel. *Israel Journal of Zoology,* 40: 135-143.
- Winkler A.J. (1994). The middle/upper Miocene dispersal of major rodent groups between southern Asia and Africa. In (Y. Tomida, C. Li, T. Setoguchi, Eds) *Rodent and Lagomorph Families of Asian Origins and Diversification*, pp. 173–184. Tokyo: National Science Museum Monographs No. 8.
- Winkler A.J. (2002). Neogene paleobiogeography and East African paleoenvironments: contributions from the Tugen Hills rodents and lagomorphs. *Journal of Human Evolution*, 42(1-2), 237-256.
- Wube T., Fares F., Haim A. (2008b). A differential response in the reproductive system and energy balance of spiny mice *Acomys* populations to vasopressin treatment. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, *151*(4), 499-504.

- Wube T., Haim A., Fares F. (2008a). Reproductive response of xeric and mesic populations of the spiny mouse *Acomys* to photoperiod acclimation. *Journal of arid environments*, *72*(4), 440-447.
- Wube T., Haim A., Fares F. (2009). Effect of increased dietary salinity on the reproductive status and energy intake of xeric and mesic populations of the spiny mouse, *Acomys*. *Physiology and behavior*, 96(1), 122-127.
- Zachos J., Pagani M., Sloan L., Thomas E., Billups K. (2001). Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science*, 292(5517), 686-693.

# 8 Závěr

Předložená disertační práce přináší nové poznatky o distribuci a fylogenezi vzájemných vztahů bodlinatých myší rodu *Acomys*. Bodlinaté myši rodu *Acomys* jsou rozšířeny hlavně na suchých otevřených stanovištích v Africe a na Středním východě. V poslední době jsou široce využívány jako modelová zvířata pro různé biologické výzkumy. Je tedy důležité znát vzájemné fylogenetické vztahy jednotlivých druhů.

Z dosažených výsledků vyplývá, že bodlinaté myši rodu *Acomys* mají své kořeny ve východní Africe, odkud se postupně šířily (v pozdním miocénu) a speciovaly nejen v rámci Afriky, ale i na blízký východ a středomoří. K těmto šířícím se událostem mohlo dojít za současného vzniku vhodných klimatických a vegetačních podmínek a někdy i za vzniku pevninského mostu, nebo rozvojem námořní dopravy a tím k šíření společně s lidmi. Pravděpodobně došlo celkem ke třem nezávislým kolonizačním událostem mimo Afriku. Nejstarším kolonizátorem mimo Afriku byl *A. russatus,* v současnosti se vyskytující na Sinajském poloostrově a na Středním východě. Mnohem později kolonizoval Arabský poloostrov *A. dimidiatus* pravděpodobně přes úžinu Bab al-Mandab, ležící mezi Afrikou a Jemenem. Poslední vlna kolonizátorů byla pravděpodobně z Egypta, kdy komenzální populace *A. cahirinus* zamířila na středomořské ostrovy a pobřeží Turecka. Velmi složité a proměnlivé prostředí, jež bylo ve východní Africe, zapříčinilo vznik několika druhů bodlinatých myší na malém území.

Složitost tohoto území se odráží i na sekvenční variabilitě „Džibutské“ podskupiny druhu *A.louisae* sensu lato. v severozápadním Somalilandu a přilehlých oblastech Džibuti a Etiopie. Ta kontrastuje s překvapivou uniformitou tohoto druhu na území středního a východního Somalilandu.

Z celkového, dnes dostupného souboru molekulárních znaků byla stanovena existence celkem pěti hlavních linií bodlinatých myší rodu *Acomys*: *cahirinus*, *wilsoni, russatus*, *spinosissimus, subspinosus*, z nichž jsme rozlišili 27-28 výlučných genetických linií pravděpodobně odpovídajícím jednotlivým druhům rodu *Acomys*.

Bodlinaté myši rodu *Acomys* ve většině případů sdílí velmi podobnou morfologii a jejich rozpoznání přímo v terénu je podle dostupných klíčů téměř nemožné. Jejich správné druhové určení se zatím neobejde bez předchozí molekulární analýzy.

Složitost geologické historie některých afrických území velmi znesnadňuje pochopení historie areálů jednotlivých druhů / linií myší. Obzvláště složitá je situace v oblasti Východoafrické příkopové propadliny a jí přilehlých území, jako je Africký roh Somálska nebo spojení Arabského poloostrova s Afrikou přes blok Danakilu.

Během svého studia jsem participovala na výzkumu sekundárního poměru vrhu pohlaví a velikosti vrhu u bodlinatých myší rodu *Acomys*. Zároveň jsem také dělala podobné molekulární práce na dalších zvířatech: *Anolis*, *Cuora*, *Mauremys*, *Orlitia*, což dokresluje můj odborný profil viz publikace v příloze 1.

# 9 Příloha č. 1: Publikace dokreslující odborný profil

Následující publikace nejsou součástí této disertační práce zpravidla proto, že tematicky nezapadají do vymezení disertačního projektu. Jsou zde přiloženy výhradně pro dokreslení odborného profilu uchazečky.

Práce jsou seřazeny chronologicky od nejmladší po nejstarší. Poslední dvě publikace se týkají bodlinatých myší rodu *Acomys*, kde jsem pomáhala sbírat data pro statistiku v rámci výzkumu poměru pohlaví a velikosti vrhu. Zbylé čtyři práce jsou z větší či menší části molekulárního charakteru a právě tuto část jsem zajistila v celé říši společně s jinou toho času studentkou, bakalářského studia, později magisterského studia Barborou Somerovou.

## 9.1 Holáňová Zahradníčková V., Abramjan A., Palupčíková K., Rehák I., Frynta D. (2017) Discovering an Antillean Anolis (Squamata: Polychrotidae) with contrasting sexual dichromatism in otherwise sexually monomorphic "chamaeleolis" group. *Acta Societatis Zoogicae Bohemicae*. 81: 31-47.

The anole (genus Anolis Daudin, 1802) dewlap is a rapidly evolving trait. Sexually dichromatic anole species usually occur in the mainland, while the island species display only little dichromatism in particular. The so-called "chamaeleolis" group of anoles endemic to Cuba Island, traditionally classified as the 'twig giant' ecomorph, consists of large, slow and very cryptic species with very similar sexes. Our study describes a new population of "chamaeleolis" anoles which, unlike other related species, display a surprising sexual dichromatis in dewlaps. Males have conspicuously red dewlaps, while the dewlaps of females are whitish. We compared the specimens from the newly discovered populations with related Anolis barbatus Garrido, 1982, A. chamaeleonides Duméril et Bibron, 1837, A. guamuhaya Garrido, Pérez-Beato et Moreno, 1991 and A. porcus Cope, 1864 through the means of spectrophotometry, visual modelling, morphology and mtDNA analysis. The results show that the red coloration substantially increases both chromatic and achromatic contrasts, while the dichromatism in the remaining species is only in the achromatic channel, if any. Both genetic and morphometric comparisons suggest distinctness of the dichromatic populations which may represent a separate species. The reason for the unusual dewlap coloration remains unclear, though an ecological explanation is discussed.

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# Discovering an Antillean *Anolis* (Squamata: Polychrotidae) with contrasting sexual dichromatism in otherwise sexually monomorphic "chamaeleolis" group

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**Abstract**. The anole (genus *Anolis* Daudin, 1802) dewlap is a rapidly evolving trait. Sexually dichromatic anole species usually occur in the mainland, while the island species display only little dichromatism in particular. The so-called "chamaeleolis" group of anoles endemic to Cuba Island, traditionally classified as the 'twig giant' ecomorph, consists of large, slow and very cryptic species with very similar sexes. Our study describes a new population of "chamaeleolis" anoles which, unlike other related species, display a surprising sexual dichromatis in dewlaps. Males have conspicuously red dewlaps, while the dewlaps of females are whitish. We compared the specimens from the newly discovered populations with related *Anolis barbatus* Garrido, 1982, *A. chamaeleonides* Duméril et Bibron, 1837, *A. guamuhaya* Garrido, Pérez-Beato et Moreno, 1991 and *A. porcus* Cope, 1864 through the means of spectrophotometry, visual modelling, morphology and mtDNA analysis. The results show that the red coloration substantially increases both chromatic and achromatic contrasts, while the dichromatism in the remaining species is only in the achromatic channel, if any. Both genetic and morphometric comparisons suggest distinctness of the dichromatic populations which may represent a separate species. The reason for the unusual dewlap coloration remains unclear, though an ecological explanation is discussed.

**Key words**. Sexual size dimorphism, body shape, mtDNA, reflectance, *Anolis*, *Chamaeleolis*, Cuba, Neotropical Region.

## INTRODUCTION

Coloration of males and females differs considerably in many animal species. This sexual dichromatism is a specific form of sexual dimorphism. Sexual dichromatism has attracted attention of researchers and theoreticians for centuries (e.g., Darwin 1871), but the phylogenetic studies analysing macroevolutionary patterns of sexual dichromatism within major clades of terrestrial vertebrates appeared only recently (frogs: Bell & Zamudio 2012; lizards: Stuart–Fox & Ord 2004; snakes: Shine & Madsen 1994; turtles: Liu et al. 2013; birds: Badayev & Hill 2003, Stoddard & Prum 2011).

The evolution of sexual dichromatism is usually explained by the two following opposing selective forces: (1) sexual selection favouring conspicuous coloration of the sex with higher variance in its reproductive output, typically males; (2) predation pressure favouring cryptic coloration in both sexes, but more strongly in females which are vulnerable to predation during gravidity and/or maternal care (Darwin 1871, for a review see also Stutchbury & Morton 2001). Risks for females associated with mating and male harassment were currently proposed as a driving force leading to the reduction of conspicuous ornamentation in females (Hosken et al. 2016).

Anoles are one of the best model systems for the evolutionary, ecological and phylogenetic studies of morphological traits including sexual dimorphism and dichromatism (for a review see Losos 2009). The most important part of anole body for the study of dichromatism is the dewlap. Dewlap is a flap of skin under the throat of the lizard that is extended and retracted rapidly during signalling and has an important species recognition functions (Ord & Martins 2006, Ng & Glor 2011, Ng et al. 2013). In many anole species females have smaller and/or less conspicuously coloured dewlaps than males (Harrison & Poe 2012). Nevertheless, fundamental sexual differences in dewlap coloration and pattern are rare and such cases are mostly reported from mainland anoles (Köhler 2003, Ugueto et al. 2007, 2009, Köhler et al. 2014).

Intersexual differences in dewlaps could be understood as a result of different pressures on males and females. While the reproductive success of males is determined mostly by success in mate acquisition which is positively associated with signalling, female success depends mainly on egg production and survival. This results in different design of dewlaps between sexes (Vanhooydonck et al. 2009). Females generally use their dewlaps much less than males and rarely in reproductive context (Nunez et al. 1997, Losos 2009, Johnson & Wade 2010). Females also exhibit no relationship between display behaviour and dewlap morphology.

Thus, the use of the dewlap is not associated with the size of the cartilaginous structures that support its movement (Johnson et al. 2011). Harrison & Poe (2012) focused on females dewlap size variation in huge comparative study and found evidence that females have large dewlaps in species with little sexual size dimorphism, while having small or no dewlaps in species with wider sexual size dimorphism. From all Antillean ecomorphs, the largest dewlaps are found in crown-giants and twig anoles. One hypothesis for this phenomenon is that large dewlaps are essential in dense habitats as they would be seen better, and second hypothesis is based on low densities of these two ecomorphs – larger dewlaps facilitate long distance communication (Harrison & Poe 2012).

In this study we focus on the "chamaeleolis" group endemic to Cuba Island (Schettino 2003, Losos 2009). They used to have their own ecomorph class named "twig giants" (Haas et al. 1993) referring to their unique body shape and lifestyle but they were moved to wide-size ecomorph class "twig anoles" (Beuttell & Losos 1999, Mahler et al. 2016). Nevertheless these striking lizards show list of unusual characteristics among other anoles- large body size, short limbs, huge bony casques on heads, lack of tail autotomy (Garrido & Schwartz 1968), independent eye movement, cryptic coloration and extraordinary scalation, molariform teeth in adults (Estes & Williams 1984, Schwartz & Henderson 1991) specialized for crushing snails (Schettino 1999, 2003, Herrel & Holáňová 2008) and cryptic and slow motion lifestyle (Leal & Losos 2000). Consequently, these anoles have been traditionally recognized as a distinct genus *Chamaeleolis* Cocteau, 1838. Because this group forms a derived internal clade of the genus *Anolis* Daudin, 1802 sensu lato, it is usually treated as a junior synonym of *Anolis*. The studies discovering the cladogenesis of the anoles have placed the genus *Chamaeleolis* within the main body of the tree of Antillean anoles. These form a sister group of a clade consisting of the Puerto Rican species *Anolis cuvieri* Merrem, 1820 and Hispaniolan *A. barahonae* Williams, 1962 and *A. christophei* Williams, 1960 (e.g., Haas et al. 1993, Poe 2004, Nicholson et al. 2005, 2012). In this study we discovered a population exhibiting fundamental sexual dichromatism, males possessing red dewlap strongly contrasting with white dewlaps of conspecific females.

The aim of this paper was (1) to describe dewlap coloration and reflectance spectra in the discovered dichromatic population and other four species/populations of anoles belonging to the "chamaeleolis" group; (2) to assess sexual dichromatism and sexual size dimorphism in the studied "chamaeleolis" species; (3) to analyse morphometric and genetic variation in this group and related anoles in order to discuss evolution of the dimorphic traits.

## MATERIAL AND METHODS

**Species determination**

The examined material (see below) was assigned to species according to morphological criteria (Holáňová et al. 2012). We provisionally determined the specimens of dichromatic population from vicinity of San German (Holguín province, Cuba) and Gran Piedra (Santiago de Cuba province, Cuba; Figs. 1–3) as *Anolis porcus*. Considering multiple distinct morphometric and genetic characters of these animals (see under the results), we further refer to this population as *Anolis* sp. In original description of *A. porcus* Cope, 1864 there is no information about dewlap coloration nor about type locality. We avoid taxonomic discussions concerning species identity of these specimens until a thorough revision of *A. porcus* sensu lato including properly localized materials will be performed. Clarification of the geographic origin of the holotype is needed prior to any nomenclatural suggestion.

**Spectrophotometry**

We measured 3 males and 2 females of *Anolis* sp. from San German population together with *Anolis barbatus* Garrido, 1982 (Soroa), *A. chamaeleonides* Duméril et Bibron, 1837 (Viñales), *A. guamuhaya* Garrido, Pérez-Beato et Moreno, 1991 (Topes de Collantes) and *A. porcus* (Baracoa), each represented by a single male and a single female. All specimens were obtained from collections of private European and Russian breeders. All were captive bred after parental animals with known original localities.

The dewlap colour reflectance was determined between 300 and 700 nm with an OceanOptics USB4000 spectrophotometer, using a PX-2 Pulsed Xenon lamp source. We used the Ocean Optics WS-1 white standard for calibration, which was performed after every three measurements. The probe was held in a constant perpendicular 5mm distance from the gently stretched dewlap skin and the measurements were performed in a darkened room. Each colour patch was measured 3 times and then calculated its mean reflectance value. For visual modelling, we used the photoreceptor sensitivity data for *Anolis lineatopus* Gray, 1840 (Loew et al. 2002, Marshall & Stevens 2014). We calculated both chromatic and achromatic

Figs. 1–3. 1 – Original locality of *Anolis* sp. at Gran Piedra, Santiago de Cuba province, Cuba (photo by VHZ). 2 and 3 – Wild adult male of *Anolis* sp. at locality Gran Piedra, Santiago de Cuba province, Cuba (both photos by VHZ).

Table 1. Genetic samples and their GenBank accession numbers

| samples | origin | GenBank Accession Numbers |
|---|---|---|
| *Anolis barbatus* AB 1 | Soroa | MF157534 |
| *Anolis barbatus* AB 2 | Soroa | MF157535 |
| *Anolis porcus* AP 1 | El Yunque | MF157536 |
| *Anolis porcus* AP 2 | Duaba | MF157537 |
| *Anolis porcus* AP 3 | Duaba | MF157538 |
| *Anolis guamuhaya* AG 1 | Topes de Collantes | MF157539 |
| *Anolis* sp. APS 6 | Gran Piedra | MF157540 |
| *Anolis* sp. APS 4 | Gran Piedra | MF157541 |
| *Anolis* sp. APS 5 | Gran Piedra | MF157542 |
| *Anolis sierramaestrae* AS | La Mula | MF157543 |
| *Anolis chamaeleonides* AC 3 | Viñales | MF157544 |
| *Anolis chamaeleonides* AC 2 | Viñales | MF157545 |
| *Anolis chamaeleonides* AC 1 | Viñales | MF157546 |
| *Anolis* sp. APS 1 | San German | MF157547 |
| *Anolis* sp. APS 2 | San German | MF157548 |
| *Anolis* sp. APS 3 | San German | MF157549 |
| *Anolis guamuhaya* AG 2 | Topes de Collantes | MF157550 |

contrasts between (1) "males and females" dewlaps (the pale colour), and (2) the pale and red colour within the dewlap of the males of *A.* sp. from San German. The chromatic contrast expressed in "just noticeable differences" (JND) was calculated according to Vorobyev & Osorio (1998); values below 1 indicate that two colours are unrecognizable within the particular visual system, values between 1–3 are considered to be distinguishable under ideal lighting conditions and with the increasing value the colours gradually become more distinct. Data for relative photoreceptor densities and the Weber fraction value 0.05 were taken from Marshall & Stevens (2014). The calculation of the achromatic contrast was performed after the model of Siddiqi et al. (2004) and Loyeau et al. (2007). All calculations were performed in Avicol v.6 (Gomez 2006).

**Morphometric traits**

We examined 128 adult anoles of the "chamaeleolis" group of which 83 were live animals or preserved specimens provided by European private breeders (18 *A. barbatus* from Soroa, Cuba; 23 *A. chamaeleonides* from Viñales, Cuba; 19 *A. guamuhaya* from Topes de Collantes, Cuba; 17 *A. porcus* Baracoa, Cuba and 6 *Anolis* sp. from San German, Cuba) and 44 were museum specimens from the herpetological collection of the National Museum in Prague (NMP), Czech Republic (list of museum specimens in Appendix 1).

The following measurements were made with digital callipers to the nearest 0.1 mm: snout-to-vent length (SVL: measured from the tip of the snout to the vent); body length (LIE: longitudo interextremitatis – the distance between front and hind legs); jaw out-lever distance (JOL: the distance between the jaw articulation and the tip of the jaw); head length (HL: measured from the edge of the head casque to tip of the snout); head width (HW: measured at the intersection with the angle of the jaws); head height (HH: measured just posterior to the orbits); snout-orbit distance (SO: the distance between the tip of the snout to the nearest point of the orbit ); snout-nostril distance (SN: the distance between the tip of the snout to the edge of the left nostril); snout-mouth end (SME: the distance from the tip of the snout to the corner of the mouth); lower jaw length (LJL: the distance from the back of the retroarticular process to the tip of the lower jaw); snout length (SL: the length of the snout measured from the back of the jugal bone to the tip of the upper jaw); closing in-lever (CL: the distance between the jaw articulation and the back of the jugal bone; this distance was calculated by substracting the snout length from the distance measured from the jaw articulation to the tip of the jaw = QT); opening in-lever (OL: the distance from the jaw articulation to the back of the retroarticular proces; this distance was calculated by substracting QT from lower jaw length); internasal distance (IN: the distance between the nostrils); orbit-casque distance (OC: the distance between the posterior-most point of the orbit and the highest point of the casque); interorbital distance (IO: the shortest distance between the orbits); ear opening (EO: the maximum vertical length of an ear opening); tibia (TB: the length of the left tibia); femur (FEM: the lenght of the left femur); hind metatarsus (HM: the lenght of the left hind metatarsus); hind finger (HF: the lenght of the longest –the fourth- hind finger excluding the claw); humerus (HU: the lenght of the left humerus); radius (RA: the lenght of the left radius); front metatarsus (FM: the lenght of the left front metatarsus); barb scales (BS: the maximum length of the barb-like scales on a dewlap).

**Statistical analysis of morphometric data**

Size component of morphometric variation may mask differences in body shape (e.g., Frýdlová et al. 2011). Thus, we performed size adjustment of morphometric traits prior further analyses comparing the sexes and/or populations. For this purpose, we adopted a method published by Somers (1986, 1989) as implemented in the Size analysis v02 (Thompson & Withers 2005a, b, c). This software computes from original untransformed measurements not only generalised (multivariate) isometric size, but also partial isometric size-adjusted measurements. The latter ones were further treated by univariate and/or multivariate statistical procedures. We used STATISTICA, version 6.0, StatSoft Inc., 2001, for these calculations.

**Genetic characteristics**

We sampled 17 individuals of genus *Anolis* (see Table 1 for GenBank accession numbers of samples). Total genomic DNA was isolated from tissue samples with DNAeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. DNA amplification was performed with two previously published primers: L4437 (Macey et al. 1997) and H5730 (Glor et al. 2004). These primers were used to sequence the entire 1035 bp fragment, which includes complete sequence for the gene encoding ND2.

Polymerase chain reactions (PCR) were carried out in 50 µl including 2.5 µl of each 10 µM primer, 5 µl of 10× PCR buffer (Fermentas), 5 µl of 10 mM dNTP, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of 5 U/ml Fermentas Taq DNA polymerase, 100 ng of DNA and 27 µl of $ddH_2O$. The PCR amplification protocol consisted of 30 cycles of denaturation at 95 °C for 35 s, annealing at 48–51 °C for 35 s, and extension at 72 °C for 150 s; a further 7 min elongation step at 72 °C followed the last cycle. All PCR products were purified with the Qiaquick® purification kit (Qiagen, Hilden, Germany) and directly sequenced using the same primers as for the amplification.

Bayesian analysis (BA) was conducted with MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003). The optimal model of DNA sequence evolution was selected using the AIC criterion in Modeltest 3.7 (Posada & Crandall 1998). Two independent runs of Bayesian analyses were conducted with a random starting tree and run for $6\times10^6$ generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 6,000 trees (600,000 generations). Posterior-probability values were used to indicate support for nodes of the Bayesian topology.

The outgroup is composed of ND2 sequences of *Anolis argenteolus* Cope, 1861 (GenBank Accession Number AY296154.1), *A. lucius* Duméril et Bibron, 1837 (GenBank Accession Number AF055962.2), *A. etheridgei* Williams, 1962 (GenBank Accession Number AF055934.2), *A. insolitus* Willians et Rand, 1969 (GenBank Accession Number AF055933.2), *A. cuvieri* (GenBank Accession Number AF055973.2), *A. christophei* (GenBank Accession Number AF055957.2), *A. ricordii* Duméril et Bibron, 1837 (GenBank Accession Number AY367138.1), *A. barahonae* (GenBank Accession Number AF055972.2) and *A. baleatus* Cope, 1864 (GenBank Accession Number AY296155.1).

## RESULTS

### Sexual dichromatism

Pictorial comparison of heads and dewlaps of males and females in each of five examined species/populations of anoles belonging to "chamaeleolis" group is provided in Fig. 4. Our spectrophotometric measurements confirmed the white/gray/brown character of the dewlaps in *Anolis barbatus* (Soroa), *A. chamaeleonides* (Viñales), *A. guamuhaya* (Topes de Collantes) and *A. porcus* (Baracoa), without any significant peak in the UV (see Table 2). Certain UV reflectance (320–400 nm) was detected in both males and females in these four "non-red" species, but was absent in red-throated *Anolis* sp. from San German (Fig. 5). Chromatic contrast suggested little intersexual differences in the pale colour: 2.09–4.28 JND in "non-red" species; 4.02–6.70 JND in *Anolis* sp. from San German (JND values <1, 1–3 and >3 indicate indistinguishable, barely distinguishable and clearly distinguishable stimuli respectively; see Methods). The only sexual difference in "non-red" species, if present, was manifested mainly through the overall brightness of the pale colour, which was brighter in male dewlaps. In *A. porcus*, the reflectance curve differed at longer wavelengths between males and females, suggesting a possible chromatic difference. It is however hard to evaluate this difference with only a single pair of individuals. The achromatic contrasts between male and female dewlaps spanned from nearly non-dimorphic (3.5 JND in *A. chamaeleonides*) to strongly distinct (22 JND in *A. barbatus*) and the same range was present among the individuals of *Anolis* sp. from San German. The red spot in the males from this popu-

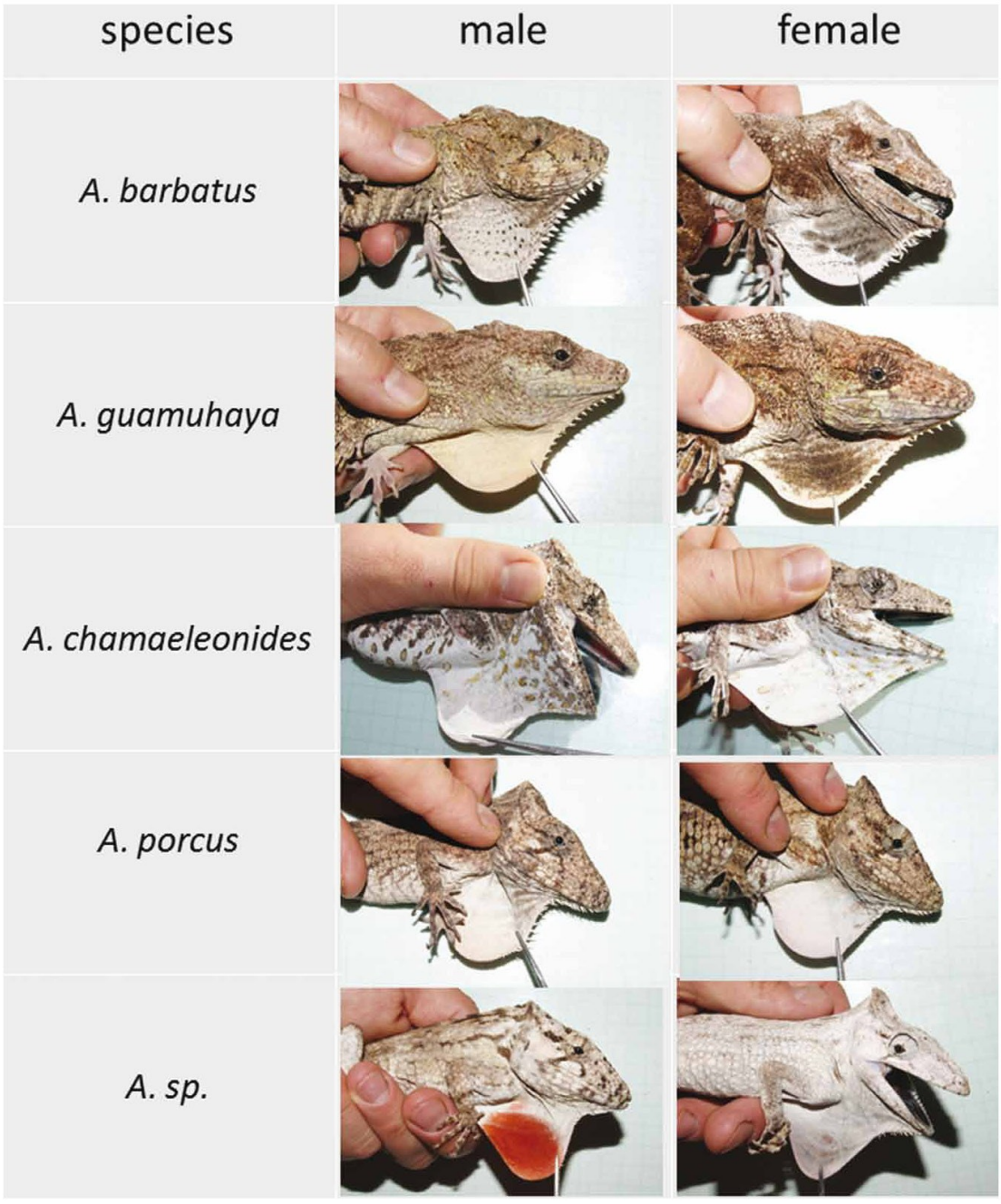


Fig. 4. Lateral view of heads and dewlaps in males and females of five examined *Anolis* species/populations of the "chamaeleolis" group.

lation strongly contrasted with its white background in both chromatic (mean=12.35±0.05 JND) and achromatic (mean=21.40±0.07 JND) visual channels (Table 1).

Table 2. Chromatic and achromatic contrasts between (1) males' and females' dewlaps (the pale color), and (2) the pale and red color within the dewlap of the males of *Anolis* sp. from San German

| | chromatic contrast (JND) | achromatic contrast (JND) |
|---|---|---|
| *Anolis barbatus* white M-F | 4.193 | 22.013 |
| *Anolis chamaeleonides* white M-F | 2.094 | 3.490 |
| *Anolis guamuhaya* white M-F | 4.286 | 6.759 |
| *Anolis porcus* white M-F | 2.107 | 20.087 |
| *Anolis* sp. white M1-F1 | 6.698 | 19.956 |
| *Anolis* sp. white M1-F2 | 4.496 | 9.174 |
| *Anolis* sp. white M2-F1 | 6.397 | 9.907 |
| *Anolis* sp. white M2-F2 | 4.085 | 3.467 |
| *Anolis* sp. white M3-F1 | 6.071 | 15.350 |
| *Anolis* sp. white M3-F2 | 4.017 | 4.569 |
| *Anolis* sp. red-white M1 | 12.149 | 20.742 |
| *Anolis* sp. red-white M2 | 12.849 | 21.921 |
| *Anolis* sp. red-white M3 | 12.065 | 21.539 |

**Sexual dimorphism in body size and shape**

Multivariate analysis of variance (MANOVA) showed strong effect of species ($F_{100,327}$=6.70, P<0.0001) on 25 size-adjusted morphometric variables, but revealed neither the effect of sex ($F_{25,82}$=1.14, P=0.3221) nor sex*species interaction ($F_{100,327}$=1.26, P=0.0704). This result was confirmed by Analysis of variance examining the effects of species/populations, sex and its interaction on isometric multivariate body size (PC1 produced by the Size analysis v02 software, Thompson & Withers 2005a). Body size differed among species/populations ($F_{4,106}$=8.20, P<0.0001), but not between the sexes (sex: $F_{1,106}$=1.01, P=0.3174; sex*species interaction: $F_{4,106}$=1.39, P=0.2415). Almost the same results were obtained for PC2 and PC3 reflecting body shape (Species: both P<0.0001, for sex and interaction all P>0.20). This allowed us to pool sexes for further analyses.

**Morphometric differentiation among populations/species**

We performed discriminant function analysis (DFA) to visualize differences among examined species/populations (except *A. sierramaestrae* Holáňová, Frynta et Rehák, 2012 for which only the holotype specimen was available) in size-free morphometric traits. We applied stepwise forward selection method which resulted in inclusion of 21 of variables (four variables were excluded) and high classification success (Wilks' Lambda=0.0161, $F_{84,361}$=7.94, P<0.0001). 108 of 116 specimens (93%) were assigned to proper species/population. All *A. chamaeleonides* (25) and *A. porcus* (17) were reclassified correctly. Two of 40 specimens of *A. barbatus* were misclassified as *A. guamuhaya* while five of 28 *A. guamuhaya* as *A. barbatus*. One of six *Anolis* sp. was misclassified as *A. porcus*. The type specimen of *A. sierramaestrae* was closest to *A. porcus* according to the classification equations derived from DFA.

Cluster analysis (CA) performed by Ward method from the matrix of squared Mahalanobis distances visualized similarities between *A. barbatus* and *A. guamuhaya* as well as between *A. porcus* and *Anolis* sp.; *A. chamaeleonides* was morphometrically least similar to remaining examined species (Fig. 6, Appendix 2).

Canonical analysis revealed four significant (P<0.0005) multivariate axes (Appendix 3). The position of examined specimens in the morphospace of the first two canonical axes is plotted on Fig. 7.

**Genetic differentiation among populations/species**

The phylogenetic analysis was based on sequences of mitochondrial ND2 gene (alignment consisting of 1035 bp). The sequence divergences between haplotypes of "red dewlap" (here referred to *Anolis* sp.) and "whitish-yellowish dewlap" (*A. porcus*) populations belonging to *A. porcus* sensu lato (*p*-distances ranging from 5.25% to 6.11%, mean=5.62%), is comparable to those among haplotypes of currently recognized species of the "chamaeleolis" group of anoles (i.e.,

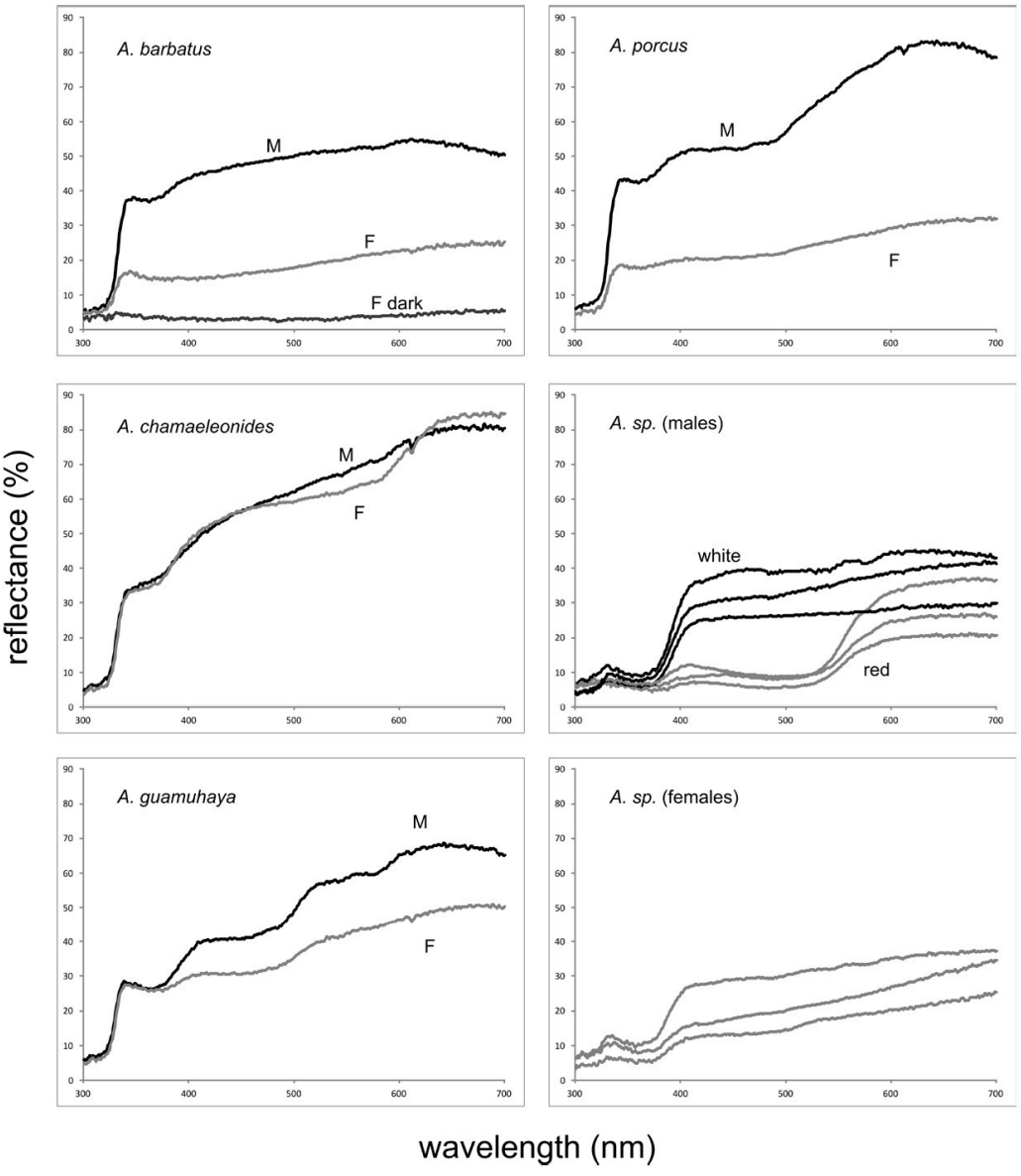


Fig. 5. The dewlap color reflectance between 300 and 700 nm in males and females of five species/populations of anoles of "chamaeleolis" group.

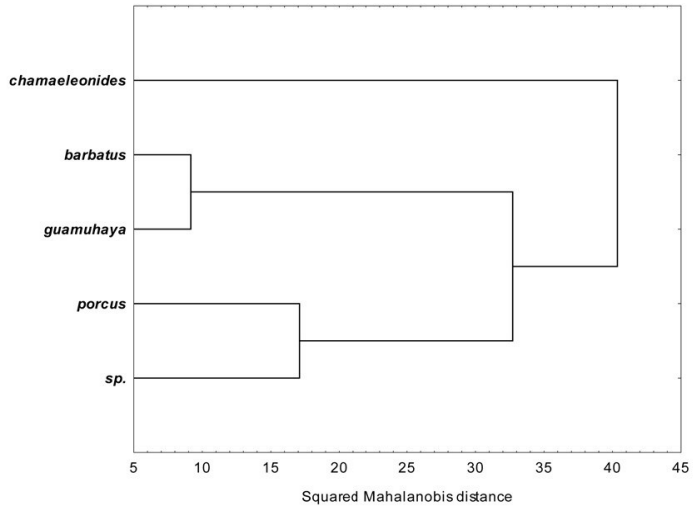


Fig. 6. Phenetic tree of five *Anolis* species based on morphometric data. It is constructed from matrix of Mahalanobis distances clustered by Ward's method.

*A. barbatus*, *A. chamaeleonides*, *A. guamuhaya*, *A. porcus* and *A. sierramaestrae*). The uncorrected P-distances of these between species comparisons vary within the range of 4.44–10.20%, mean=6.58%, while within species distances are much smaller; maximum values were 0.59, 0.59,

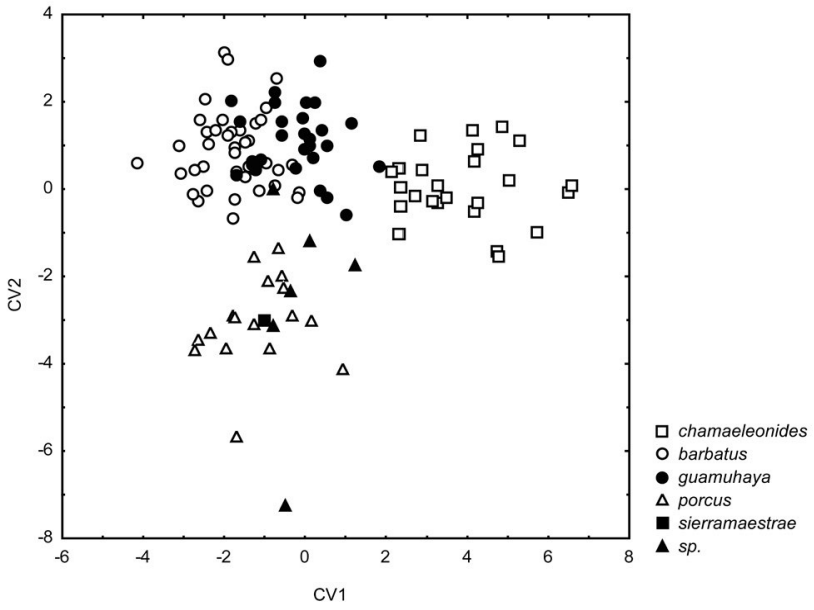


Fig. 7. Position of *Anolis* species/populations of the "chamaeleolis" group in the morphospace of the first two canonical axes (CVA1 and CVA2).

1.19, 0.39, and 2.21 for *A. barbatus*, *A. chamaeleonides*, *A. guamuhaya*, *A. porcus* and *Anolis* sp., respectively. The Bayessian analysis revealed that examined haplotypes belonging to *A. barbatus*, *A. chamaeleonides* and *A. guamuhaya* (all posterior probabilities = 1) form monophyletic groups, but this is not true for *A. porcus sensu lato* including populations of both colour forms. In contrast to this the haplotypes from *A.* sp. (= "red dewlap") and "whitish-yellowish dewlap" (= *A. porcus*) populations formed mutually exclusive monophyletic groups (posterior probabilities were 0.99 and 1.00 respectively). As expected, monophyletic status of entire "chamaeleolis" group of anoles was strongly supported (Fig. 8).

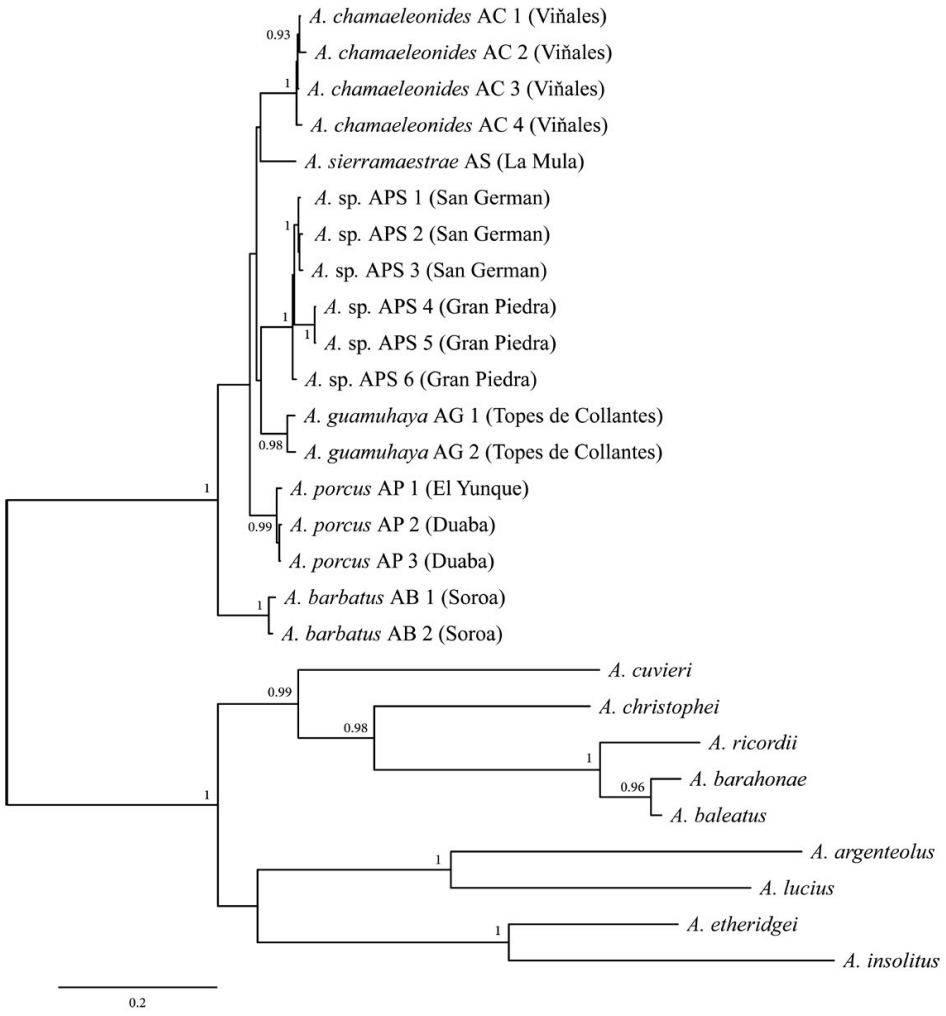


Fig. 8. Bayesian tree of mitochondrial ND2 gene including six species/populations of Cuban anoles belonging to the "chamaeleolis" group as well as nine related anoles introduced as outgroups.

**Dewlap dichromatism**

Our study showed that in comparison to other species/populations of the "chamaeleolis" group, dewlaps of *Anolis* sp. are clearly sexually dichromatic. Taking into account the tetrachromatic vision of the anoles, spectrophotometry and visual modelling revealed no such intersexual difference which would be perceived only by the anoles, but not by humans. While the "dichromatism" in the "non-red" anoles, if present, can be manifested mainly through the lightness of the dewlap, there is both chromatic and achromatic contrast in the red-throated *Anolis* sp., making their dewlaps more conspicuous. Males of the "chamaeleolis" anoles perform the courtship behaviour style as other anoles do, including dewlap displays and head bobs (pers. obs.). The red coloration may therefore represent an honest signal, possibly mediated by carotenoid pigments, which are often involved as indicators of males' quality (Steffen & McGraw 2009, Steffen et al. 2010). The other frequent honest signal in lizards is the ultraviolet colouration (Font et al. 2009, Fleishman et al. 2011), but although our measurements detected some UV reflectance in the "non-red" species (and none in *Anolis* sp.), this did not seem to be the case. Firstly, the UV reflectance was the lowest within the whole spectrum and secondly, when the sexes differed, they did by the overall shape of the reflectance curve, not just by the UV reflectance itself. However, larger samples are needed to determine reliable statistics of possible trends in this or other traits.

**Dewlap function and evolution**

There are multiple hypotheses about the function and evolution of the dewlap (Losos 2009, Steffen & Guyer 2014, Hagman & Ord 2016). It may play a role in species recognition, territorial signalling (intrasexual selection), honest signalling of male quality, or in predator deterrence.

Huge dewlap evolution in lizards with clearly allopatric distribution, like in the "chamaeleolis" anoles, does not suggest that it should have function in species recognition as it has in sympatrically living anoles of the same ecomorph. Lizards of the "chamaeleolis" group are classified as the "twig anoles" ecomorph which displays active foraging mode. Species belonging to this ecomorph have slow locomotion but can search for usually cryptic prey for greater distances (Irschick & Losos 1996). These authors suppose that it is possible for this reason that twig anoles have wide size habitats and so it is difficult to defend such a large space. That could be the reason why intrasexual selection usually does not impact twig anoles and dimorphism is not noticeable. But this assumption could be applicable in case of low density of the species. In the species Anlis sp., there can be higher local population density which leads to stronger male competition and therefore to stronger intrasexual component of sexual selection. Unfortunately population density has not been measured for this population. The dichromatism subsequently makes the courtship and aggressive communication more unambiguous, unlike in monochromatic species (Regalado 2015).

The habitat type has also considerable influence on the use of the dewlap. Species inhabiting habitats with higher visibility display more frequently. Anole species extend their dewlaps in sunny habitats more often than in shady ones (Ord & Martins 2006), but light conditions appear to be unrelated to the evolution of dewlap colour and signal detectability (Fleishman et al. 2009). Moreover, all "chamaeleolis" anoles live in more or less the same type of habitat (personal observation of the first author), it is therefore unlikely that the dewlap colour is directly associated with their environment.

Different pressures on sexes can also lead to different use and size of dewlaps in some anole species. Johnson & Wade (2010) showed in their comparative study that males have larger dewlaps (and cartilage and muscular components controlling dewlap movement) and use them

more frequently than females. Dewlap size is a significant predictor of the winner in male fights in species with low but not high SSD. Neither the dewlap display rate is associated with SSD (Lailvaux & Irschick 2007). But as we detected no significant SSD in *Anolis* sp., this explanation is unlikely as well.

According to the pursuit-deterrence hypothesis, when the prey is detected by the predator, it gives him an "I am aware of you" signal (shows the dewlap), which might deter the predator from pursuing its prey (Caro 1995). This hypothesis predicts that mainland species living under higher predation risk would display more often. Mainland *A. carolinensis* Voigt, 1832 exhibit elevated rate of dewlap use compared to its island relatives (Vanhooydonck et al. 2009, Johnson & Wade 2010). However, this explanation would be doubtful in the case of "chamaeleolis" anoles, as they are not presumably predated much, being considerably cryptic. Moreover, if the conspicuous dewlap was to deter potential predators, it should be present in both sexes and not just the males.

In conclusion, because of lack of data on the population density of *Anolis* sp. and its related species, it is difficult to determine the evolutionary causes of the dewlap dichromatism.

**Sexual size monomorphism/dimorphism**

The differences between the sexes in morphometric traits have been thoroughly and repeatedly analysed in anoles (see Losos 2009). Sexual differences in body size are positively correlated with those in body shape (Losos et al. 2003). Sexual size dimorphism is traditionally explained by three causes: (1) sexual selection or competition for reproductive success, (2) intersexual resource differences and (3) different reproductive roles (Losos et al. 2003). Nevertheless, the relative clutch size in anoles is considerably reduced compared to the other clades of squamates (Kratochvíl & Kubička 2007). This limits a peak load of maternal investment and thus, the effect of sex differences in reproductive roles have in this particular group of lizards. Contribution of the strength of sexual selection was repeatedly confirmed even by intraspecific comparisons, e.g., sexual size dimorphism increases with population density (Stamps et al. 1997). Empirical studies revealed that habitat use of a particular species belongs to key predictors the sexual size dimorphism in anoles (Butler et al. 2000, Losos et al. 2003). Size of defended habitat, foraging style, food source dispersion – these habitat characteristics influence sexual dimorphism.

In examined material of the "chamaeleolis" anoles, we failed to detect sexual size dimorphism neither in the multivariate body size nor in the body shape. Although, the available sample of the dichromatic Anolis sp. was too small to allow a separate analysis, the sexes were of comparable size and shape even in this population/species. The entire absence of the sexual size and shape dimorphism in this group is not surprising. Lizards of group are arranged to the "twig anoles" ecomorph which displays active foraging mode. Species belonging to this ecomorph have slow locomotion but can search for usually cryptic prey for greater distances (Irschick & Losos 1996). These authors suppose that it is possible for this reason that twig anoles have wide size habitats and so it is difficult to defend such a large space. That could be the reason why intrasexual selection usually does not impact twig anoles and dimorphism is typically not noticeable.

**Morphometric and genetic divergence of "chamaeleolis" species**

Anoles of the "chamaeleolis" group are slowly moving lizards with limited home ranges. Thus, they are most likely poor dispersers. This suggestion is supported by distribution patterns of these species in Cuba (Garrido et al. 1991, Díaz et al. 1998, Schettino 1999, Holáňová et al. 2012). Each species is typically restricted to a local mountainous area and its surroundings. The isolation of local populations may explain a considerable degree of morphological divergence we observed. Considering the reports of morphologically suspect individuals from other localities (e.g., Rancho Velaz, Villa Clara province, Sierra de Banao, Sancti Spiritus province) (Garrido 1982, Garrido

et al. 1991), discoveries of additional species of the chamaeleolis group can be expected in the near future.

Our multivariate analysis of morphometric traits showed that the most distinct species is *A. chamaeleonides*, while *A.barbatus* resembles *A. guamuhaya* and non-dichromatic *A. porcus* is similar to dichromatic *Anolis* sp. In this context it is interesting that a morphotype resembling "chamaeleolis group" evolved entirely independently in Hispaniola in a group of related anole species (Mahler et al. 2016).

Genetically, all studied species/populations seem to differ in mitochondrial DNA. But except of finding that *A. barbatus* is the most distinct species of all we cannot determine their phylogenetic relationship. It seems that in view of the fact that they have low dispersion ability there will be separated species/population on every mountainous locality. It only confirms that there is extensive genetical diversity in Cuba, which is known also in other species (Starostová et al. 2010). The sequence divergence of a mitochondrial gene we found among species/populations of "chamaeleolis" anoles is considerable and this supports the view that each of examined form, including dichromatic *A.* sp., represents distinct species. Nevertheless, recent studies suggest that reproductive isolation and thus speciation process are sometimes not fully completed even between lizards with roughly two times higher genetic distances calculated from the sequence divergence of mitochondrial genes (Jančúchová-Lásková et al. 2015a, b).

## CONCLUSIONS

In conclusion, local populations of the "chamaeleolis" groups are distinct genetically as well as morphologically. They are non-dimorphic in size but one population is sexually dichromatic (*Anolis* sp.). Such conspicuous dichromatism is unusual among the island anoles.


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## REFERENCES


Badyaev A. V. & Hill G. E. 2003: Avian sexual dichromatism in relation to phylogeny and ecology. *Annual Review of Ecology, Evolution, and Systematics* **34**: 27–49.

Bell R. C. & Zamudio K. R. 2012: Sexual dichromatism in frogs: natural selection, sexual selection and unexpected diversity. *Proceedings of the Royal Society of London B, Biological Sciences* **279**: 4687–4693.

Beuttell K. & Losos J. B. 1999: Ecological morphology of Caribbean anoles. *Herpetological Monographs* **13**: 1–28.

Butler M. A., Schoener T. W. & Losos J. B. 2000: The relationship between sexual size dimorphism and habitat use in Greater Antillean Anolis lizards. *Evolution* **54**: 259–272.

Caro T. M. 1995: Pursuit-deterrence revisited. *Trends in Ecology & Evolution* **10**: 500–503.

Cope E. D. 1864: Contributions to the herpetology of tropical America. *Proceedings of the Academy of Natural Sciences of Philadelphia* **1864**: 166–181.

Darwin C. 1871: *The Descent of Man, and Selection in Relation to Sex. Volume 2*. London: John Murray, 450 pp.

Díaz L., Navarro N. & Garrido O. H. 1998: Nueva especie de Chamaeleolis (Sauria: Iguanidae) de la Meseta de Cabo Cruz, Granma, Cuba. *Avicennia* **8–9**: 27–34.

Estes R. & Williams E. E. 1984: Ontogenetic variation in the molariform teeth of lizards. *Journal of Vertebrate Paleontology* **4**: 96–107.
Fleishman L. J., Leal M. & Persons M. H. 2009: Habitat light and dewlap color diversity in four species of Puerto Rican anoline lizards. *Journal of Comparative Physiology A* **195**: 1043–1060.
Fleishman L. J., Loew E. R. & Whiting M. J. 2011: High sensitivity to short wavelengths in a lizard and implications for understanding the evolution of visual systems in lizards. *Proceedings of the Royal Society of London B, Biological Sciences* **278**: 2891–2899.
Font E., Pérez i de Lanuza G. & Sampedro C. 2009: Ultraviolet reflectance and cryptic sexual dichromatism in the ocellated lizard, Lacerta (Timon) lepida (Squamata: Lacertidae). *Biological Journal of the Linnean Society* **97**: 766–780.
Frýdlová P., Velenský P., Šimková O., Cikánová V., Hnízdo J., Rehák I. & Frynta D. 2011: Is body shape of mangrove-dwelling monitor lizards (Varanus indicus; Varanidae) sexually dimorphic? *Amphibia-Reptilia* **32**: 27–37.
Garrido O. H. & Schwartz A. 1968: Cuban lizards of the genus Chamaeleolis. *Quarterly Journal of Florida Academy of Sciences* **30**: 197–220.
Garrido O. H. 1982: Descripción de una nueva especie cubana de Chamaeleolis (Lacertilia: Iguanidae), con notas sobre su comportamiento. *Poeyana* **236**: 1–25.
Garrido O. H., Perez-Beato O. & Moreno L. V. 1991: Nueva especie de Chamaeleolis (Lacertilia: Iguanidae) para Cuba. *Caribbean Journal of Science* **27**: 162–168.
Glor R. E., Gifford M. E., Larson A., Losos J. B., Schettino R. L., Ada R., Lara C. & Jackman T. R. 2004: Partial island submergence and speciation in an adaptive radiation: a multilocus analysis of the Cuban green anoles. *Proceedings of the Royal Society of London B, Biological Sciences* **271**: 2257–2265.
Gomez D. 2006: *AVICOL, A Program to Analyse Spectrometric Data*. Last update October 2013. Free executable available at http://sites.google.com/site/avicolprogram/
Hagman M. & Ord T. J. 2016: Many paths to a common destination: morphological differentiation of a functionally convergent visual signal. *American Naturalist* **188**: 306–318.
Harrison A. & Poe S. 2012: Evolution of an ornament, the dewlap, in females of the lizard genus Anolis. *Biological Journal of the Linnean Society* **106**: 191–201.
Hass C. A., Hedges S. B. & Maxson L. R. 1993: Molecular insights into the relationships and biogeography of West Indian anoline lizards. *Biochemical Systematics and Ecology* **21**: 97–114.
Herrel A. & Holáňová V. 2008: Cranial morphology and bite force in Chamaeleolis lizards: Adaptations to molluscivory? *Zoology* **111**: 467–475.
Holáňová V., Rehák I. & Frynta D. 2012: Anolis sierramaestrae sp. nov. (Squamata: Polychrotidae) of the "chamaeleolis" species group from Eastern Cuba. *Acta Societatis Zoologicae Bohemicae* **76**: 45–52.
Hosken D. J., Alonzo S. H. & Wedell N. 2016: Why aren't signals of female quality more common? *Animal Behaviour* **114**: 199–201.
Irschick D. J. & Losos J. B. 1996: Morphology, ecology, and behavior of the twig anole, Anolis angusticeps. Pp.: 291–301. In: Powell R. & Henderson R. W. (eds.): *Contributions to West Indian Herpetology: A Tribute to Albert Schwartz.* Ithaca, NY: Society for the Study of Amphibians and Reptiles, 457 pp.
Jančúchová-Lásková J., Landová E. & Frynta D. 2015a: Are genetically distinct lizard species able to hybridize? A review. *Current Zoology* **61**: 155–180.
Jančúchová-Lásková J., Landová E. & Frynta D. 2015b: Experimental crossing of two distinct species of leopard geckos, Eublepharis angramainyu and E. macularius: viability, fertility and phenotypic variation of the hybrids. *Publlic Library of Science One* **10**(12): 1–27.
Johnson M.A. & Wade J. 2010: Behavioural display systems across nine Anolis lizard species: sexual dimorphisms in structure and function. *Proceedings of the Royal Society of London B, Biological Sciences* **277**: 1711–1719.
Johnson M. A., Cohen R. E., Vandeca R., j.r. & Wade J. 2011: Relationships among reproductive morphology, behavior, and testosterone in a natural population of green anole lizards. *Physiology & Behavior* **104**: 437–445.
Köhler G. 2003: *Reptiles of Central America*. Offenbach, Germany: Herpeton, 380 pp.
Köhler G., Vargas J. & Lotzkat S. 2014: Two new species of the Norops pachypus complex (Squamata, Dactyloidae) from Costa Rica. *Mesoamerican Herpetology* **1**: 254–280.
Kratochvíl L. & Kubička L. 2007: Why reduce clutch size to one or two eggs? Reproductive allometries reveal different evolutionary causes of invariant clutch size in lizards. *Functional Ecology* **21**: 171–177.
Lailvaux S. P. & Irschick D. J. 2007: The evolution of performance-based male fighting ability in Caribbean Anolis lizards. *American Naturalist* **170**: 573–586.
Leal M. & Losos J. B. 2000: Behavior and ecology of the Cuban "Chipojos Bobos" Chamaeleolis barbatus and C. porcus. *Journal of Herpetology* **34**: 318–322.
Liu Y. X., Davy C. M., Shi H. T. & Murphy R. W. 2013: Sex in the half-shell: a review of the functions and evolution of courtship behavior in freshwater turtles. *Chelonian Conservation and Biology* **12**: 84–100.

Loew E. R., Fleishman L. J., Foster R. G. & Provencio I. 2002: Visual pigments and oil droplets in diurnal lizards: a comparative study of Caribbean anoles. *Journal of Experimental Biology* **205**: 927–938.
Losos J. B. 2009: *Lizards in an Evolutionary Tree: Ecology and Adaptive Radiation of Anoles*. Berkeley: University of California Press, 528 pp.
Losos J. B., Butler M. & Schoener T. W. 2003: Sexual dimorphism in body size and shape in relation to habitat use among species of Caribbean Anolis lizards. Pp.: 356–380. In: Fox S. F., McCoy J. K. & Baird T. A. (eds.): *Lizard Social Behavior*. Baltimore: The Johns Hopkins University Press, 544 pp.
Loyau A., Gomez D., Moureau B., Théry M., Hart N. S., Saint Jalme M., Bennett A. T. D. & Sorci G. 2007: Iridescent structurally based coloration of eyespots correlates with mating success in the peacock. *Behavioral Ecology* **18**:1123–1131.
Macey J. R., Larson A., Ananjeva N. B., Fang Z. & Papenfuss T. J. 1997: Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. *Molecular Biology and Evolution* **14**: 91–104.
Mahler D. L., Lambert S. M., Geneva A. J., Ng J., Hedges S. B., Losos J .B. & Glor R. E. 2016: Discovery of a giant chameleon-like lizard (Anolis) on Hispaniola and its significance to understanding replicated adaptive radiations. *American Naturalist* **188**: 357–364.
Marshall K. L. A. & Stevens M. 2014: Wall lizards display conspicuous signals to conspecifics and reduce detection by avian predators. *Behavioral Ecology* **25**: 1325–1337.
Ng J. & Glor R. E. 2011: Genetic differentiation among populations of a Hispaniolan trunk anole that exhibit geographical variation in dewlap colour. *Molecular Ecology* **20**: 4302–4317.
Ng J., Landeen E. L., Logsdon R. M. & Glor R. E. 2013: Correlation between Anolis lizard dewlap phenotype and environmental variation indicates adaptive divergence of a signal important to sexual selection and species recognition. *Evolution* **67**: 573–582.
Nicholson K. E., Glor R. E., Kolbe J. J, Larson A., Hedges S. B. & Losos J. B. 2005: Mainland colonization by island lizards. *Journal of Biogeography* **32**: 929–938.
Nicholson K. E., Crother B. I., Guyer C. & Savage J. M. 2012: It is time for a new classification of anoles (Squamata: Dactyloidae). *Zootaxa* **3477**: 1–108.
Nunez S. C., Jenssen T. A. & Ersland K. 1997: Female activity profile of a polygynous lizard (Anolis carolinensis): evidence of intersexual asymmetry. *Behavior* **134**: 205–223.
Ord T. J. & Martins E. P. 2006: Tracing the origins of signal diversity in anole lizards: phylogenetic approaches to inferring the evolution of complex behaviour. *Animal Behaviour* **71**: 1411–1429.
Poe S. 2004: Phylogeny of anoles. *Herpetological Monographs* **18**: 37–89.
Posada D. & Crandall K. A. 1998: MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
Regalado R. 2015: Does dichromatism variation affect sex recognition in dwarf geckos? *Ethology Ecology & Evolution* **27**: 56–73.
Ronquist F. & Huelsenbeck J. P. 2003: MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
Schettino L. R. 1999: *The Iguanid Lizards of Cuba*. Florida, United States: University of Florida Press, 428 pp.
Schettino L. R. 2003: *Anfibios y reptiles de Cuba*. Vaasa, Finlandia: UPC Print, 169 pp.
Schwartz A. & Henderson R. W. 1991: *Amphibians and Reptiles of the West Indies: Descriptions, Distributions, and Natural History*. Gainesville, FL: University of Florida Press, 714 pp.
Shine R. & Madsen T. 1994: Sexual dichromatism in snakes of the genus Vipera: a review and a new evolutionary hypothesis. *Journal of Herpetology* **28**: 114–117.
Siddiqi A., Cronin T. W., Loew E. R., Vorobyev M. & Summers K. 2004: Interspecific and intraspecific views of color signals in the strawberry poison frog Dendrobates pumilio. *Journal of Experimental Biology* **207**: 2471–2485.
Somers K. M. 1986: Multivariate allometry and removal of size with principal components analysis. *Systematic Biology* **35**: 359–368.
Somers K. M. 1989: Allometry, isometry and shape in principal components analysis. *Systematic Biology* **38**: 169–173.
Stamps J. A., Losos J. B. & Andrews R. M. 1997: A comparative study of population density and sexual size dimorphism in lizards. *American Naturalist* **149**: 64–90.
Starostová Z., Rehák I. & Frynta D. 2010: New haplotypes of Cyclura nubila nubila from Cuba changed the phylogenetic tree of rock-iguanas: a challenge for conservation strategies? *Amphibia-Reptilia* **31**: 134–143.
Steffen J. E. & McGraw K. J. 2009: How dewlap color reflects its carotenoid and pterin content in male and female brown anoles (Norops sagrei). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **154**: 334–340.
Steffen J. E., Hill G. E. & Guyer C. 2010: Carotenoid access, nutritional stress, and the dewlap color of male brown anoles. *Copeia* **2010**: 239–246.

Steffen J. E. & Guyer C. C. 2014: Display behaviour and dewlap colour as predictors of contest success in brown anoles. *Biological Journal of the Linnean Society* **111**: 646–655.
Stoddard M. C. & Prum R. O. 2011: How colorful are birds? Evolution of the avian plumage color gamut. *Behavioral Ecology* **22**: 1042–1052.
Stuart-Fox D. M. & Ord T. J. 2004: Sexual selection, natural selection and the evolution of dimorphic coloration and ornamentation in agamid lizards. *Proceedings of the Royal Society of London B, Biological Sciences* **271**: 2249–2255.
Stutchbury B. J. & Morton E. S. 2001: *Behavioral Ecology of Tropical Birds*. San Diego: Academic Press, 165 pp.
Thompson G. G. & Withers P. C. 2005a: Size-free shape differences between male and female Western Australian dragon lizards (Agamidae). *Amphibia-Reptilia* **26**: 55–63.
Thompson G. G. & Withers P. C. 2005b: The relationship between size-free body shape and choice of retreat of Western Australian Ctenophorus (Agamidae) dragon lizards. *Amphibia-Reptilia* **26**: 65–72.
Thompson G. G. & Withers P. C. 2005c: Shape of Western Australian dragon lizards (Agamidae). *Amphibia-Reptilia* **26**: 73–85.
Ugueto G. N., Fuenmayor G. R., Barros T., Sánchez-Pacheco S. J. & García-Pérez J. E. 2007: A revision of the Venezuelan Anoles I: A new *Anolis* species from the Andes of Venezuela with the redescription of Anolis jacare Boulenger 1903 (Reptilia: Polychrotidae) and the clarification of the status of Anolis nigropunctatus Williams 1974. *Zootaxa* **150**: 1–30.
Ugueto G.N., Rivas G., Barros T. & Smith E. N. 2009: A revision of the Venezuelan anoles II: redescription of Anolis squamulatus Peters 1863 and Anolis tigrinus Peters 1863 (Reptilia: Polychrotidae). *Caribbean Journal of Science* **45**: 30–51.
Vanhooydonck B., Herrel A., Meyers J. J. & Irschick D. J. 2009: What determines dewlap diversity in Anolis lizards? An among-island comparison. *Journal of Evolutionary Biology* **22**: 293–305.
Vorobyev M. & Osorio D. 1998: Receptor noise as a determinant of colour thresholds. *Proceedings of the Royal Society of London B, Biological Sciences* **265**: 351–358.

## APPENDIX 1

List of museum specimens from the herpetological collection of the National Museum in Prague (NMP), Czech Republic used for morphometric examination.

*Anolis barbatus*, 29 specimens: NMP6j 25/1992, NMP6V 34520/1-11, NMP6V 71872/1–3, NMP6V 71873, NMP6V 73148/1–4, NMP6d 279/03, NMP6V 34504;
*Anolis guamuhaya*, 10 specimens: NMP6V 71871, NMP6V 71870/1–8, NMP6V 34517;
*Anolis chamaeleonides*, 3 specimens: NMP6d 81/06, NMP6V 34505, NMP6V 34518;
*Anolis porcus*, 1 specimen: NMP6V 34519;
*Anolis sierramaestrae*, 1 specimen: NMP6V 74453.

## APPENDIX 2

Matrix of squared Mahalanobis distances between examined species/populations of "chamaeleolis" group revealed by DFA.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 *A. chamaeleonides* : *A. chamaeleonides* | 0.000 | 34.394 | 23.326 | 38.285 | 34.384 |
| 2 *A. barbatus* : *A. barbatus* | 34.394 | 0.000 | 9.163 | 19.005 | 24.287 |
| 3 *A. guamuhaya* : *A. guamuhaya* | 23.326 | 9.163 | 0.000 | 20.939 | 27.466 |
| 4 *A. porcus* : *A. porcus* | 38.285 | 19.005 | 20.939 | 0.000 | 17.099 |
| 5 *A. sierramaestrae* : *Anolis* sp. | 34.384 | 24.287 | 27.466 | 17.099 | 0.000 |

## APPENDIX 3

Loadings of roots revealed by canonical analysis subroutine of DFA.

| root | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| SVL | –0.136 | –0.623 | –0.164 | –0.185 |
| OL | 0.370 | –0.132 | 0.039 | 0.232 |
| FM | –0.301 | –0.085 | 0.192 | 0.468 |
| HM | 0.026 | –0.181 | 0.053 | 0.481 |
| IN | 0.204 | –0.204 | 0.034 | –0.347 |
| HL | 0.228 | 0.071 | 0.228 | –0.183 |
| OC | 0.062 | 0.086 | –0.400 | –0.129 |
| SN | 0.119 | 0.261 | –0.089 | 0.141 |
| RA | –0.002 | 0.173 | –0.035 | –0.197 |
| SL | –0.107 | 0.002 | 0.273 | –0.267 |
| HF | –0.086 | 0.107 | 0.087 | 0.072 |
| IO | 0.207 | –0.014 | 0.187 | 0.109 |
| HH | 0.147 | 0.220 | 0.121 | 0.139 |
| LIE | 0.042 | 0.313 | –0.078 | –0.065 |
| HW | –0.090 | 0.122 | 0.343 | –0.385 |
| SO | –0.075 | –0.100 | –0.139 | 0.024 |
| JOL | 0.077 | –0.008 | 0.080 | –0.213 |
| TB | –0.037 | –0.060 | –0.245 | –0.171 |
| LJL | 0.102 | 0.239 | –0.065 | 0.080 |
| HU | –0.064 | –0.036 | –0.013 | –0.020 |
| SME | 0.053 | 0.245 | 0.096 | 0.052 |

## 9.2 Protiva T., Gunalen D., Bauerová A., Palupčíková K., Somerová B., Frýdlová P., Jančúchová-Lásková J., Šimková O., Frynta D., Rehák I. (2016) Shell shape and genetic variability of Southeast Asian Box Turtles (*Cuora amboinensis*) from Borneo and Sumatra. *Vertebrate Zoology*. 66(3): 387-396.

Distinguishing between species is an essential aspect of animal research and conservation. For turtles, morphology and genetic analysis are potentially valuable tools for identification. Shell shape is an important component of phenotypic variation in turtles and can be easily described and quantified by geometric morphometrics (GM). Here, we focus on carapace and plastron shape discrimination of immature Southeast Asian box turtles (*Cuora amboinensis*) from two of the Greater Sunda Islands with partially distinct faunas. GM analysis identified significant differences in carapace and plastron shape between turtles from Borneo and Sumatra. The discrimination success amounted to 90% and 83.7% for carapace and plastron, respectively. The correlations of carapace and plastron shapes were high for Sumatra (0.846), and less pronounced for Borneo (0.560). We detected no differences in the ontogenetic trajectories of the shell shape between the two islands. We conclude that shell shape can be used for reliable geographic assignment of *C. amboinensis* of unknown origin. In addition to the comparison of shell shapes, turtles from Borneo, Sumatra, Seram, and turtles of unknown origin from two Czech zoos were studied genetically. Analysis of the complete mitochondrial cytochrome b gene confirmed the distinctness of turtles from Borneo and Sumatra, with p-distance 2.68 – 4.09% sequence difference. Moreover, we discovered considerable genetic difference in Seram turtles of previously unknown haplogroup (p-distance 6.00 – 8.68%) revealing the need for the revision of the whole species complex of *Cuora amboinensis*.

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# Shell shape and genetic variability of Southeast Asian Box Turtles (*Cuora amboinensis*) from Borneo and Sumatra

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## Abstract

Distinguishing between species is an essential aspect of animal research and conservation. For turtles, morphology and genetic analysis are potentially valuable tools for identification. Shell shape is an important component of phenotypic variation in turtles and can be easily described and quantified by geometric morphometrics (GM). Here, we focus on carapace and plastron shape discrimination of immature Southeast Asian box turtles (*Cuora amboinensis*) from two of the Greater Sunda Islands with partially distinct faunas. GM analysis identified significant differences in carapace and plastron shape between turtles from Borneo and Sumatra. The discrimination success amounted to 90% and 83.7% for carapace and plastron, respectively. The correlations of carapace and plastron shapes were high for Sumatra (0.846), and less pronounced for Borneo (0.560). We detected no differences in the ontogenetic trajectories of the shell shape between the two islands. We conclude that shell shape can be used for reliable geographic assignment of *C. amboinensis* of unknown origin. In addition to the comparison of shell shapes, turtles from Borneo, Sumatra, Seram, and turtles of unknown origin from two Czech zoos were studied genetically. Analysis of the complete mitochondrial cytochrome *b* gene confirmed the distinctness of turtles from Borneo and Sumatra, with p-distance 2.68 – 4.09% sequence difference. Moreover, we discovered considerable genetic difference in Seram turtles of previously unknown haplogroup (p-distance 6.00 – 8.68%) revealing the need for the revision of the whole species complex of *Cuora amboinensis*.

## Key words

Geometric morphometrics, Geoemydidae, Cytochrome b, *Cuora amboinensis*, Conservation biology.

## Introduction

The Southeast Asian box turtle *Cuora amboinensis* (Riche *in* Daudin, 1801), belongs to the most diversified and widespread taxon of the genus *Cuora* with a distribution range including a major part of Southeast Asia (Iverson 1992). Unfortunately, it is also the most abundant hard-shelled turtle in Chinese markets and frequently used in traditional Chinese medicine (Cheung & Dudgeon 2006; Chen *et al.* 2009). Thus, it is exploited in huge numbers, especially from Indonesia and Malaysia, despite export quotas and even a total export ban in some regions. As a result, its numbers are rapidly declining and some populations are already extinct (Ives *et al.* 2008; Schoppe 2008, 2009). *Cuora amboinensis* is listed in Appendix II of CITES and globally red-listed as 'Vulnerable' (IUCN 2013). In the face of the current Asian Turtle Crisis (Cheung & Dudgeon 2006) and the

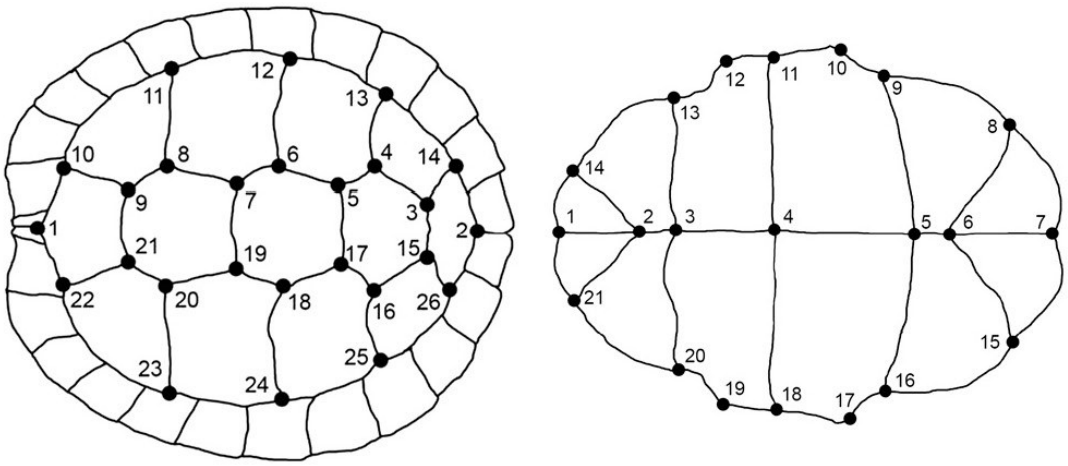


**Fig. 1.** Carapace and plastron of *Cuora amboinensis* showing the landmarks used in this study.

unsuccessful protection of the species in the wild, ex situ captive breeding programs inside and outside its distribution range are gaining increasing importance. For such captive breeding efforts, the correct identification of subspecies and the geographic provenance of turtles is of paramount importance. Although adults of currently recognized subspecies can be distinguished by standard morphometrics and coloration (Rummler & Fritz 1991; McCord & Philippen 1998), the accurate determination of juveniles still poses serious problems.

Currently, there are four subspecies classified according to morphology and coloration (Rhodin *et al.* 2010). Turtles from Sumatra and Java are considered to belong to *C. a. couro* (Schweigger, 1812). The subspecies *C. a. kamaroma* (Rummler and Fritz 1991) occurs in Borneo, the Malay Peninsula, Cambodia, Laos, Thailand, and Vietnam. Turtles from Myanmar are identified as *C. a. lineata* (McCord and Philippen, 1998) and specimens from the Philippines, Celebes, the Mollucas and Timor are regarded as *C. a. amboinensis* (Riche *in* Daudin, 1801) (Rummler & Fritz 1991; Schoppe & Das 2011). Some authors consider the turtles from Borneo as a distinct form (Schoppe 2009). Taxonomy of the Geoemydidae family is still in flux, especially in light of recent molecular genetic studies (e.g. Spinks *et al.* 2004; Stuart & Parham 2004; Diesmos *et al.* 2005; Spinks & Shaffer 2006; Le *et al.* 2007; Praschag *et al.* 2006, 2007; Fritz *et al.* 2008; Tiedemann *et al.* 2014), and several species and subspecies have been described as new to science or resurrected from synonymy (e.g. Blanck *et al.* 2006, Praschag *et al.* 2007, 2009; Spinks *et al.* 2012; Ihlow *et al.* 2016). A comprehensive genetic study is still lacking for the wide-ranging and polytypic *C. amboinensis*. The situation is further complicated by the frequent hybridization of geoemydid turtles (Wink *et al.* 2001; Buskirk *et al.* 2005; Stuart & Parham 2006; Fong *et al.* 2007; Shi *et al.* 2008; Fong & Chen 2010) often involving members of the genus *Cuora* (Wink *et al.* 2001; Stuart & Parham 2004), including *C. amboinensis* (Fritz & Mendau 2002; Galgon & Fritz 2002).

Measuring external morphology using geometric morphometrics (GM) is a practical tool for assessing phenotypic variation of shell shape. This approach is easily applied and yields immediate results, independent from any laboratory work, thus making it highly suitable for taxonomic determination in the field (Zelditch *et al.* 2004).

We therefore used GM to analyse the shell shapes of immature *C. amboinensis* box turtles from Borneo and Sumatra. In addition, we used the mitochondrial cytochrome *b* gene to genetically investigate the turtles from these islands, and specimens from other locations, in order to gather more information about these species and to compare the morphological results with the genetic findings.

## Materials and Methods

### Geometric morphometrics

A total of 195 photographs of *C. amboinensis* were examined (69 turtles from Borneo and 126 from Sumatra) and 132 (69 Borneo, 63 Sumatra) were chosen for further study. These included only immature individuals of unknown sex, with carapace lengths between 70 and 120 mm. Photographs of carapaces or plastra with abnormalities were discarded as well as photos of closed plastra to avoid perspective bias leaving 130 carapaces (68 Borneo, 62 Sumatra) and 98 plastra (69 Borneo, 29 Sumatra) for analysis.

For each turtle, standard dimensions of the shell (carapace length, carapace width, plastron length, plastron width) were measured using a calliper (0.1 mm precision). The digital images of carapace and plastron of each

**Table 1.** Genetic samples of *Cuora amboinensis* species complex and its closely related species used in this study.

| Number of sample | Accession number | Provenance | Taxonomic identification | Source |
|---|---|---|---|---|
| 40 | | Borneo | *Cuora amboinensis* | this study |
| 41 | | Borneo | *Cuora amboinensis* | this study |
| 43 | | Borneo | *Cuora amboinensis* | this study |
| 38 | | Sumatra | *Cuora amboinensis* | this study |
| 39 | | Sumatra | *Cuora amboinensis* | this study |
| 55 | | Sumatra | *Cuora amboinensis* | this study |
| 57 | | Sumatra | *Cuora amboinensis* | this study |
| 134 | | Seram | *Cuora amboinensis* | this study |
| 135 | | Seram | *Cuora amboinensis* | this study |
| 49 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 50 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 51 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 52 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 53 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 54 | | unknown | *Cuora amboinensis* | Zoo Prague |
| 131 | | unknown | *Cuora amboinensis* | Zoo Ústí nad Labem |
| 132 | | unknown | *Cuora amboinensis* | Zoo Ústí nad Labem |
| 133 | | unknown | *Cuora amboinensis* | Zoo Ústí nad Labem |
| | AY434575 | pet trade | *Cuora amboinensis kamaroma* | Spinks *et al.* 2004 |
| | AY434581 | pet trade | *Cuora amboinensis couro* | Spinks *et al.* 2004 |
| | AY434580 | pet trade | *Cuora amboinensis amboinensis* | Spinks *et al.* 2004 |
| | AY434620 | pet trade | *Cuora amboinensis lineata* | Spinks *et al.* 2004 |
| | JN232524 | India, Assam | *Cuora amboinensis* | Baruah *et al.*[1] |
| | AY434570 | pet trade | *Cuora flavomarginata sinensis* | Spinks *et al.* 2004 |
| | AY434604 | pet trade | *Cuora mouhotii* | Spinks *et al.* 2004 |
| | AY434574 | pet trade | *Cuora pani* | Spinks *et al.* 2004 |
| | AY434627 | pet trade | *Cuora trifasciata* | Spinks *et al.* 2004 |

individual were obtained using a digital camera (Canon EOS 30D with Canon 50/1.8 lens) mounted on a tripod. Twenty-one anatomical landmarks of type 1 on plastron and twenty-five landmarks of type 1 and one of type 3 on carapace following the classification of Bookstein (1997) were recorded (Fig. 1.) using TPSdig software (Rohlf 2008). Each set was then symmetrised and one half was removed using the BigFix6 program (Sheets 2003). Statistical examination was performed on half of the landmark sets. We employed the Procrustes superimposition method (Zelditch *et al.* 2004) using the CoordGen6 program (Sheets 2003) to remove the effects of position, orientation and scale, employing sets of x, y coordinates of landmarks from each specimen. We used the standardization on mean carapace length (for each population separately) to remove the size related shell shape differences in the program Standard6 (Sheets 2003). Visualization was performed using CVAGen6 software (Sheets 2003). The vectors of the shell shape ontogeny between turtles from Borneo and Sumatra were compared to the variability of the ontogeny vector inside these two samples using the VecCompare6 program (Sheets 2003) and 400 permutations. When the vector between the samples is bigger than the 95$^{th}$ percentile of the ranges of within-sample angles, we can assume that it is not expected that the samples significantly differ in the shell shape vector of the ontogeny randomly. The correlation between carapace and plastron shape was examined using PLSMaker6 software (Sheets 2003). The partial warp scores for the further statistical analysis were generated using PCAGen6 software (Sheets 2003). The differences in shell shape between turtles from Borneo and Sumatra were tested in the program Statistica 6 (Weiss 2007) using Discriminant Analysis.

## DNA samples and mitochondrial DNA sequencing

Nine turtles of known geographical provenance (three from Borneo, four from Sumatra, and two from Seram) were studied genetically. Additionally, nine individuals of unknown geographical provenance from zoological gardens (six samples from Zoological Garden Prague, Czech Republic, three samples from Zoological Garden Ústí nad Labem, Czech Republic) were included in this analysis (Table 1).

For each turtle a claw tip was removed and stored in an Eppendorf tube with 96% ethanol prior to DNA extraction. Total genomic DNA was then isolated using the DNAeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines.

The DNA amplification was performed with the primers suggested by Spinks *et al.* (2004) for a total length of

[1] Baruah, C., Shanker, K., & Sharma, D.K. (2011): Phylogenetic relationships of Indian freshwater turtles and tortoises based on mitochondrial cytochrome *b* sequences. - unpublished.

1140 bp of cytochrome *b*. The PCR reactions were carried out in 25 µl including 1 µl of each 10 µM primer, 12.5 µl Combi PPP Master Mix (Top-Bio), 1 µl of DNA and 9,5 µl of $ddH_2O$. The PCR protocol started with a denaturation step at 94 °C for 180 s, followed by 35 cycles composed of denaturation at 94 °C for 45 s, annealing at 46 °C for 45 s, and extension at 72 °C for 1 min and 20 s; and finishing with a further 7 min elongation step at 72 °C after the last cycle. For some of the samples, the temperature of annealing had to be increased to 50 °C to obtain usable PCR products. PCR products were purified using a Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) and directly sequenced in both directions with substantial overlap with the same primers that were used in the PCR reaction. Newly obtained haplotypes of *C. amboinensis* were merged with previously published ones and sequences of additional *Cuora* species and four outgroup species: *Cuora amboinensis* (GenBank accession number: JN232524), *Cuora amboinensis amboinensis* (AY434580), *Cuora amboinensis couro* (AY434581), *Cuora amboinensis kamaroma* (AY434575), *Cuora amboinensis lineata* (AY434620), *Cuora mouhotii* (AY434604), *Cuora flavomarginata sinensis* (AY434570), *Cuora pani* (AY434574) and *Cuora trifasciata* (AY434627).

Chromatograms of newly generated sequence data were manually checked using Chromas Lite 2.01 software (http://www.technelysium.com.au/chromas_lite.html), BioEdit (HALL 1999) and sequences were aligned in the Clustal X 1.81 program (THOMPSON *et al.* 1997).

### Phylogenetic analyses

We used our sequence data to construct a bootstrapped maximum likelihood (ML) tree using RAxML software (version 7.2.8-alpha) (STAMATAKIS 2006). The relationship between the subspecies was examined using 1,000 bootstrap replicates and the GTRGAMMA model. The average distances between haplotypes from particular groups were calculated in the mega 7.0.18 program (KUMAR *et al.* 2016) using uncorrected p-distances model.

## Results

### Geometric morphometrics

We found significant differences in the shape of the carapace (Wilks' Lambda = 0.3764, $F_{(25,104)} = 6.8916$, $p < 0.0001$) and plastron (Wilks' Lambda = 0.5815, $F_{(17,80)} = 3.3867$, $p < 0.0001$) between the samples from Borneo and Sumatra. In total, 90% of the turtles could be discriminated (97.1% for Borneo, 82.3% Sumatra) by carapacial shape and 83.7% (91.3% for Borneo, 65.5% Sumatra) by plastral shape. The differences are presented in a canonical plot (Fig. 2A) and in a thin plate spline diagram (Fig. 2B) for carapacial shape and in Fig. 3A and B for plastral shape, respectively.

The correlation for carapace length and centroid size (geometric size) differed between the samples from Sumatra and Borneo (Fig. 4). However, we did not find any significant differences with respect to the growth vectors of carapacial (angle between populations 40, angle within Borneo 43.3, angle within Sumatra 26.6) and plastral shape (angle between populations 32, angle within Borneo 45.3, angle within Sumatra 35.3) between the samples from these two islands.

The analysis revealed a weak correlation (0.5597) between carapacial and plastral shape for turtles from Borneo, and a much stronger correlation (0.8458) for turtles from Sumatra.

### DNA analysis

We sequenced the mitochondrial cytochrome *b* gene (1140 bp) in 18 individuals and found 14 distinct haplotypes. ML revealed two clearly distinct groups. The first group contained *C. amboinensis* haplotypes from Seram which were deeply divergent from all other haplotypes of *C. amboinensis* (uncorrected p distances 6.00–8.68%). Among the remaining haplotypes, uncorrected p distances ranging from 0.00% to 5.36% were observed. The phylogenetic analyses placed the Seram haplotypes as a sister group of a monophyletic group including the remaining *C. amboinensis* sequences (Fig. 5). The latter group exhibits a clear structure, with sequences from Borneo and Sumatra in distinct parts of the tree. Uncorrected p-distances between haplotypes belonging to the Borneo and Sumatra groups varied within the range of 2.68–4.09% (Table 2.). The haplotypes from the same island were similar (uncorrected p distances: 0.51–1.53% for Borneo and 0.13–0.26% for Sumatra) and formed monophyletic groups (Borneo: bootstrap support 87) and, for the sequences from Sumatra (bootstrap support 98), contained additional sequences of unknown geographic origin. The sister relationship between the "Sumatra clade" and the "132 Zoo Ústí nad Labem and 51 Zoo Prague clade" is moderately supported (bootstrap support 70) with uncorrected p-distances varying within the range of 0.64–1.15%. The relative position of additional sequences from GenBank of *C. amboinensis*, *C. kamaroma*, *C. cuoro* and *C. lineata* in the tree was not resolved because the phylogenetic relationships within this clade were poorly supported.

## Discussion

Shell shape variation shows a clear distinctiveness between Borneo and Sumatra populations of *C. amboinensis,* which corresponds with our DNA analyses. Geometric morphometrics therefore provides sufficient

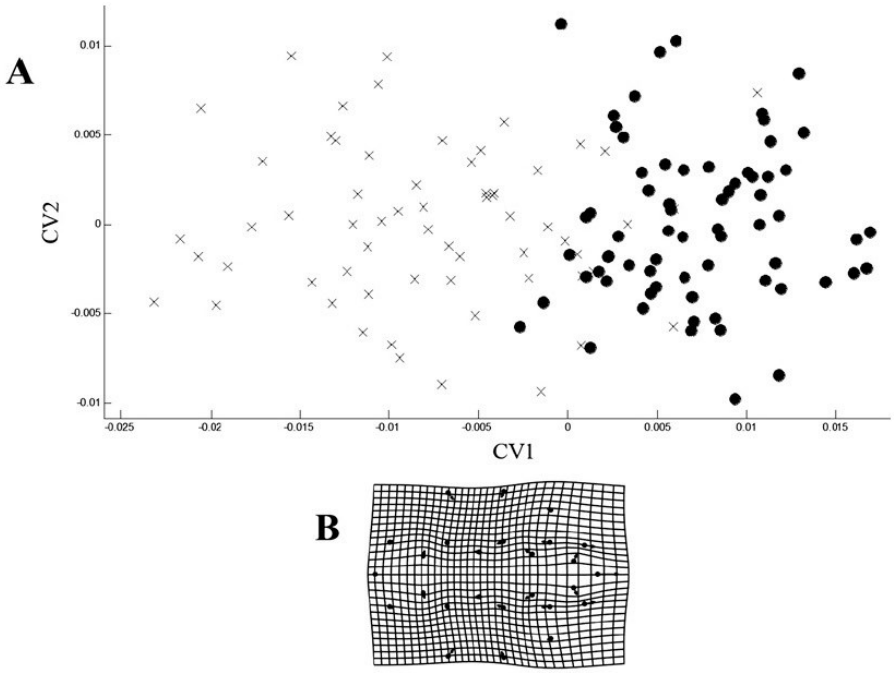


**Fig. 2.** Canonical plot for carapacial shape. Each carapace is represented by a symbol: × – Sumatra, ● – Borneo (A). The first axis accounts for 79.05% of the total between group variation. The thin plate spline diagram shows a change in carapacial shape along the first axis (in direction of arrows) (B).

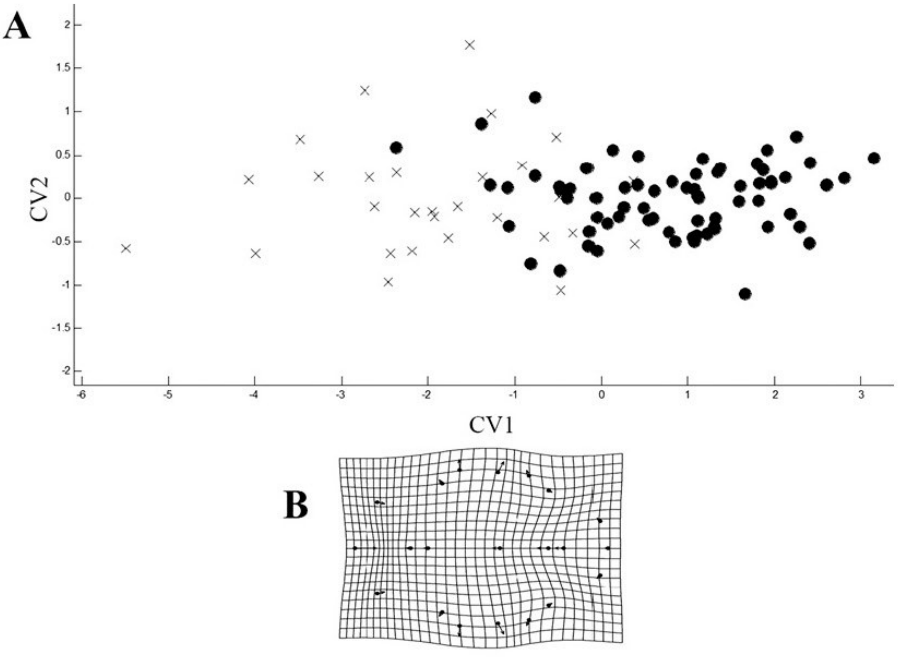


**Fig. 3.** Canonical plot for plastral shape. Each plastron is represented by a symbol: × – Sumatra, ● – Borneo (A). The first axis accounts for 55.25% of the total between group variation. The thin plate spline diagram shows a change in plastral shape along the first axis (in direction of arrows) (B).

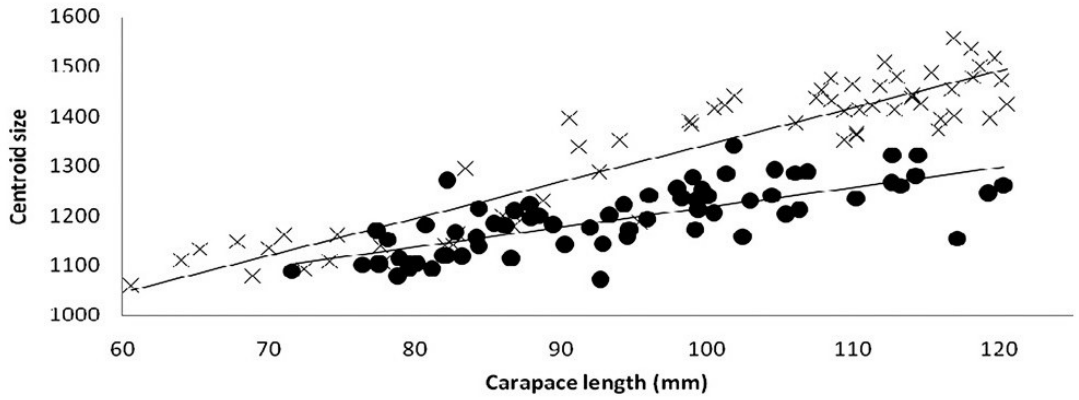


**Fig. 4.** Correlation of carapacial length and centroid size. × – Sumatra, ● – Borneo

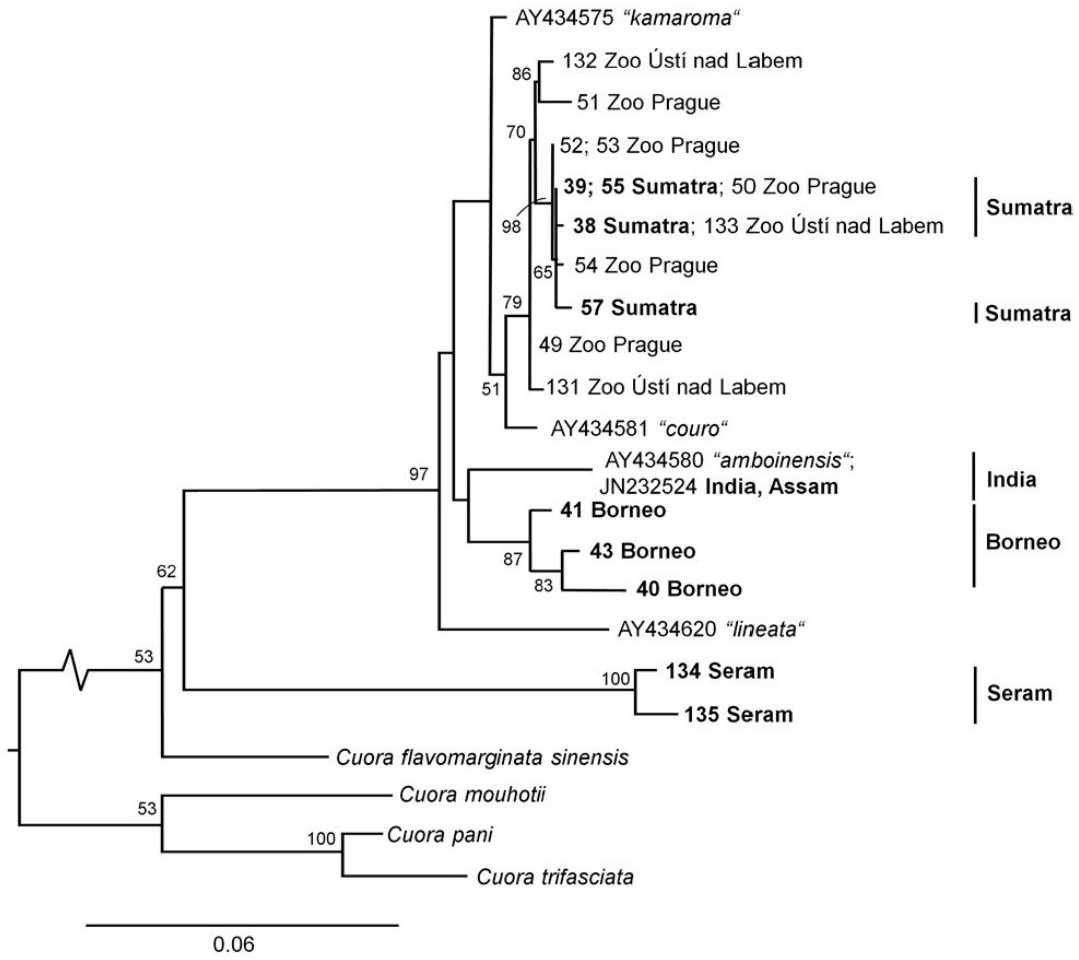


**Figure 5.** Phylogenetic tree by maximum likelihood (ML) method based on alignment of the complete cytochrome *b* gene (1140 bp). Numbers at branches are bootstrap values > 50. Samples sequenced in this study are labelled with sample numbers and are in bold, remaining samples were sequenced by previous authors and are shown with full taxonomic names or with GenBank accession numbers. For sequences with reliable geographic provenance, the countries are given. Subspecies names in apostrophes are from SPINKS *et al.* (2004). Root length shortened by 75%.

**Table 2.** Uncorrected *p* distances (percentages) based on the mitochondrial cytochrome *b* gene (1140 bp).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 132 Zoo Ústí nad Labem | | | | | | | | | | | | | | | | | | | | | | |
| **2** | 51 Zoo Prague | 0.64 | | | | | | | | | | | | | | | | | | | | | |
| **3** | 52, 53 Zoo Prague | 0.51 | 0.89 | | | | | | | | | | | | | | | | | | | | |
| **4** | 39, 55 Sumatra; 50 Zoo Prague | 0.64 | 1.02 | 0.13 | | | | | | | | | | | | | | | | | | | |
| **5** | 38 Sumatra; 133 Zoo Ústí nad Labem | 0.77 | 1.15 | 0.26 | 0.13 | | | | | | | | | | | | | | | | | | |
| **6** | 54 Zoo Prague | 0.64 | 1.02 | 0.13 | 0.00 | 0.13 | | | | | | | | | | | | | | | | | |
| **7** | 57 Sumatra | 0.77 | 1.15 | 0.26 | 0.13 | 0.26 | 0.13 | | | | | | | | | | | | | | | | |
| **8** | 49 Zoo Prague | 0.38 | 0.77 | 0.38 | 0.51 | 0.64 | 0.51 | 0.64 | | | | | | | | | | | | | | | |
| **9** | 131 Zoo Ústí nad Labem | 0.64 | 1.02 | 0.64 | 0.77 | 0.89 | 0.77 | 0.89 | 0.26 | | | | | | | | | | | | | | |
| **10** | AY434581 *"couro"* | 1.02 | 1.15 | 1.02 | 1.15 | 1.28 | 1.15 | 1.28 | 0.64 | 0.89 | | | | | | | | | | | | | |
| **11** | AY434575 *"kamaroma"* | 1.40 | 1.53 | 1.40 | 1.53 | 1.66 | 1.53 | 1.66 | 1.02 | 1.02 | 0.89 | | | | | | | | | | | | |
| **12** | AY434580 *"amboinensis"*, JN232524 India, Assam | 3.58 | 3.96 | 3.32 | 3.45 | 3.58 | 3.45 | 3.32 | 3.19 | 3.19 | 3.45 | 2.81 | | | | | | | | | | | |
| **13** | 41 Borneo | 2.68 | 2.55 | 2.68 | 2.81 | 2.68 | 2.81 | 2.68 | 2.81 | 2.81 | 2.43 | 2.55 | 3.58 | | | | | | | | | | |
| **14** | 43 Borneo | 2.94 | 2.55 | 2.94 | 3.07 | 2.94 | 3.07 | 2.94 | 3.07 | 3.07 | 2.68 | 2.55 | 3.58 | 0.51 | | | | | | | | | |
| **15** | 40 Borneo | 3.96 | 3.58 | 3.96 | 4.09 | 3.96 | 4.09 | 3.96 | 4.09 | 4.09 | 3.70 | 3.58 | 4.85 | 1.53 | 1.28 | | | | | | | | |
| **16** | AY434620 *"lineata"* | 3.45 | 3.83 | 3.45 | 3.32 | 3.45 | 3.32 | 3.19 | 3.32 | 3.32 | 3.83 | 3.70 | 4.47 | 3.96 | 4.09 | 5.36 | | | | | | | |
| **17** | 134 Seram | 7.15 | 7.28 | 7.41 | 7.28 | 7.28 | 7.28 | 7.15 | 7.28 | 7.28 | 7.15 | 7.02 | 8.17 | 6.90 | 7.02 | 6.00 | 8.17 | | | | | | |
| **18** | 135 Seram | 7.92 | 8.05 | 8.17 | 8.05 | 8.05 | 8.05 | 7.92 | 7.79 | 7.79 | 7.66 | 7.54 | 8.68 | 7.41 | 7.54 | 6.51 | 8.68 | 1.02 | | | | | |
| **19** | *Cuora flavomarginata sinensis* | 6.90 | 7.28 | 7.15 | 7.02 | 6.90 | 7.02 | 6.90 | 7.02 | 7.02 | 6.90 | 6.51 | 7.02 | 6.39 | 6.39 | 6.39 | 7.41 | 6.90 | 7.28 | | | | |
| **20** | *Cuora mouhotii* | 6.13 | 6.13 | 6.39 | 6.51 | 6.39 | 6.51 | 6.39 | 6.00 | 6.00 | 6.13 | 5.49 | 6.00 | 6.00 | 5.62 | 6.39 | 7.15 | 6.39 | 6.90 | 5.62 | | | |
| **21** | *Cuora pani* | 7.28 | 7.41 | 7.54 | 7.66 | 7.79 | 7.66 | 7.54 | 7.41 | 7.41 | 7.28 | 6.90 | 7.41 | 6.77 | 6.77 | 6.90 | 8.05 | 7.54 | 7.92 | 6.26 | 5.49 | | |
| **22** | *Cuora trifasciata* | 7.92 | 8.05 | 7.92 | 8.05 | 7.92 | 8.05 | 7.92 | 7.79 | 7.79 | 7.66 | 7.02 | 7.79 | 6.90 | 6.90 | 7.02 | 8.43 | 7.28 | 7.66 | 6.13 | 4.85 | 1.66 | |

geographical specific population recognition according to shell shape. Thus, it is a useful method for accurate turtle classification even in early ontogenetic stages. Similar studies of the other subspecies, populations and ontogenetic stages are needed to map all phenotypic variability in this species. GM can then serve as a simple yet effective practical tool for the determination of specimens of unknown geographical origin, with particular application in fieldwork.

From our genetic analyses, we discovered that the mitochondrial cytochrome *b* sequences of *Cuora amboinensis* turtles from Borneo and Sumatra are clearly distinct, which corresponds with the currently accepted taxonomy of this species (Rummler & Fritz 1991; Rhodin *et al.* 2010), with Bornean turtles being identified with another subspecies (*C. a. kamaroma*) rather than with Sumatran turtles (*C. a. couro*). Yet, in our phylogenetic analyses, previously published sequences identified with these subspecies (Spinks 2004) were distinct from ours, perhaps due to misidentification or geographic variation of these taxa.

A notable finding of our study was that the haplotypes from Seram, geographically corresponding to the subspecies of *C. a. amboinensis*, are highly divergent. However, it was not possible to prove the congruence between morphology and genetic divergences due to the low number of individuals (n=2) for morphometric analysis. We are therefore not yet able to draw taxonomic conclusions for turtles from the Seram provenience. More detailed DNA analyses, including the use of nuclear markers, are needed to clarify the affinities of this population. Moreover, *Cuora amboinensis* could in fact be a species complex with similar patterns as in other turtle species with vast geographical distribution which have been studied recently (Fritz *et al.* 2012; Kindler *et al.* 2012; Kindler *et al.* 2016; Vargas-Ramírez *et al.* 2010; Vargas-Ramírez *et al.* 2013; Petzold *et al.* 2014; Edwards *et al.* 2016).

The shell-shape and genetic differences between Borneo and Sumatra samples are in compliance with the general differences and separation of the majority of these island faunas. The isolation of the Greater Sunda Islands in the Tertiary caused deep genetic divergences between the reptilian fauna (Keogh et al. 2001) and even in mammals (Thinh *et al.* 2010; Nater *et al.* 2011) inhabiting Borneo, Sumatra and the mainland. The similar pattern of distribution in *C. amboinensis* could be the result of a low dispersion ability due to the semiaquatic lifestyle in marshes, ponds and small streams, as seen in the ecologically close genus *Cyclemys* (Fritz *et al.* 2008) and the other *Cuora* species (Spinks & Shaffer 2006) with limited distribution ranges. In contrast, a big river species like the Malaysian giant turtle (*Orlitia borneensis*) with a similar distribution range as *C. amboinensis* could have benefited from geomorphologic conditions during the glacial periods when the Malay Peninsula, Sumatra and Borneo were connected by a system of rivers i.e.the Siam and West Sunda River drainages (Voris 2000). It allowed continuous gene flow between the now isolated areas (Palupcikova *et al.* 2012). The central location of moun-

tains in Borneo may act as an effective natural barrier between the suitable lowland habitats of *C. amboinensis*. A low correlation of the carapace and plastron shape in the Borneo turtles could be a consequence of geographically isolated populations. A similar pattern was observed in the genetic variation found in the Mediterranean turtle (*Mauremys leprosa*) (Fritz *et al.* 2006).

Our results revealing distinct morphological and genetic differences between Sumatra and Borneo box turtles are congruent with the recent findings of Ernst *et al.* (2016), who performed classical morphometric analysis on *C. amboinensis* throughout its distribution range. Their research supported the validity of only two subspecies, *amboinensis* and *kamaroma*. According to these authors, *amboinensis* occupy a range that includes Sumatra, while *kamaroma* turtles inhabit Borneo. Placement of Borneo populations into continental subspecies *C. a. kamaroma* sensu lato is confirmed by the clustering of the examined continental haplotype into the Bornean cluster. Nevertheless, the positions of the additional haplotypes from Genbank in our phylogenetic tree suggest that preliminary taxonomic conclusions made by Ernst *et al.* (2016) need to be confirmed by genetic analyses covering the whole range of the species. In light of our results, the use of the name *C. a. amboinensis* also for Sumatra populations, formerly classified as *C. a. couro*, is especially problematic. Although we analysed just two samples from the Molucca archipelago, where the typical habitat of *C. amboinensis* is found, these haplotypes are strongly different not only from those collected in Sumatra, but also from all other examined ones.

In conclusion, the high identification success of immature *C. amboinensis* specimens based on the phenotypic variation of shell shape using GM clearly demonstrates the usefulness of this method. It could be used on other forms of this species as a practical and effective tool for the determination of *C. amboinensis* of unknown origin thus contributing to the conservation strategies for this taxon as well as benefitting general scientific research.

From our genetic analyses we have discovered not only that the congruence between morphology and genetic distinctness for Borneo and Sumatra box turtles support deep divergent lineages, but moreover, we uncovered previously unknown haplotypes from Seram suggesting that the species status of this population should be reconsidered.

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## References

Blanck, T., McCord, W. P. & Le, M. (2006): On the variability of *Cuora trifasciata* (Bell, 1825). – Edition Chimaira (Frankfurt am Main), 152 pp.

Bookstein, F.L. (1997): Morphometric tools for landmark data: geometry and biology. – Cambridge University Press (New York), 455 pp.

Buskirk, J.R., Parham, J.F. & Feldman, C.R. (2005): On the hybridisation between two distantly related Asian turtles (Testudines: *Sacalia* × *Mauremys*). – Salamandra, **42**: 21–26.

Chen, T.H., Chang, H.C. & Lue, K.Y. (2009): Unregulated Trade in Turtle Shells for Chinese Traditional Medicine in East and Southeast Asia: The Case of Taiwan. – Chelonian Conservation and Biology, **8**: 11 – 18.

Cheung, S.M. & Dudgeon, D. (2006): Quantifying the Asian turtle crisis : market surveys in southern China, 2000–2003. – Ecology, **770**: 751–770.

Diesmos, A.C., Parham, J.F., Stuart, B.L. & Brown, R.M. (2005): The Phylogenetic Position of the Recently Rediscovered Philippine Forest Turtle (Bataguridae: *Heosemys leytensis*). – Proceedings of the California Academy of Sciences, **56**: 31–41.

Edwards, T., Karl, A.E., Vaughn, M., Rosen, P.C., Torres, C.M. & Murphy, R.W. (2016): The desert tortoise trichotomy: Mexico hosts a third, new sister-species of tortoise in the *Gopherus morafkai-G. agassizii* group. – ZooKeys, **562**: 131–158.

Ernst, C.H., Laemmerzahl, A. & Lovich, J.E. (2016): A morphological review of subspecies of the Asian box turtle, *Cuora amboinensis* (Testudines, Geomydidae). – Proceedings of the Biological Society of Washington, **129**(1): 144–156.

Felsenstein, J. (1985): Confidence limits on phylogeny: an approach using the bootstrap. – Evolution, **39**: 783–789.

Fong, J.J. & Chen, T.H. (2010): DNA evidence for the hybridization of wild turtles in Taiwan: possible genetic pollution from trade animals. – Conservation Genetics, **11**: 2061–2066.

Fong, J.J., Parham, J.F., Shi, H., Stuart, B.L. & Carter, R.L. (2007): A genetic survey of heavily exploited, endangered turtles: caveats on the conservation value of trade animals. – Animal Conservation, **10**: 452–460.

Fritz, U. & Mendau, D. (2002): Ein Gattungsbastard zweier südostasiatischer Schildkröten: *Cuora amboinensis kamaroma* Rummler & Fritz, 1991 × *Mauremys annamensis* (Siebenrock, 1903). – Salamandra, **38**: 129–134.

Fritz, U., Barata, M., Busack, S.D., Fritzsch, G. & Castilho, R. (2006): Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwa-

ter turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). – Zoologica Scripta, **35**: 97–108.

Fritz, U., Guicking, D., Auer, M., Sommer, R.S., Wink, M. & Hundsdörfer, A.K. (2008): Diversity of the Southeast Asian leaf turtle genus *Cyclemys*: how many leaves on its tree of life? – Zoologica Scripta, **37**: 367–390.

Fritz, U., Branch, W.R., Hofmeyr, M.D., Maran, J., Prokop, H., Schleicher, A., Široký, P., Stuckas, H., Vargas-Ramírez, M., Vences, M. & Hundsdörfer, A.K. (2010): Molecular phylogeny of African hinged and helmeted terrapins (Testudines: Pelomedusidae: *Pelusios* and *Pelomedusa*). – Zoologica Scripta, **40**: 115–125.

Fritz, U., Stuckas, H., Vargas-Ramírez, M., Hundsdörfer, A.K., Maran, J. & Päckert, M. (2012): Molecular phylogeny of Central and South American slider turtles: implications for biogeography and systematics (Testudines: Emydidae: *Trachemys*). – Journal of Zoological Systematics and Evolutionary Research, **50**: 125–136.

Galgon, F. & Fritz, U. (2002): Captive bred hybrids between *Chinemys reevesii* (Gray, 1831) and *Cuora amboinensis kamaroma* Rummler & Fritz, 1991. – Herpetozoa, **15**: 137–148.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucleic Acids Symposium Series, **41**: 95–98.

Huelsenbeck, J.P. & Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogeny. – Bioinformatics, **17**: 754–755.

Ihlow, F., Vamberger, M., Flecks, M., Hartmann, T., Cota, M., Makchai, S., Meewattana, P., Dawson, J.E., Kheng, L., Rödder, D. & Fritz U. (2016): Integrative taxonomy of Southeast Asian snail-eating turtles (Geoemydidae: *Malayemys*) reveals a new species and mitochondrial introgression. – PLoS ONE, 11(4): e0153108.

Iverson, J.B. (1992): A revised checklist with distribution maps of the turtles of the world. – Privately Printed (Richmond, Indiana), xiii + 363 pp.

Ives, I.E., Platt, S.G., Tasirin, J.S., Hunowu, I., Siwu, S. & Rainwater, T.R. (2008): Field Surveys, Natural History Observations, and Comments on the Exploitation and Conservation of *Indotestudo forstenii, Leucocephalon yuwonoi,* and *Cuora amboinensis* in Sulawesi, Indonesia. – Chelonian Conservation and Biology, **7**: 240–248.

Keogh, J.S., Barker, D.G. & Shine, R. (2001): Heavily exploited but poorly known: systematics and biogeography of commercially harvested pythons (*Python curtus* group) in Southeast Asia. – Biological Journal of the Linnean Society, **73**: 113–129.

Kindler, C., Branch, W.R., Hofmeyr, M.D., Maran, J., Široký, P., Vences, M., Harvey, J., Hauswaldt, J.S., Schleicher, A., Stuckas, H. & Fritz, U. (2012): Molecular phylogeny of African hinge-back tortoises (*Kinixys*): implications for phylogeography and taxonomy (Testudines: Testudinidae). – Journal of Zoological Systematics and Evolutionary Research, **50**: 192–201.

Kindler, C., Moosig, M., Branch, W.R. Harvey, J., Kehlmaier, C., Nagy, Z.T., Prokop, H., Široký, P. & Fritz, U. (2016): Comparative phylogeographies of six species of hinged terrapins (*Pelusios* spp.) reveal discordant patterns and unexpected differentiation in the *P. castaneus*/*P. chapini* complex and *P. rhodesianus*. – Biological Journal of the Linnean Society, **117**: 305–321.

Kumar, S., Stecher, G. & Tamura, K. (2016): MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. – Molecular Biology and Evolution, **33**(7): 1870–1874.

Le, M., McCord, W.P. & Iverson, J.B. (2007): On the paraphyly of the genus *Kachuga* (Testudines: Geoemydidae). – Molecular Phylogenetics and Evolution, **45**: 398–404.

McCord, W.P. & Philippen, H.D. (1998): A new subspecies of box turtle, *Cuora amboinensis lineata*, from northern Myanmar (Burma), with remarks on the distribution and geographic variation of the species. – Reptile Hobbyist, **3**: 51–58.

Nater, A., Nietlisbach, P., Arora, N., van Schaik, C.P., van Noordwijk, M.A., Willems, E.P., Singleton, I., Wich, S.A., Goossens, B., Warren, K.S., Verschoor, E.J., Perwitasari-Farajallah, D., Pamungkas, J. & Krutzen, M. (2011): Sex-biased dispersal and volcanic activities shaped phylogeographic patterns of extant orangutans (genus: *Pongo*). – Molecular Biology and Evolution, **28**: 2275–2288.

Palupčíková, K., Somerová, B., Protiva, T., Rehák, I., Velenský, P., Hulva, P., Gunalen. D. & Frynta, D. (2012): Genetic and shell-shape analyses of *Orlitia borneensis* (Chelonia: Geoemydidae) reveal limited divergence among founders of the European zoo population. – Zootaxa, **3280**: 56–66.

Petzold, A., Vargas-Ramírez, M., Kehlmaier, C., Vamberger, M., Branch, W.R., Du Preez, L., Hofmeyr, M.D., Meyer, L., Schleicher, A., Široký, P. & Fritz, U. (2014): A revision of African helmeted terrapins (Testudines: Pelomedusidae: *Pelomedusa*), with descriptions of six new species. – Zootaxa, **3795**: 523–548.

Posada, D. & Crandall, K.A. (1998): MODELTEST: testing the model of DNA substitution. – Bioinformatics, **14**: 817–818.

Praschag, P., Schmidt, C., Fritzsch, G., Müller, A., Gemel, R. & Fritz, U. (2006): *Geoemyda silvatica*, an enigmatic turtle of the Geoemydidae (Reptilia: Testudines), represents a distinct genus. – Organisms, Diversity & Evolution, **6**: 151–162.

Praschag, P., Hundsdörfer, A.K. & Fritz, U. (2007): Phylogeny and taxonomy of endangered South and South-east Asian freshwater turtles elucidated by mtDNA sequence variation (Testudines: Geoemydidae: *Batagur, Callagur, Hardella, Kachuga, Pangshura*). – Zoologica Scripta, **36**: 429–442.

Praschag, P., Holloway, R., Georges, A., Päckert, M., Hundsdörfer, A.K. & Fritz, U. (2009): A new subspecies of *Batagur affinis* (Cantor, 1847), one of the world's most critically endangered chelonians. – Zootaxa, **2233**: 57–68.

Rhodin, A.G.J., Paul van Dijk, P., Iverson, J.B. & Shaffer, H.B. (2010): Turtles of the World, 2010 Update: Annotated Checklist of Taxonomy, Synonymy, Distribution, and Conservation Status. – Chelonian Research Monographs, 5.

Rohlf, R.J. (2008): TPSDIG, version 2.12. Stony Brook: Department of Ecology and Evolution, State University of New York.

Rummler, H.J. & Fritz, U. (1991): Geographische Variabilität der Amboina-Scharnierschildkröte *Cuora amboinensis* (Daudin, 1802), mit Beschreibung einer neuen Unterart, *C. a. kamaroma* subsp. nov. – Salamandra, **27**: 17–45.

Sheets, H.D. (2003): IMP, version 6a.

Shi, H., Fong, J.J., Parham, J.F., Pang, J., Wang, J., Hong, M. & Zhang, Y.P. (2008): Mitochondrial variation of the "eyed" turtles (*Sacalia*) based on known-locality and trade specimens. – Molecular Phylogenetics and Evolution, **49**: 1025–9.

Schoppe, S. (2008): Science in CITES: The biology and ecology of the Southeast Asian Box Turtle *Cuora amboinensis* and its local uses and trade in Malaysia. – TRAFFIC Southeast Asia, Petaling Jaya, Selangor, Malaysia.

Schoppe, S. (2009): Status, trade dynamics and management of the Southeast Asian Box Turtle *Cuora amboinensis* in Indonesia. – TRAFFIC Southeast Asia, Petaling Jaya, Selangor, Malaysia.

Schoppe, S. & Das, I. (2011): *Cuora amboinensis* (Riche in Daudin 1801) – Southeast Asian Box Turtle. In: Rhodin, A.G.J., Pritchard, P.C.H., van Dijk, P.P., Saumure, R.A., Buhlmann, K.A., Iverson, J.B. & Mittermeier, R.A. (Eds.). Conservation Biology of Freshwater Turtles and Tortoises: A Compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group. – Chelonian Research Monographs No. 5, pp. 053.1–053.13, doi:10.3854/crm.5.053.amboinensis.v1.2011, http://www.iucn-tftsg.org/cbftt/.

Spinks, P.Q., Shaffer, H.B., Iverson, J.B. & McCord, W.P. (2004): Phylogenetic hypotheses for the turtle family Geoemydidae. – Molecular Phylogenetics and Evolution, **32**: 164–82.

Spinks, P.Q. & Shaffer, H.B. (2006): Conservation phylogenetics of the Asian box turtles (Geoemydidae, *Cuora*): mitochondrial introgression, numts, and inferences from multiple nuclear loci. – Conservation Genetics, **8**: 641–657.

Spinks, P.Q., Thomson, R.C., Hughes, B., Moxley, B., Brown, R., Diesmos, A. & Shaffer, H.B. (2012): Cryptic variation and the tragedy of unrecognized taxa: the case of international trade in the spiny turtle *Heosemys spinosa* (Testudines: Geoemydidae). – Zoological Journal of the Linnean Society, **164**: 811–824.

Stamatakis, A. (2006): RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. – Bioinformatics.

Stuart, B.L. & Parham, J.F. (2004): Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). – Molecular Phylogenetics and Evolution, **31**: 164–77.

Stuart, B.L. & Parham, J.F. (2006): Recent hybrid origin of three rare Chinese turtles. – Conservation Genetics, **8**: 169–175.

Swofford, D.L. (2002): PAUP*. Phylogenetic analysis using parsimony. Version 4.0b10. – Sinauer Associates, Sunderland, Massachusetts.

Thinh, V.N., Moontrick, A.R., Geissmann, T., Li, M., Ziegler, T., Agil, M., Moisson, P., Nadler, T., Walter, L. & Roos, C. (2010): Mitochondrial evidence for multiple radiations in the evolutionary history of small apes. – BMC Evolutionary Biology, **10**: 74–87.

Thompson, D.J., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997): The CLUSTALX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tool. – Nucleic Acids Research, **24**: 4876–4882.

Vargas-Ramírez, M., Vences, M., Branch, W.R., Daniels, S.R., Glaw, F., Hofmeyr, M.D., Kuchling, G., Maran, J., Papenfuss, T.J., Široký, P., Vieites, D.R. & Fritz, U. (2010): Deep genealogical lineages in the widely distributed African helmeted terrapin: evidence from mitochondrial and nuclear DNA (Testudines: Pelomedusidae: *Pelomedusa subrufa*). – Molecular Phylogenetics and Evolution, **56**: 428–440.

Vargas-Ramírez, M., Carr, J.L. & Fritz, U. (2013): Complex phylogeography in *Rhinoclemmys melanosterna*: conflicting mitochondrial and nuclear evidence suggests past hybridization (Testudines: Geoemydidae). – Zootaxa, **3670**: 238–254.

Voris, H.K. (2000): Maps of Pleistocene Sea Levels in South East Asia: Shorelines, River Systems, Time Durations. – Journal of Biogeography, **27**: 1153–1167.

Weiss, C.H. (2007): StatSoft, Inc., Tulsa, OK.: STATISTICA, Version 8. – AStA Advances in Statistical Analysis, **91**: 339–341.

Wink, M., Guicking, D. & Fritz, U. (2001): Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard et McCord, 1991, and *Mauremys pritchardi* McCord, 1997. – Zoologische Abhandlungen, **51**: 41–49.

Zelditch, M.L., Swiderski, D.L., Sheets, H.D. & Fink, W.L. (2004): Geometric morphometrics for biologists: a primer. – Elsevier Academic Press (New York and London) 437pp.

## 9.3 Somerová B., Rehák I., Velenský P., Palupčíková K., Protiva T., Frynta D. (2015) Haplotype variation in founders of the *Mauremys annamensis* population kept in European Zoos. *Acta Herpetologica*. 10(1): 7-15.

The critically endangered Annam leaf turtle *Mauremys annamensis* faces extinction in nature. Because of that, the conservation value of the population kept in European zoos becomes substantial for reintroduction programmes. We sampled 39 specimens of M. annamensis from European zoos and other collections (mainly founders, imports and putatively unrelated individuals), and also four specimens of *Mauremys mutica* for comparison. In each animal, we sequenced 817 bp of the mitochondrial ND4 gene and 940 bp of the nuclear R35 intron that were used as phylogenetic markers for *Mauremys mutica-annamensis* group by previous authors. The sequences of the R35 intron, which are characteristic for M. annamensis and which clearly differ from those characteristic for *M. mutica* and/or other Mauremys species, were mutually shared by all of the examined *M. annamensis*. They also possessed mitochondrial haplotypes belonging to the *annamensis* subclades I and II, distinctness of which was clearly confirmed by phylogenetic analyses. Thus, both nuclear and mitochondrial markers agreed in the unequivocal assignment of the examined individuals to *M. annamensis*. Although no obvious hybrids were detected within the founders of the captive population, further careful genetic evaluation using genom-wide markers is required to unequivocally confirm this result.

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# Haplotype variation in founders of the *Mauremys annamensis* population kept in European Zoos

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**Abstract.** The critically endangered Annam leaf turtle *Mauremys annamensis* faces extinction in nature. Because of that, the conservation value of the population kept in European zoos becomes substantial for reintroduction programmes. We sampled 39 specimens of *M. annamensis* from European zoos and other collections (mainly founders, imports and putatively unrelated individuals), and also four specimens of *Mauremys mutica* for comparison. In each animal, we sequenced 817 bp of the mitochondrial ND4 gene and 940 bp of the nuclear R35 intron that were used as phylogenetic markers for *Mauremys mutica-annamensis* group by previous authors. The sequences of the R35 intron, which are characteristic for *M. annamensis* and which clearly differ from those characteristic for *M. mutica* and/or other *Mauremys* species, were mutually shared by all of the examined *M. annamensis*. They also possessed mitochondrial haplotypes belonging to the *annamensis* subclades I and II, distinctness of which was clearly confirmed by phylogenetic analyses. Thus, both nuclear and mitochondrial markers agreed in the unequivocal assignment of the examined individuals to *M. annamensis*. Although no obvious hybrids were detected within the founders of the captive population, further careful genetic evaluation using genom-wide markers is required to unequivocally confirm this result.

**Keywords.** Mauremys, Geoemydidae, conservation, mt gene ND4, nuclear intron R35, Vietnam, hybridization.

## INTRODUCTION

Asian turtles face an extinction crisis due to habitat destruction and high demands from the Chinese markets (van Dijk et al., 2000; Le et al., 2004; Cheung and Dudgeon, 2006; Turtle Conservation Coalition, 2011). One of the heavily exploited species is *Mauremys annamensis*, the Annam leaf turtle. (Siebenrock, 1903). This species of the family Geoemydidae has a very limited and fragmented distribution and is restricted only to central Vietnam (Le et al., 2004; Parham et al. 2006). *Mauremys annamensis* is almost extinct in the wild, with limited numbers in *ex-situ* populations in Vietnam, Europe and the USA. It is listed in the Appendix II of CITES and is globally red-listed as critically endangered by the IUCN (2013). Captive breeding seems to be one of the long-term solutions for the survival of Asian turtles (Hudson and Buhlmann, 2002; Turtle Conservation Coalition, 2011). *Mauremys annamensis* has been repeatedly bred in some European zoos, including Prague Zoo (Velenský, 2006; Raffel and Meier, 2013). Currently, these zoos have started co-ordinated *ex situ* conservation breeding of the species associated with a repatriation project. Among the programmes' top priorities at present is the repatriation of the best captive-bred specimens.

The situation of conservation breeding is complicated by hybridization among distinct species and even genera of the geoemydids (Galgon and Fritz, 2002; Fritz and Mendau, 2002; Fritz et al., 2004; Schilde et al., 2004; Spinks et al., 2004; Buskirk et al., 2005; Stuart and Par-

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ham, 2006; Shi et al., 2008). Hybridization among *Mauremys annamensis*, *M. mutica*, *M. sinensis*, *M. nigricans*, *Cuora amboinensis* and *C. trifasciata* was reported both in captivity and in the wild (Parham et al., 2001; Shi and Parham, 2001; Fong and Chen, 2010). The current events of natural hybridization between *M. mutica* and *M. sinensis* on Taiwan Island (Fong and Chen, 2010) represent an especially interesting case.

The phylogenetically closest species of *M. annamensis* is *M. mutica* (Barth et al., 2004; Feldman and Parham, 2004; Spinks et al., 2004) and these species may interbreed (Fong et al., 2007). This represents a serious problem for the efforts to build sustainable *ex-situ* breeding programs enabling the reintroduction and establishment of sustainable populations of *M. annamensis* in the wild. Hybridization events in the *annamensis-mutica* complex were demonstrated by striking incongruence among phylogenies of the individual genes, i.e., the mitochondrial and nuclear markers. Some of these incongruences may result from recent translocation and consequent hybridization; however, hybridization events that took place in the past are even more likely. Fong et al. (2007) clearly demonstrated such incongruence in the Hainan population of *M. mutica*, which differs from the "true *mutica*" of the Eastern continental China by the presence of mitochondrial haplotypes forming a clade branching within those belonging to the *M. annamensis*. In contrast, sequences of R35 intron of Hainan *M. mutica* are even less related to the corresponding sequences of the *M. annamensis* than those of the "true *mutica*". Moreover, it was clearly demonstrated that mitochondrial haplotypes of the *M. annamensis* was split into two deeply divergent haplogroups, which are referred to as the *annamensis* subclade I and II (Fong et al., 2007; Fong, 2008). The phylogeographic pattern of these subclades is, however, unclear due to the extinction of most of the original populations in the nature.

To organize proper *ex-situ* captive breeding and to remove potential hybrids from the rescue population, it is necessary to examine the genetic variation of the founders of the *M. annamensis* population. In this study, we focused on the founders, imported and putatively unrelated individuals of the *M. annamensis* kept in European zoos and other collections. We sequenced mitochondrial (ND4 gene) and nuclear (R35 intron) parts of DNA to (1) verify the species determination of the founders, (2) assess sequence variation of the captive population, (3) assign captive specimens into the main haplogroups (subclades I and II) and to (4) exclude the discovered interspecific species hybrids from the breeding pool. For comparison, we also included a few specimens of *M. mutica* into the analyses.

## MATERIAL AND METHODS

In this paper, we examined 39 specimens of *Mauremys annamensis* from European zoos and other collections (founders, imported and putatively unrelated individuals, i.e., captive born specimens having no shared maternal ancestors in their pedigree), and also four specimens of *M. mutica* were included for comparison (Table 1). For all individuals, we sequenced a combination of mitochondrial (mtDNA) and nuclear DNA (nuDNA).

For sampling of individuals, we used a non-invasive method: we took the tip of the claw from each sampled animal and stored in Eppendorf tubes with 96% ethanol at -20°C prior DNA extraction. We isolated the total genomic DNA with DNAeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations.

Using standard conditions and the primers L-ND4 and H-Leu, we amplified an 892 bp fragment of mtDNA containing the NADH dehydrogenase subunit 4 (ND4) gene and parts of tRNA (Stuart and Parham, 2004). Following the conditions in Fujita et al. (2004), and using the primers R35Ex1 and R35Ex2, we amplified the fragment of nuDNA containing 1133 bp of the RNA fingerprint protein 35 (R35) gene intron 1.

Patterns from the sequencing chromatograms indicated that at the R35 locus, some individuals were heterozygous for a length polymorphism, which usually corrupts the sequence reads downstream of the indel site (see Bhangale et al., 2005, Fig. 1B). For sequencing the R35 intron, we used internal forward and reverse primers (Spinks and Shaffer, 2007) in combination with external primers (Fujita et al., 2004) for the putative length-polymorphic individuals (Spinks and Shaffer, 2007).

Sequences of both mtDNA and nuDNA fragments were aligned and manually checked using Chromas Lite 2.01 (Technelysium Pty Ltd), BioEdit (Hall, 1999) and Clustal X 1.81 (Thompson et al., 1997).

Analyses of the estimates of evolutionary divergence between the sequences of ND4 gene and R35 intron were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The included codon positions were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Bayesian analysis (BA) was conducted with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best-fit model (HKY+G) was selected by hLRT in Modeltest 3.7 (Posada and Crandall, 1998). Two independent runs of Bayesian analyses were conducted with a random starting tree and run for $30x10^6$ generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 10% of trees (3,000,000 generations), which were generated before the chain reached equilibrium in the distribution of trees.

For these phylogenetic analyses, we also included some mtDNA and nuDNA sequence data used in intrageneric studies about the *Mauremys mutica-annamensis* complex (Fong et al., 2007; Fong and Chen, 2010) and some species from the family Geoemydidae, which were used as outgroups (GenBank numbers are listed in Appendix 1).

## RESULTS

In an alignment of the mitochondrial ND4 gene (817 bp), we detected 16 haplotypes, 25 variable sites and 17 parsimony-informative sites. All individuals of *M. annamensis* examined in this study possessed the mitochondrial ND4 gene (p-distaces ranging from 0.127% to 1.826%) typical for this species (Fong et al., 2007).

Phylogenetic analyses containing our sequences in the context of those available in the GenBank confirmed haplogroups and the general topology of previously published trees (Fong et al., 2007). The BA tree (Fig. 1) suggests a principal split between the "true *mutica* clade" (BA posterior probability = 1.00) and a clade (BA = 1.00) containing both the *M. annamensis* and Hainan *M. mutica*. The latter clade further splits into three distinct clades (all BA probabilities = 1.00). These are an "*annamensis* subclade I", "*annamensis* subclade II" and the "Hainan *mutica* clade". Average uncorrected p-distance between the "*annamensis* subclade I" and "*annamensis* subclade II was 1.968%". The sister relationship between the "*annamensis* subclade I" and the "Hainan *mutica* clade" is moderately supported (BA = 0.82).

ND4 sequences of our *M. annamensis* samples belong to the haplogroups previously described as the "*annamensis* subclade I" and "*annamensis* subclade II" (13 and 26 cases, respectively). Out of four examined samples of the putative *M. mutica*, ND4 sequences branch within the "true *mutica* clade" and one within the "*annamensis* subclade I". P-distances among these four clades computed from all available sequences (including GenBank sources) suggest low mutual divergence among both the "*annamensis*" and "Hainan *mutica*" clades (Table 1).

In an alignment of nuclear R35 intron (918 bp), we detected 25 haplotypes, 20 variable sites and 7 parsimony-informative sites. All 39 specimens putatively belonging to the *M. annamensis* shared mutually similar sequences of R35 intron (p-distaces from 0.132% to 0.932%). The R35 sequences in three of four *M. mutica* samples clearly differed from those of the *M. annamensis*.

Phylogenetic analysis of these sequences and those available in the GenBank (alignment of 940 bp, see Fig. 2) confirmed the presence of the three previously described clades (Fong et al., 2007) within the *annamensis-mutica* complex: the "Hainan *mutica* clade" (BA posterior probability = 0.98) is the sister group of the true *mutica-annamensis* clade (BA = 1.00), which contains a group of *mutica* sequences corresponding to the "true *mutica* clade" (BA = 0.53) and a well-supported "*annamensis* clade" (BA = 1.00). In BA tree, the "true *mutica*" is paraphyletic with respect to the "*annamensis* clade", however, most of the sequences of this group form a single branch with low support (BA = 0.53).

The BA analysis placed all 39 examined sequences of the *M. annamensis* into the "*annamensis* clade". Out of four of the *M. mutica* sequences, one belongs to the "Hainan *mutica* clade", one into the "*annamensis* clade" and the remaining two into the "true *mutica*" (Table 2).

## DISCUSSION

We have no evidence suggesting the presence of the interspecific hybrids among the examined founders of the *M. annamensis* kept in European collections. Of course, without an application of expensive genome-wide markers (like SNPs, extensive number of microsatellites), it is impossible to entirely rule out partial introgression of the genomes of other related geoemydids into some founders of the European population of the *M. annamensis* (i.e., presence of hybrids of a higher order - $F_2$ and higher generations and backcrosses). Also, without cloning, we are unable to evaluate the affinity of potential heterozygots of the R35 intron to individual mitochondrial subclades. Nevertheless, when considering other supportive evidence (age, origin), the presence of hybrids seems to be fairly unlikely.

The original geographic distribution of the *Mauremys annamensis* is unknown, only few records document it. That is why it is hard to understand the significance of the two distinct mitochondrial clades, which we, as well as previous authors, detected in the *M. annamensis*. It is unclear whether these clades occur or occurred in the wild in syntopy or allopatrically. The sequence divergence

**Table 1.** Average values of estimates of evolutionary divergence between sequences (the p-values are expressed in per cents).

| | *annamensis* subclade II | *annamensis* subclade I | Hainan *mutica* clade | true *mutica* clade |
|---|---|---|---|---|
| *annamensis* subclade II | 0.083-0.167 | | | |
| *annamensis* subclade I | 0.844-1.193 | 0.167-0.420 | | |
| Hainan *mutica* clade | 1.020-1.281 | 0.589-0.934 | 0.083-0.167 | |
| true *mutica* clade | 3.952-4.895 | 3.549-4.782 | 3.952-4.782 | 0.083-1.554 |

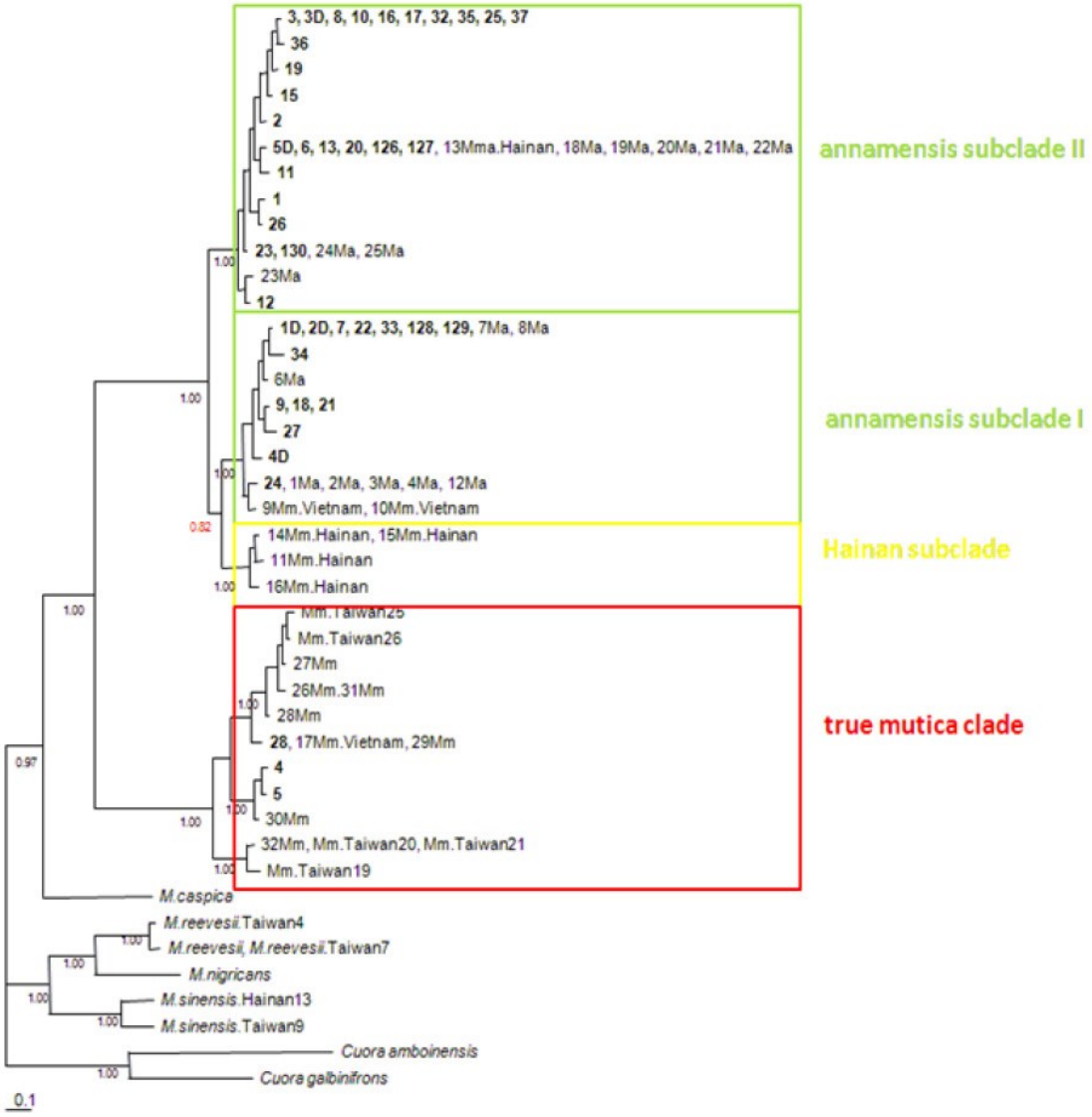


**Fig. 1.** Bayesian tree of mitochondrial DNA (ND4) of the genus *Mauremys*. Numbers at branches are support values, only values > 0.95 are shown. Samples sequenced in this study, which correspond to Table 1, in bold, remaining samples were sequenced by previous authors (Fong et al., 2007; Fong and Chen, 2010), Mm = *Mauremys mutica*, Ma = *Mauremys annamensis*. Countries of the origins of samples are shown at individuals with reliable locality.

between the two clades is only about 0.84-1.19 %. Thus, we cannot reject the possibility that retention of ancestral polymorphism is a cause of a simultaneous occurrence of these related, but still distinct clades, in the sampled population of the *M. annamensis*. Ancestral polymorphism may be irrelevant to an original population structure of the species prior to its recent decline leading to near extinction in the wild.

The distinction between the mitochondrial haplotype groups I and II has been recognized only recently and thus, the species has been treated as a single conservation unit in most zoos and collections. However, it is possible to keep the animals of the two groups apart. This would be recommended especially in the case of animals producing offspring suitable for repatriation projects. Nevertheless, such a precaution cannot sub-

**Table 2.** List of samples used in this study containing information about species, breeder, nuclear and mitochondrial haplotype subclades.

| Nr. | | Species | Breeder | ND4 | R35 |
|---|---|---|---|---|---|
| ***Mauremys annamensis* mtDNA subclade II** | | | | | |
| 1 | | *Mauremys annamensis* | H. Becker | *annamensis* subclade II | *annamensis* clade |
| 2 | | *Mauremys annamensis* | H. Becker | *annamensis* subclade II | *annamensis* clade |
| 3 | | *Mauremys annamensis* | H. Becker | *annamensis* subclade II | *annamensis* clade |
| 3D | | *Mauremys annamensis* | D. Frynta | *annamensis* subclade II | *annamensis* clade |
| 5D | | *Mauremys annamensis* | D. Frynta | *annamensis* subclade II | *annamensis* clade |
| 6 | 704774 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade II | *annamensis* clade |
| 8 | 704525 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade II | *annamensis* clade |
| 10 | 704524 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade II | *annamensis* clade |
| 11 | 705067 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade II | *annamensis* clade |
| 12 | 3 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 13 | 4 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 15 | 9 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 16 | 1 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 17 | 2 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 19 | 6 | *Mauremys annamensis* | Münster | *annamensis* subclade II | *annamensis* clade |
| 20 | | *Mauremys annamensis* | M. Schilde | *annamensis* subclade II | *annamensis* clade |
| 23 | M53 | *Mauremys annamensis* | Praha | *annamensis* subclade II | *annamensis* clade |
| 25 | F21 | *Mauremys annamensis* | Praha | *annamensis* subclade II | *annamensis* clade |
| 26 | F9 | *Mauremys annamensis* | Praha | *annamensis* subclade II | *annamensis* clade |
| 32 | ROO718 | *Mauremys annamensis* | Leipzig | *annamensis* subclade II | *annamensis* clade |
| 35 | 32 | *Mauremys annamensis* | Panuška | *annamensis* subclade II | *annamensis* clade |
| 36 | 33 | *Mauremys annamensis* | Panuška | *annamensis* subclade II | *annamensis* clade |
| 37 | 34 | *Mauremys annamensis* | Panuška | *annamensis* subclade II | *annamensis* clade |
| 126 | COS679 | *Mauremys annamensis* | Chester | *annamensis* subclade II | *annamensis* clade |
| 127 | COS678 | *Mauremys annamensis* | Chester | *annamensis* subclade II | *annamensis* clade |
| 130 | COS349 | *Mauremys annamensis* | Chester | *annamensis* subclade II | *annamensis* clade |
| ***Mauremys annamensis* mtDNA subclade I** | | | | | |
| 1D | | *Mauremys annamensis* | D. Frynta | *annamensis* subclade I | *annamensis* clade |
| 2D | | *Mauremys annamensis* | D. Frynta | *annamensis* subclade I | *annamensis* clade |
| 4D | | *Mauremys annamensis* | D. Frynta | *annamensis* subclade I | *annamensis* clade |
| 7 | 704212 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade I | *annamensis* clade |
| 9 | 704523 | *Mauremys annamensis* | Rotterdam | *annamensis* subclade I | *annamensis* clade |
| 18 | 8 | *Mauremys annamensis* | Münster | *annamensis* subclade I | *annamensis* clade |
| 21 | | *Mauremys annamensis* | M. Schilde | *annamensis* subclade I | *annamensis* clade |
| 22 | | *Mauremys annamensis* | M. Schilde | *annamensis* subclade I | *annamensis* clade |
| 24 | M7 | *Mauremys annamensis* | Praha | *annamensis* subclade I | *annamensis* clade |
| 33 | ROO720 | *Mauremys annamensis* | Leipzig | *annamensis* subclade I | *annamensis* clade |
| 34 | ROO719 | *Mauremys annamensis* | Leipzig | *annamensis* subclade I | *annamensis* clade |
| 128 | CZ/921 | *Mauremys annamensis* | Chester | *annamensis* subclade I | *annamensis* clade |
| 129 | CZ/922 | *Mauremys annamensis* | Chester | *annamensis* subclade I | *annamensis* clade |
| ***Mauremys mutica*** | | | | | |
| 4 | | *Mauremys mutica* | H. Becker | true *mutica* clade | true *mutica* clade |
| 5 | | *Mauremys mutica* | H. Becker | true *mutica* clade | true *mutica* clade |
| ***Animals of hybrid origin*** | | | | | |
| 28 | 3 | *Mauremys mutica* | Praha | true *mutica* clade | *annamensis* clade |
| 27 | 2 | *Mauremys mutica* | Praha | *annamensis* subclade I | Hainan *mutica* clade |

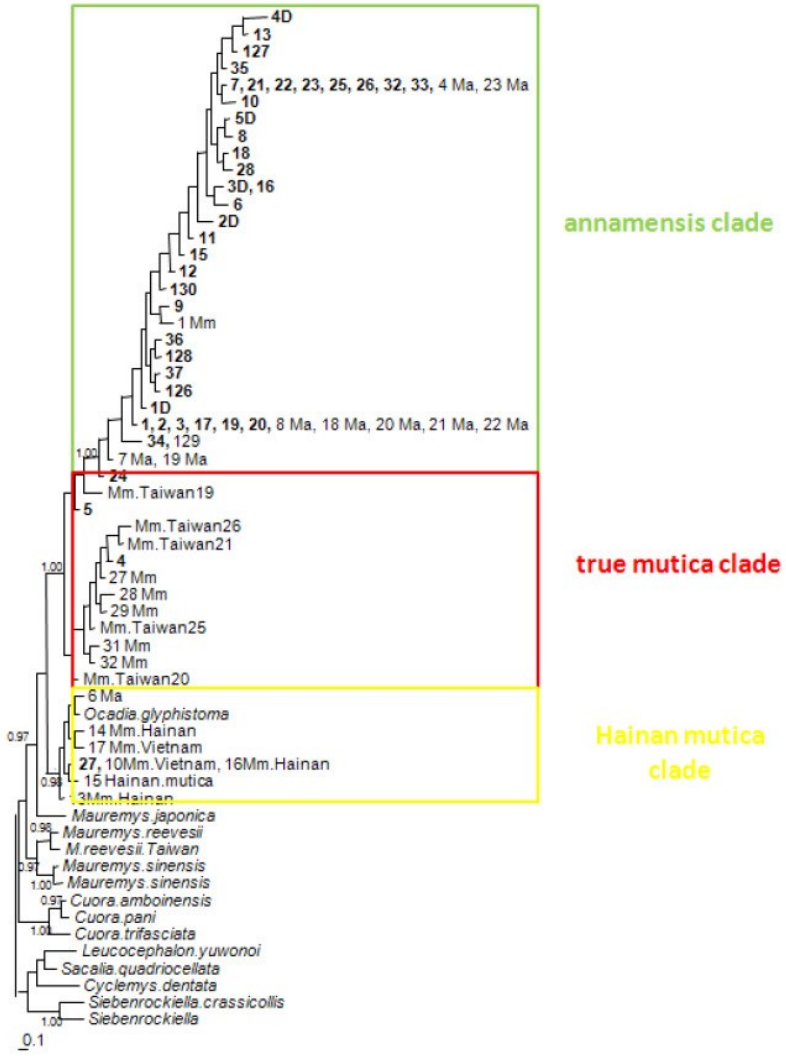


**Fig. 2.** Bayesian tree of nuclear DNA (R35) of the genus *Mauremys*. For further explanation, see Fig. 1

stantiate the elimination of the descendants of parents belonging to different clades from the studbook population. There is an urge call for further research of the genetic variation in the *M. annamensis* using multiple nuclear markers and/or advanced genomic methods, especially to enable a better understanding of the divergence of the two distinct subclades.

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## REFERENCES

Barth, D., Bernhard, D., Fritzsch, G., Fritz, U. (2004): The freshwater turtle genus *Mauremys*: a textbook example of an east-west disjunction or a taxonomic misconcept? Zool. Scr. **33**: 213-221.

Bhangale, T.R., Rieder, M.J., Livingston, R.J., Nickerson, D.A. (2005): Comprehensive identification and characterization of diallelic insertion–deletion polymorphisms in 330 human candidate genes. Hum. Mol. Genet. **14**: 59-69.

Buskirk, J.R., Parham, J.F., Feldman, C. R. (2005): On the hybridisation between two distantly related Asian turtles (Testudines: *Sacalia* x *Mauremys*). Salamandra **41**: 21.

Feldman, C.R., Parham, J.F. (2004): Molecular systematics of Old World stripe-necked turtles (Testudines: *Mauremys*). Asiat. Herpetol. Res. **10**: 28-37.

Felsenstein, J. (1985): Confidence limits on phylogeny: an approach using the bootstrap. Evolution **39**: 783-789.

Fong, J.J. (2008): Linnaeus Fund Research Report: Conservation genetics of the Yellow Pond Turtle (*Mauremys mutica*) and the Annam Leaf Turtle (*Mauremys annamensis*). Editor's Note, 21.

Fong, J.J., Chen, T.H. (2010): DNA evidence for the hybridization of wild turtles in Taiwan: possible genetic pollution from trade animals. Conserv. Genet. **11**: 2061-2066.

Fong, J.J., Parham, J.F., Shi, H., Stuart, B.L., Carter, R.L. (2007): A genetic survey of heavily exploited, endangered turtles: caveats on the conservation value of trade animals. Anim. Conserv. **10**: 452-460.

Fritz, U., Ayaz, D., Buschbom, J., Kami, H.G., Mazanaeva, L.F., Aloufi, A.A., Hundsdörfer, A.K. (2008): Go east: phylogeographies of *Mauremys caspica* and *M. rivulata*–discordance of morphology, mitochondrial and nuclear genomic markers and rare hybridization. J. Evol. Biol. **21**: 527-540.

Fritz, U., Mendau, D. (2002): Ein Gattungsbastard zweier südostasiatischer Schildkröten: *Cuora amboinensis kamaroma* Rummler and Fritz, 1991 x *Mauremys annamensis* (Siebenrock, 1903). Salamandra **38**: 129-134.

Fujita, M.K., Engstrom, T.N., Starkey, D.E., Shaffer, H.B. (2004): Turtle phylogeny: insights from a novel nuclear intron. Mol. Phylogenet. Evol. **31**: 1031-1040.

Galgon, F., Fritz, U. (2002): Captive bred hybrids between *Chinemys reevesii* (Gray, 1831) and *Cuora amboinensis kamaroma* Rummler & Fritz, 1991. Herpetozoa **15**: 137-148.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. **41**: 95-98.

Hudson, R., Buhlmann, K. (2002): Turtle rescue - turtle survival alliance executive summary. Turtle Tort. Newsl. **6**: 12-14.

Huelsenbeck, J.P., Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**: 754-755.

Cheung, S.M., Dudgeon, D. (2006): Quantifying the Asian turtle crisis: market surveys in southern China, 2000–2003. Aquat. Conserv. **16**: 751-770.

IUCN. (2013): The IUCN Red List of threatened species. Version 2013.2.

Le, M., Hoang, T., Le, D. (2004): Trade data and some comments on the distribution of *Mauremys annamensis* (Siebenrock, 1903). Asiat. Herpetol. Res. **10**: 110-113.

Parham, J. F., Simison, W. B., Kozak, K. H., Feldman, C. R., Shi, H. (2001): New Chinese turtles: endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens. Anim. Conserv. **4**: 357-367.

Parham, J. F., Stuart, B. L., Orlov, N. L. (2006): *Mauremys annamensis*: Distribution record for Vietnam. Herpetol. Rev. **37**: 239.

Posada, D., Crandall, K.A. (1998): MODELTEST: testing the model of DNA substitution. Bioinformatics **14**: 817-818.

Raffel, M., Meier, E. (2013): Best year ever at the International Centre for the Conservation of Turtles (IZS) at Muenster Zoo. Turtle Survival 2013, Turtle Survival Alliance, Fort Worth, Texas, USA.

Ronquist, F., Huelsenbeck, J. P. (2003): MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**: 1572-1574.

Shi, H., Fong, J. J., Parham, J. F., Pang, J., Wang, J., Hong, M., Zhang, Y. P. (2008): Mitochondrial variation of the "eyed" turtles (*Sacalia*) based on known-locality and trade specimens. Mol. Phylogenet. Evol. **49**: 1025-1029

Shi, H., Parham, J. F. (2001): Preliminary observations of a large turtle farm in Hainan Province, People's Republic of China. Turtle Tort. Newsl. **3**: 4-6.

Schilde, M., Barth, D., Fritz, U. (2004): An *Ocadia sinensis* x *Cyclemys shanensis* hybrid (Testudines: Geomydidae). Asiat. Herpetol. Res. **10**: 120-125

Siebenrock, F. (1903): Schildkröten des östlichen Hinterindien. Sitzung. Akad. Wissen. Wien Math.-Natur. Klasse **112**: 333-353.

Spinks, P. Q., Shaffer, H. B. (2007): Conservation phylogenetics of the Asian box turtles (Geoemydidae, *Cuora*): mitochondrial introgression, numts, and inferences from multiple nuclear loci. Conserv. Gen. **8**: 641-657.

Spinks, P. Q., Shaffer, H.B., Iverson, J. B., McCord, W. P. (2004): Phylogenetic hypotheses for the turtle family Geoemydidae. Mol. Phylogenet. Evol. **32**: 164-182.

Stuart, B. L., Parham, J. F. (2004): Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). Mol. Phylogenet. Evol. **31**: 164-177.

Stuart, B. L., Parham, J. F. (2007): Recent hybrid origin of three rare Chinese turtles. Conserv. Gen. **8**: 169-175.

Tamura, K., Nei, M., Kumar, S. (2004): Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS **101**: 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski A., Kumar S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. **30**: 2725-2729.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G. (1997): The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. **25**: 4876-4882.

Turtle Conservation Coalition (2011): Turtles in trouble: The world's top 25+ most endangered tortoises and freshwater turtles - 2011. Available from: http://www.iucn-tftsg.org/top-25-2011/ (May 11, 2014).

van Dijk, P.P., Stuart, B.L., Rhodin, A.G.J. (2000): Asian Turtle Trade: Proceedings of a Workshop on Conservation and Trade of Freshwater Turtles and Tortoises in Asia, Chelonian Research Monographs, Chelonian Research Foundation, Lunenburg, Massachusetts.

Velenský, P. (2006): Chovatelství – plazi. Výroční zpráva 2006, Zoo Praha. Available from: http://www.zoopraha.cz/vse-o-zoo/vyrocni-zpravy/7572-vyrocni-zprava-2006 (May 31, 2014).

*Appendix 1*

List of sequences containing their GenBank numbers used in this study.

| Name of sequence | Species | ND4 | R35 |
|---|---|---|---|
| 1Ma | *M. annamensis* | EF034098 | EF587934 |
| 10Ma | *M. annamensis* | AF348280 | DQ386668 |
| 11Mm.Hainan | *M. annamensis* | EF034096 | EF87929 |
| 12Ma | *M. annamensis* | EF034105 | — |
| 13Ma.Hainan | *M. annamensis* | EF034104 | EF587915 |
| 14Mm.Hainan | *M. mutica* | EF034097 | EF587925 |
| 15.Mm.Hainan | *M. mutica* | EF034101 | EF587917 |
| 16.Mm.Hainan | *M. mutica* | EF034095 | EF587930 |
| 17Mm.Vietnam | *M. mutica* | AF348278 | DQ386664 |
| 18Ma | *M. annamensis* | EF034104 | EF587923 |
| 19Ma | *M. annamensis* | EF034106 | EF587928 |
| 20Ma | *M. annamensis* | EF034107 | EF587924 |
| 21Ma | *M. annamensis* | EF034112 | DQ386656 |
| 22Ma | *M. annamensis* | EF587914 | EF587921 |
| 23Ma | *M. annamensis* | EF034099 | EF587919 |
| 24Ma | *M. annamensis* | EF034100 | — |
| 25Ma | *M. annamensis* | EF034108 | — |
| 26Mm | *M. mutica* | EF034092 | — |
| 27Mm | *M. mutica* | EF034093 | EF587931 |
| 28Mm | *M. mutica* | EF034089 | EF587932 |
| 29Mm | *M. mutica* | AF348278 | DQ386666 |
| 2Ma | *M. annamensis* | AY337338 | EF587933 |
| 30Mm | *M. mutica* | EF034090 | — |
| 31Mm | *M. mutica* | EF034092 | EF587916 |
| 32Mm | *M. mutica* | EF034094 | EF587927 |
| 3Ma | *M. annamensis* | EF034103 | — |
| 4Ma | *M. annamensis* | EF034105 | EF587922 |

| Name of sequence | Species | ND4 | R35 |
|---|---|---|---|
| 6Ma | *M. annamensis* | EF034102 | EF587929 |
| 7Ma | *M. annamensis* | EF034109 | EF587926 |
| 8Ma | *M. annamensis* | EF034113 | DQ386655 |
| 9Mm.Vietnam | *M. mutica* | AF348279 | ---- |
| Cuora amboinensis | *Cuora amboinensis* | EF011357 | HQ442382 |
| Cuora galbinifrons | *Cuora galbinifrons* | AY364617 | ---- |
| Cuora pani | *Cuora pani* | — | EF011442 |
| Cuora trifasciata | *Cuora trifasciata* | — | JQ596437 |
| Cyclemys dentata | *Cyclemys dentata* | — | AM931697 |
| Leucocephalon yuwonoi | *Leucocephalon yuwonoi* | — | AM931708 |
| M. nigricans | *M. nigricans* | EF034111 | ---- |
| M. reevesii | *M. reevesii* | EF034110 | ---- |
| *M. reevesii*.Taiwan4 | *M. reevesii* | GQ259438 | GQ259459 |
| *M. reevesii*.Taiwan7 | *M. reevesii* | GQ259441 | GQ259464 |
| *M. sinensis*.Hainan13 | *M. sinensis* | AY337345 | DQ386678 |
| *M. sinensis*.Taiwan9 | *M. sinensis* | GQ259443 | GQ259465 |
| M. caspica | *M. caspica* | AY337340 | ---- |
| Mauremys japonica | *Mauremys japonica* | — | HQ442386 |
| Mm.Taiwan19 | *M. mutica* | GQ259452 | GQ259471 |
| Mm.Taiwan20 | *M. mutica* | GQ259453 | GQ259472 |
| Mm.Taiwan21 | *M. mutica* | GQ259454 | GQ259473 |
| Mm.Taiwan25 | *M. mutica* | GQ259457 | GQ259474 |
| Mm.Taiwan26 | *M. mutica* | GQ259458 | GQ259475 |
| Ocadia glyphistoma | *Ocadia glyphistoma* | — | DQ386663 |
| Sacalia quadriocellata | *Sacalia quadriocellata* | — | HQ442384 |
| Siebenrockiella | *Siebenrockiella leytensis* | — | AM931708 |
| Siebenrockiella crassicollis | *Siebenrockiella crassicollis* | — | AY954913 |

## 9.4 Palupčíková K., Somerová B., Protiva T., Rehák I., Velenský P., Hulva P., Gunalen D., Frynta D. (2012) Genetic and shell-shape analyses of *Orlitia borneensis* (Testudines: Geoemydidae) reveal limited divergence among founders of the European zoo population. *Zootaxa*. 3280: 56-66.

The Malaysian Giant Turtle (*Orlitia borneensis*) is a poorly known turtle with rapidly decreasing numbers in nature in spite of its strong protection on paper. Most individuals of this species kept in European zoos and included in captive breeding programs are confiscates from the illegal trade for food consumption and their geographic provenance is unknown. This study was aimed to assess genetic and phenotypic variation of the founders of this captive population. We sequenced the mitochondrial cytochrome b gene and found 23 haplotypes. We constructed a haplotype network and examined demographic changes by Bayesian skyline plots of the effective population size. The maximum sequence divergence was less than 1.5% and the phylogenetic structure of the haplotypes was supported poorly. A close genetic similarity among sampled turtles was further confirmed by sequencing the nuclear R35 gene, while the geometric morphometrics of the shell-shape were likewise similar. Thus, the examined captive population of *O. borneensis* may be further treated as a single conservation unit.

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**Article**

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# Genetic and shell-shape analyses of *Orlitia borneensis* (Testudines: Geoemydidae) reveal limited divergence among founders of the European zoo population

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## Abstract

The Malaysian Giant Turtle (*Orlitia borneensis*) is a poorly known turtle with rapidly decreasing numbers in nature in spite of its strong protection on paper. Most individuals of this species kept in European zoos and included in captive breeding programs are confiscates from the illegal trade for food consumption and their geographic provenance is unknown. This study was aimed to assess genetic and phenotypic variation of the founders of this captive population. We sequenced the mitochondrial cytochrome *b* gene and found 23 haplotypes. We constructed a haplotype network and examined demographic changes by Bayesian skyline plots of the effective population size. The maximum sequence divergence was less than 1.5% and the phylogenetic structure of the haplotypes was supported poorly. A close genetic similarity among sampled turtles was further confirmed by sequencing the nuclear R35 gene, while the geometric morphometrics of the shell-shape were likewise similar. Thus, the examined captive population of *O. borneensis* may be further treated as a single conservation unit.

**Key words:** Ex situ breeding, genetic variability, cytochrome *b*, nuclear gene R35, phylogeography, population expansion

## Introduction

With the ongoing Asian Turtle Crisis (Cheung & Dudgeon 2006) we are now facing the reality of decreasing numbers of many species of turtles, especially in the family Geoemydidae. This situation is a result of a combination of habitat destruction and targeted exploitation of turtles to meet the demand from Chinese markets for use in traditional medicine and especially for meat (Zhou & Jiang 2008; Chen *et al.* 2009). Proper taxonomy is an important prerequisite to efficient conservation. It is especially important to detect hidden, but possibly deep genetic variation in rare species without recognized subspecies that originally inhabited extensive geographic ranges and that are now restricted to scattered refugees, as it is the case for one of the most charismatic South Asian freshwater turtle species, *Orlitia borneensis* (Gray 1873). The local Indonesian name of this species is Kura Tuntong or Biuku. According to IUCN, *O. borneensis* is listed as Endangered A1d+2d range-wide, and was specifically considered Endangered (A1cd) in Indonesia and Vulnerable (2cd) in West Malaysia. Because it can reach up to 80 cm carapace length (Ernst & Barbour 1989), it is a preferred target of collectors and thus the numbers of individuals of this species in nature are drastically decreasing in spite of its strong legal protection on paper (e.g., Indonesian Law PP No. 7 1999).

It was attempted to develop individual countermeasures to solve this problem. In Indonesia, commercial harvesting of turtle species for food consumption is strictly regulated by a system of quota. Such harvesting is permitted exclusively to licensed foods traders and concerns a few turtle species that are believed to be still abundant (*Amyda cartilaginea, Dogania subplana, Cuora amboinensis, Cyclemys* spp.). Harvesting of the protected species like *O. borneensis* is strictly prohibited. Thus, all *O. borneensis* found outside the country (except those legally

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exported from Malaysia until the year 2009) come from illegal trade. On the data base of the Indonesian CITES Authority Department of Forestry PHKA (Ministry of Forestry Republic of Indonesia, Directorate General of Forest Protection and Nature Conservation), there is no indication for any recently issued CITES permit for *O. borneenis*. Interviews of local people performed by Danny Gunalen in November 2009 revealed an illegal harvest of *O. borneensis* to supply illegal foods traders in Riau, Pekan Baru (Central Sumatra), i.e., in the localities of the principal populations of *O. borneensis* (Samedi & Iskandar 2000). The turtles caught there are transported to Medan, capital city of North Sumatra, which is the base of most established licensed consumption turtle exporters and the biggest sea port to the neighbouring countries. This finding led to the introduction of additional countermeasures. Recently, the wildlife officers (BKSDA Conservation Agency) in Medan were trained to identify turtle species to be able to find if *O. borneensis* are mixed in with the legal shipments of the turtles under quota, or illegally transported by Sea Cargo to Malaysia or directly to Hong Kong and China.

*Ex situ* breeding programs organized by zoos and private breeders may contribute to the survival of heavily exploited species. Ten years ago, Asian turtles confiscated in Hong Kong were distributed among European and American zoos. This confiscation enabled the establishment of European captive breeding projects for several species, including *O. borneensis*.

Because the taxonomy of the family Geoemydidae continues to change (cf. Fritz *et al.* 2008a; Praschag *et al.* 2008, 2009a, b) and the relationships in this group are further complicated by frequent cases of natural and/or artificial hybridization between distinct species and even genera (Parham *et al.* 2001; Stuart & Parham 2007; Fong & Chen 2010), there is an urgent need for the genetic and morphological examination of all individuals included in breeding programs to confirm the genetic purity and compatibility of the population founders. This is especially important when the founders of rescue programs are confiscated specimens with uncertain geographic provenance.

The distribution range of *O. borneensis* covers Borneo, Sumatra and the southern part of the Malay Peninsula (Iverson 1992). From a topographical point of view, the Great Sundas are mainland fragments separated by only a shallow sea. As a result of Pleistocene glacial cycles, the islands were repeatedly connected with each other as well as to the contemporary Malay Peninsula by land bridges, especially during glacial maxima 250, 150 and 17 kya (Voris 2000). These periods allowed free faunal exchange that was facilitated by strong volcanic activities affecting the evolution of local fauna (Nater *et al.* 2011). The most important local catastrophe of this kind was the eruption of the Toba volcano 73 kya (Rampino & Ambrose 2000) that represents one of the most destructive volcanic eruptions in recent geological history of the planet (Williams *et al.* 2009). Therefore, the region has a complex biogeographic history leading to contrasting phylogeographic patterns of individual animal taxa. The Tertiary isolation of the islands caused deep genetic divergences between the populations inhabiting Borneo, Sumatra and the mainland (e.g., gibbons: Thinh *et al.* 2010; orang-utans: Nater *et al.* 2011). The *Python curtus* group provides an example of a more complex phylogeographic pattern with a deep divergence between *P. brongersmai* from the Malay Peninsula and NE Sumatra and mutually related species *P. curtus* and *P. breitensteini,* distributed in W Sumatra and Borneo, respectively (Keogh *et al.* 2001). In contrast, there are some species with surprisingly homogenous population structure across the Great Sunda Archipelago and the adjacent mainland (e.g., reticulated pythons: Auliya *et al.* 2002; tiger: Luo *et al.* 2004). Such pattern suggests a recent population expansion possibly following extinction events. Each of the above-described contrasting phylogeographic patterns recorded in different animal species of the Great Sunda Archipelago and adjacent mainland calls for specific taxonomic and conservation decisions. In the case of *O. borneensis*, it is unclear whether the species represents a single, more or less homogenous unit, or whether it should be split into distinct subspecies for taxonomic and/or conservation purposes.

The aim of this paper was to assess the genetic variation in founders of the studbook population of *O. borneensis* of all European zoos. For this purpose, (1) we sampled specimens kept in European zoos and (2) sequenced their mitochondrial cytochrome *b* gene. This gene is frequently used as a marker in taxonomic investigations of turtles and tortoises (e.g., Spinks *et al.* 2004; Fritz *et al.* 2008a, b; Praschag *et al.* 2007, 2011). As mitochondrial and nuclear gene pools may undergo different evolutionary pathways, we also (3) sequenced a nuclear marker (R35 intron) to provide comparative data for mtDNA results and (4) examined the phenotypic variation of carapace and plastron using geometric morphometrics. More specifically, we asked (5) whether the studied captive population of *O. borneensis* may be viewed as genetically homogenous and thus treated as a single unit and (6) whether the genetic data are consistent with putative origin of these animals in Indonesia. The R35 intron was chosen because it is a relatively rapidly evolving nuclear marker (Fujita *et al.* 2004).

## Material and methods

**Specimens.** We sampled 61 individuals of *Orlitia borneensis* (Table 1). A tip of claw was taken from each sampled animal and stored in Eppendorf tubes with 96% ethanol before DNA extraction. Whenever possible, standardized photographs from dorsal and ventral view were taken. Sex was determined according to the size of the animal, plastron concavity, the size of the tail and the relative size of the back sole. The size of the back sole was measured from the first toe (at the base of the claw) to the heel. We found that males have relatively bigger legs than females (compared to the carapace length) and that the back sole can be measured more easily than any other measurements on legs. Two of the examined individuals were live-trapped in Borneo (vicinity of the towns Pemangkat and Singkawang, W Kalimantan; 2 samples), and one turtle was of Sumatran origin (without precise locality). The remaining DNA samples (and photographs of additional 4 specimens) obtained from the following European zoological gardens have no locality data (most of these animals come from the Hong Kong confiscation of 2001; see Rehák 2004): Prague (4 samples), Dresden (2), Brno (6), Tiergarten Schönbrunn, Vienna (8), Haus des Meeres, Vienna (5), Budapest (2), Gdansk (6), Leipzig (1) Poznan (5) Warsaw (2), Woburn (3), Wroclaw (3), Randers (7) and Dvůr Králové nad Labem (3).

Total genomic DNA was isolated with DNAeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines.

**TABLE 1.** List of DNA and geometric morphometrics (GM) samples ordered according to haplotypes and haplotype group. Photographs and thus GM data for animals from Randers are not assigned to DNA samples of exact specimens.

| Haplotype group | Label number of haplotype | Number of speciman | Place of origin | From | DNA | GM plastron | GM carapace |
|---|---|---|---|---|---|---|---|
| 1 | 30 | 30 | Zoo Praha | confiscated 2001 | ● | ● | ● |
| | | 90 | Gdansk | confiscated 2001 | ● | | |
| | 62 | 62 | Zoo Dresden | juveniles bought in Berlin | ● | ● | ● |
| | | 68 | Brno | confiscated 2001 | ● | ● | ● |
| | 66 | 66 | Brno | confiscated 2001 | ● | ● | ● |
| | 79 | 79 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 85 | Gdansk | confiscated 2001 | ● | | |
| | | 93 | Poznan | confiscated 2001 | ● | | |
| | | 106 | Randers | confiscated 2001 | ● | ● | ● |
| | 109 | 109 | Randers | confiscated 2001 | ● | ● | |
| 2 | 47 | 47 | Zoo Praha | confiscated 2001 | ● | ● | ● |
| | | 63 | Zoo Dresden | juveniles bought in Berlin | ● | ● | ● |
| | | 64 | Brno | confiscated 2001 | ● | ● | ● |
| | | 67 | Brno | confiscated 2001 | ● | ● | ● |
| | | 84 | Budapest | confiscated 2001 | ● | | |
| | 65 | 65 | Brno | confiscated 2001 | ● | ● | |
| | | 73 | Haus des Meeres | confiscated 2001 | ● | ● | ● |
| | 89 | 89 | Gdansk | confiscated 2001 | ● | | |
| | | 110 | Randers | confiscated 2001 | ● | ● | ● |
| 33 | 29 | 29 | Zoo Praha | confiscated 2001 | ● | ● | ● |
| | | 31 | Zoo Praha | confiscated 2001 | ● | ● | ● |
| | | 60 | Borneo (Singkawang) | | ● | ● | ● |
| | | 70 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 72 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |

continued next page

TABLE 1. (continued)

| Haplotype group | Label number of haplotype | Number of specimen | Place of origin | From | DNA | GM plastron | GM carapace |
|---|---|---|---|---|---|---|---|
| | | 76 | Haus des Meeres, Vienna | confiscated 2001 | ● | ● | ● |
| | | 95 | Poznan | confiscated 2001 | ● | | |
| | | 97 | Warsaw | confiscated 2001 | ● | ● | ● |
| | | 98 | Warsaw | confiscated 2001 | ● | | |
| | | 107 | Randers | confiscated 2001 | ● | ● | ● |
| | 58 | 58 | Sumatra | | ● | ● | ● |
| | 59 | 59 | Borneo (Pemangkat) | | ● | ● | ● |
| | 69 | 69 | Brno | confiscated 2001 | ● | ● | ● |
| | 71 | 71 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 81 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 96 | Poznan | confiscated 2001 | ● | | |
| | 74 | 74 | Haus des Meeres, Vienna | confiscated 2001 | ● | ● | ● |
| | | 78 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | 77 | 77 | Haus des Meeres, Vienna | confiscated 2001 | ● | ● | |
| | | 80 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 82 | Tiergarten Schönbrunn, Vienna | confiscated 2001 | ● | ● | ● |
| | | 86 | Gdansk | confiscated 2001 | ● | | |
| | | 87 | Gdansk | confiscated 2001 | ● | | |
| | | 91 | Leipzig | confiscated 2009 | ● | | |
| | | 94 | Poznan | confiscated 2001 | ● | | |
| | | 100 | Wroclaw | confiscated 2001 | ● | | |
| | | 101 | Wroclaw | confiscated 2001 | ● | | |
| | | 102 | Wroclaw | confiscated 2001 | ● | | |
| | | 123 | Zoo Dvůr Králové nad Labem | confiscated 2001 | ● | ● | ● |
| | 92 | 92 | Poznan | confiscated 2001 | ● | | |
| | 111 | 111 | Randers | confiscated 2001 | ● | ● | ● |
| | 112 | 112 | Randers | confiscated 2001 | ● | ● | ● |
| | 121 | 121 | Zoo Dvůr Králové nad Labem | confiscated 2001 | ● | ● | |
| | 125 | 125 | Zoo Dvůr Králové nad Labem | confiscated 2001 | ● | ● | ● |
| | 61 | 61 | Zoo Děčín | confiscated 2001 | ● | ● | ● |
| | | 75 | Haus des Meeres | confiscated 2001 | ● | ● | ● |
| | | 88 | Gdansk | confiscated 2001 | ● | | |
| | | 99 | Wroclaw | confiscated 2001 | ● | | |
| | | 103 | Wroclaw | confiscated 2001 | ● | | |
| | | 104 | Wroclaw | confiscated 2001 | ● | | |
| | | 108 | Randers | confiscated 2001 | ● | ● | ● |
| | 83 | 83 | Budapest | confiscated 2001 | ● | | |
| | without DNA sample | M14 | Zoo Praha | confiscated 2001 | | ● | ● |
| | | 105 | Wroclaw | confiscated 2001 | | ● | |
| | | 122 | Zoo Dvůr Králové nad Labem | confiscated 2001 | | ● | ● |
| | | 124 | Zoo Dvůr Králové nad Labem | confiscated 2001 | | ● | ● |

**Sequencing of the cytochrome *b* gene.** DNA amplification was performed with the primers suggested by Spinks *et al.* (2004) for a total length of about 1119 bp. PCR reactions were carried out in 50 µl including 2.5 µl of each 10 µM primer, 5 µl of 10× PCR buffer (Fermentas), 5 µl of 10 mM dNTP, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of 5 U/ml Fermentas Taq DNA polymerase, 100 ng of DNA and 27 µl of $ddH_2O$. The PCR amplification protocol consisted of 35 cycles of denaturation at 94ºC for 45 s, annealing at 46ºC for 45 s, and extension at 72ºC for 1 min and 20 s; a further 7 min elongation step at 72ºC followed the last cycle. For some of the samples, the temperature of annealing had to be decreased to 50ºC to obtain usable PCR products.

**Sequencing of the R35 gene.** The RNA fingerprint protein 35 (R35) was amplified with the primers suggested by Fujita *et al.* (2004) for a total length of about 1084 bp. PCR reactions were the same as for the cytochrome *b* gene. The PCR amplification protocol consisted of 35 cycles of denaturation at 94ºC for 45 s, annealing at 50-60ºC for 45 s, and extension at 72ºC for 1 min and 20 s; a further 7 min elongation step at 72ºC followed the last cycle.

All PCR products were purified with the Qiaquick® purification kit (Qiagen, Hilden, Germany) and directly sequenced using the same primers as for the amplification.

**Phylogeographic analyses.** Chromatograms were manually checked using Chromas Lite 2.01 (http://www.technelysium.com.au/chromas_lite.html), BioEdit (Hall 1999) and sequences were aligned in Clustal X 1.81 (Thompson *et al.* 1997).

Neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* version 4.0b10 (Swofford 2002), and Bayesian analysis (BA) was conducted with MrBayes 3.1 (Huelsenbeck & Ronquist 2001). For MP, we conducted heuristic search analyses with 1,000 random replicates of taxa additions using tree-bisection and reconnection (TBR) branch swapping. The branch support was evaluated using 10,000 bootstrap pseudoreplicates (Felsenstein 1985). All characters were equally weighted and unordered. Tree search with the NJ algorithm was done with Jukes–Cantor distance and support within the final topology was assessed through 10,000 bootstrap pseudoreplicates.

The optimal model of DNA sequence evolution was selected using the AIC criterion in Modeltest 3.7 (Posada & Crandall 1998). For ML analysis we used heuristic search with 1,000 random replicates of taxa additions and TBR branch swapping. Support for the ML tree topology was assessed by bootstrap analysis with 1,000 pseudoreplicates. Two independent runs of Bayesian analyses were conducted with a random starting tree and run for $15x10^6$ generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 15,000 trees (1,500,000 generations).

Two individuals of *Malayemys subtrijuga* (GenBank accession number AY434591.1; AJ519502.1) and one each of *Geoemyda spengleri* (GenBank accession number AY434586.1) and *G. japonica* (GenBank accession number AY434602.1) were included as an outgroup.

Relationships between haplotypes were also represented by a median-joining method (Bandelt *et al.* 1999) with the software Network 4.6.0.0 (http://www.fluxus-engineering.com).

**Demographic inferences.** Polymorphism for population was worked out by the statistic software DnaSP v5 5.10.01 (Librado & Rozas 2009) which estimated the following: haplotype diversity (*h*), segregating sites (S), nucleotide diversity ($\pi$) and Tajima's D, Fu & Li's F*, Fu & Li's D*, and Fu's FS tests.

According to Russell *et al.* (2005), high values of *h* and $\pi$ indicate a constant large size of population. However, a low value of $\pi$ and high value of *h* signify recent expansion.

To estimate population dynamics through time, we have used Bayesian skyline plot approach implemented in Beast v1.6.1 (Drummond & Rambaut 2007). We have run three Markov chain Monte Carlo simulations with 30 million iterations and 10 million burn-ins using the GTR model and a strict molecular clock. We have summarized the results of particular runs in LogCombiner v1.6.1 and displayed them as Bayesian skyline plots in Tracer v1.5.

**Geometric morphometrics.** We analysed shell shape in 24 males and 20 females of *Orlitia borneensis*. Five belong to haplotype group 1, seven to haplotype group 2, seventeen to haplotype group 3 and fourteen specimens are not assigned to any haplotype group and specimen 61 is between groups 3 and 2. The carapace length and rear sole length (from first toe to heel) were measured in each turtle with callipers (0.1 mm precision). Digital images of the carapace and plastron of each specimen were obtained using a digital camera (Canon 30D with 50/1.8 lens). Following the classification of Bookstein (1997), we recorded a total of twenty-one anatomical landmarks of type 1 (cross-sections of the scutes sutures) on the plastron, and twenty-five landmarks of type 1 and one of type 3 (landmark number 1, placed in the middle of the posterior suture of nuchal scute) on the carapace (Fig. 1) using tpsDig (Rohlf 2005). Each set was then symmetrized and one half was removed using BigFix6 (Sheets 2003). The coordi-

nates Y for the landmarks 2, 3, 4, 5 and 6 on the plastra were then manually set to 0. To remove the effects of position, orientation and scale, we conducted the Procrustes superimposition method (Zelditch *et al.* 2004), performed in the CoordGen6 (Sheets 2003), using a set of the x and y coordinates of the landmarks. To remove size-related shell shape differences, we used standardization of size which was applied in the program Standard6 (Sheets 2003). Visualization was performed in the program PCAGen6 (Sheets 2003).

The differences in the shell shape between sexes and between haplotype groups were tested in Statistica 6 (StatSoft 2001).

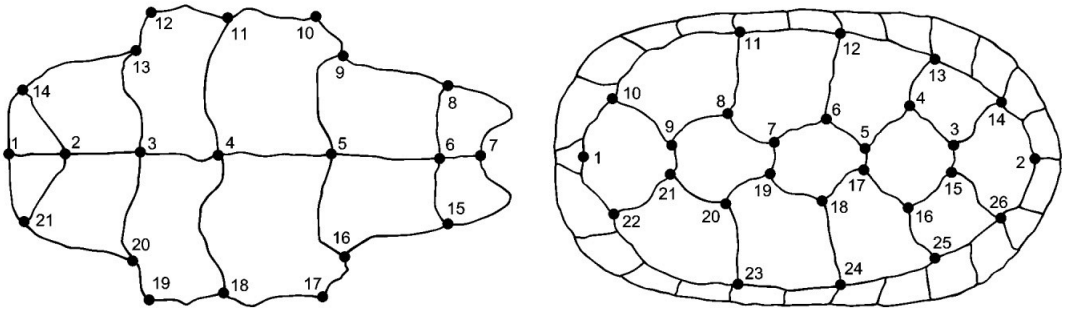

**FIGURE 1.** Ventral and dorsal view of a shell of *Orlitia borneensis* showing the landmarks used in this study.

## Results

**Mitochondrial DNA.** We sequenced the mitochondrial cytochrome *b* gene in 61 individuals (specimens) and found 23 distinct haplotypes. We obtained a nucleotide alignment of 1119 bp, of which 33 were variable and 21 were parsimony-informative. The uncorrected p-distances among the haplotypes ranged from 0.089% to 1.504%. The haplotype network revealed three main haplotype groups (Fig. 2). The phylogenetic structures of the haplotypes revealed by MP, ML and BA were congruent but only poorly supported. Using Modeltest, the GTR+G model of sequence evolution was selected. Monophyly of the haplotype group 1 was clearly supported by BA (posterior probability = 1.0), MP (bootstrap = 90) and ML (95). The group 2 was supported by MP (80) and ML (81). The third group was not supported by any method; however, its terminal part (consisting of the haplotypes 29, 77. 92, 111, 112 and 125) was weakly supported by ML (59).

Neutrality tests resulted in negative, but non-significant values (Table 2). Coalescent Bayesian approach revealed a historically stationary period in population dynamics followed by a recent expansion event (Fig. 3).

**TABLE 2.** Demographic characteristics for the *Orlitia borneensis* based on mitochondrial cytochrome *b*. Sequences: number of individuals sequenced (Ns), number of segregating sites (S), haplotype diversity (h), nucleotide diversity ($\pi$), Fu & Li's F*, Fu & Li's D*, Fu's $F_S$, Tajima's D and expansion coefficient (exp).

| $N_s$ | S | h | $\pi$ | Fu & Li's *F** | Fu & Li's *D** | Fu's *Fs* | Tajima's *D* | Exp |
|---|---|---|---|---|---|---|---|---|
| 61 | 28 | 0,908 | 0,004 | -1,16637 | -1,185 | -3,378 | -0,616 | 5,7911 |

**Nuclear marker.** We found 4 distinct sequences for the R35 gene; besides a standard sequence found in 11 individuals there were single transitions at positions 581 (samples 59 Kalimantan and 29 Prague), 652 (sample 78 Tiergarten Schönbrunn, Vienna) and 925 (samples 60 Kalimantan and 77 Haus des Meeres, Vienna).

**Geometric morphometrics.** We found no effects of sex on the shape of the carapace (Wilks' Lambda = 0.8855, $F_{(7,31)} = 0.5722$, $P < 0.7727$) and plastron (Wilks' Lambda = 0.8774, $F_{(7,36)} = 0.7185$, $P < 0.6569$). Therefore, we pooled the sexes in further analyses. We found no tendency to morphological similarity among individuals bearing haplotypes of the same group either in carapace or in plastron data sets. This is illustrated in a morphospace defined by the first two PCA axes of the plastron shape in figure 4.

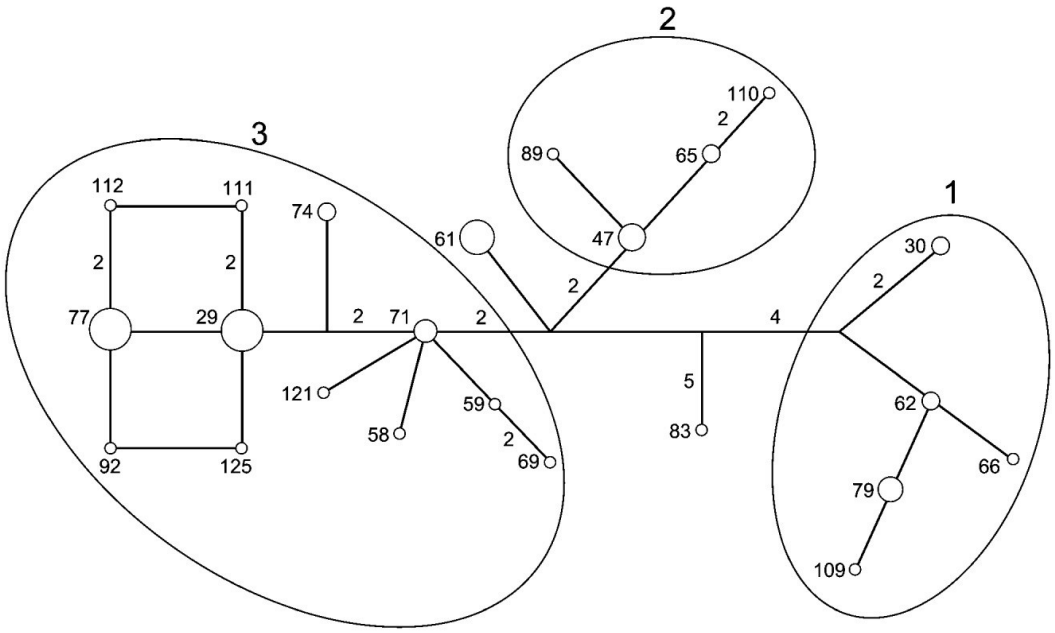


**FIGURE 2.** Median-joining network indicating relationships among haplotypes of *Orlitia borneensis* based on cytochrome *b* sequences. Haplotypes are denoted as circles, their size is proportional to number of individuals carrying respective haplotype. Numbers at branches represent numbers of mutational steps (displayed for n > 1). Three main haplogroups are marked by ovals.

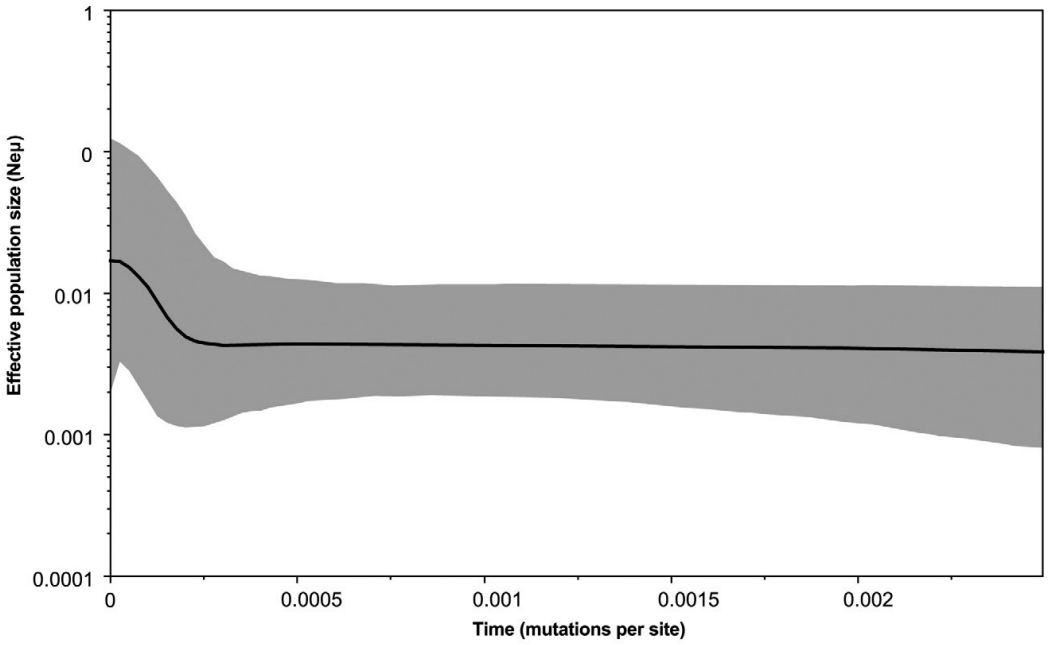


**FIGURE 3.** Bayesian skyline plot demonstrating changes in effective population size in *Orlitia borneensis* based on mitochondrial data. Thick solid line represents median of the estimate, borders of grey area delineate the highest 95% posterior density interval.

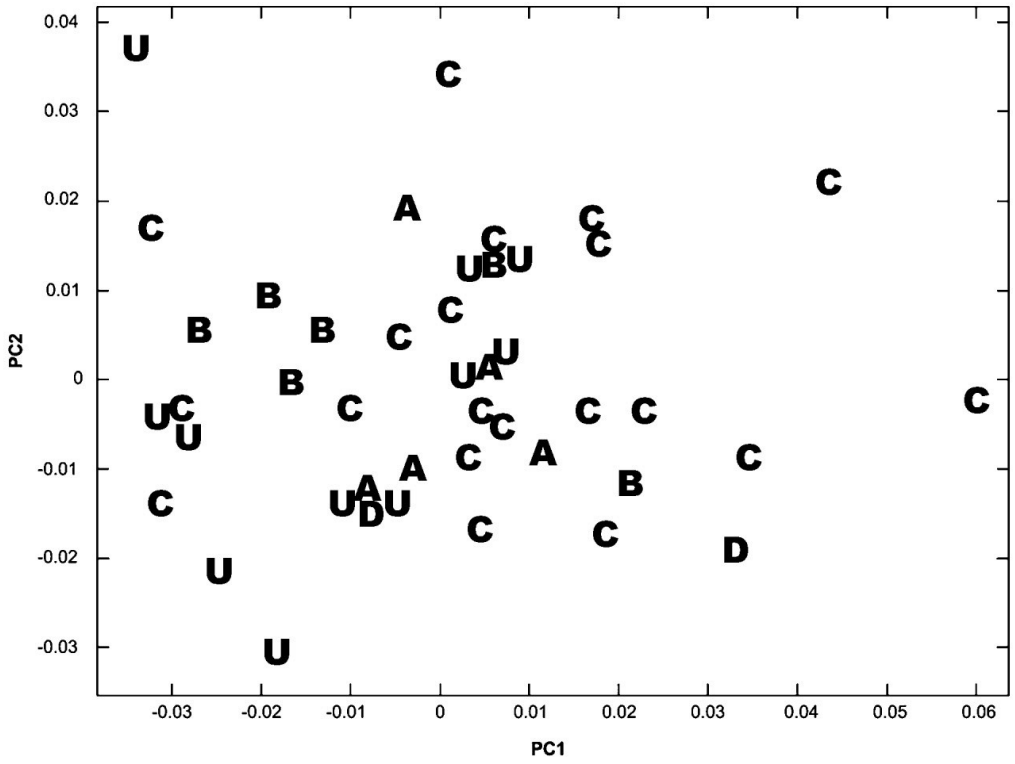


**FIGURE 4.** PCA for plastron shape. Specimens are marked according to haplotype group. A—haplotype group 1, B—group 2, C—group 3, D—haplotype 61, U—unknown. The first axis PC1 explains 29.79% and second axis PC2 explains 14.14% of the total variance in the shapes.

## Discussion

The mitochondrial cytochrome *b* sequences revealed relatively high haplotype diversity in the examined *Orlitia borneensis*. This suggests that the European zoo population covers considerable genetic variation. We may speculate that the confiscated specimens were sampled from multiple localities and represent genetically heterogeneous populations.

In contrast to haplotype diversity, the observed nucleotide diversity was surprisingly low. The maximum uncorrected p-distance was less than 1.5% for cytochrome *b* sequences; i.e., clearly below the values among most congeneric chelonian species. These typically exceed 2.8% (Vargas-Ramírez *et al.* 2010; Praschag *et al.* 2011; Stuckas & Fritz 2011; but see Fritz *et al.* 2011 for lower values in *Trachemys* spp.), supporting the hypothesis that the *O. borneensis* haplotypes represent evolution within one and the same species. The weak sequence divergence and poorly supported phylogenetic structure suggest that all examined *O. borneensis* are similar enough to be treated as a single conservation unit. This conclusion was further supported by the sequences of the nuclear R35 gene as well as by morphological similarity demonstrated by geometric morphometrics. It may be argued that the divergence rates of mitochondrial genes differ substantially among turtle clades (Martin & Palumbi 1993) and there are well-documented examples of an exceptional reduction of these rates in some North American emydids (e.g., *Graptemys* and *Pseudemys*; Wiens *et al.* 2010 but see Fritz *et al.* 2011 for the possible involvement of numts in the data set of Wiens *et al.* 2010). Nevertheless, the observed sequence divergences of the mitochondrial genes

reported in geoemydid species related to *O. borneensis* do not reveal substantial retardations of the divergence rates (Stuart & Fritz 2008; Fritz *et al.* 2008b; Gong *at al.* 2009; Jiang *at al.* 2011; Praschag *et al.* 2007).

Negative values of summary statistics suggest the recent expansion of the examined *Orlitia* population. Non-significant values may be caused by two phase population history, as revealed by the Bayesian skyline plots. It is, however, still premature to speculate about the identification of these phases with important events that affected the environment of SE Asia (e.g., climate change associated with the glacial maximum and Toba volcano eruption).

The three *O. borneensis* haplotypes from W Borneo and Sumatra cluster with the remaining haplotypes that are of unknown origin; one of these haplotypes (Singkawang, Kalimantan) was even found in another nine examined specimens. This does not contradict the putative origin of the Hong Kong confiscate including the Great Sunda islands. The geographic range of some confiscated species covers both continental and insular part of SE Asia (e.g., *Siebenrockiella crassicollis* and *Cuora amboinensis*) (Iverson 1992). Nevertheless, the confiscation also included *Heosemys grandis* and *H. annandalii,* two species distributed exclusively in the Malay Peninsula and adjacent territories located further northward (Iverson 1992). This clearly suggests that at least part of the confiscated animals were poached in continental SE Asia and that the presence of *O. borneensis* individuals collected in the Malay Peninsula is also likely. Nevertheless, having no known-locality samples from the Malay Peninsula, we cannot confirm any territories of origin.

The reconstruction of the geomorphologic conditions during the glacial periods suggests that the contemporary Malay Peninsula, Sumatra and Borneo were then joined by an interconnected system of rivers, the Siam and West Sunda River drainages (Voris 2000). Thus, an exchange of genes between nowadays isolated populations was probably enabled in this period.

Although the 1.5% divergence of the sequences for the cytochrome *b* gene is relatively small, it most probably preceded the Toba volcano explosion on Sumatra 73,000 years ago. Considering the global chelonian divergence rate of about 0.25% per million years (Avise *et al.* 1992), the onset of the divergence can be roughly dated to the Late Miocene. Obviously, the bearers of multiple haplotypes survived the Toba event. This is in agreement with the paleontological data suggesting that this catastrophe affected territories further west and north of Sumatra and surprisingly none of the Sumatran mammalian species became extinct in the period of the Toba explosion (Louys 2007). This is further supported by the demographic analyses of our sequence data suggesting a fairly stable population size of the *O. borneeensis* population in the past.

## Acknowledgements

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## References

Auliya, M., Mausfeld, P., Schmitz, A. & Böhme, W. (2002) Review of the reticulated python (*Python reticulates* Schneider, 1801) with the description of new subspecies from Indonesia. *Naturwissenschaften*, 89, 201–213.

Avise, J.C., Bowen, B.W., Lamb, T., Meylan, A.B. & Bermingham, E. (1992) Mitochondrial DNA Evolution at a Turtle's Pace: Evidence for Low Genetic Variability and Reduced Microevolutionary Rate in the Testudines. *Molecular Biology and Evolution*, 9, 457–473.

Bandelt, H.J., Foster, P. & Rohl, A. (1999) Median–joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16, 37–48.

Bookstein, F.L. (1997) *Morphometric tools for landmark data: geometry and biology.* Cambridge University Press, New York, 455 pp.

Chen, T.-H., Chang, H.-C. & Lue, K.-Y. (2009) Unregulated Trade in Turtle Shells for Chinese Traditional Medicine in East and Southeast Asia: The Case of Taiwan. *Chelonian Conservation Biology*, 8, 11–18.

Cheung, S.M. & Dudgeon, D. (2006) Quantifying the Asian turtle crisis: market surveys in southern China, 2000-2003. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 16, 751–770.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, 7, 214.

Ernst, C.H. & Barbour, R.W. (1989) *Turtles of the world*. Smithsonian Institution Press, London, 416 pp.

Felsenstein, J. (1985) Confidence limits on phylogeny: an approach using the bootstrap. *Evolution*, 39, 783–789.

Fong, J.J. & Chen, T.H. (2010) DNA evidence for the hybridization of wild turtles in Taiwan: possible genetic pollution from trade animals. *Conservation Genetics*, 11, 2061–2066.

Fritz, U., Ayaz, D., Buschbom, J., Kami, H.G., Mazanaeva, L.F., Aloufi, A.A., Auer, M., Rifai, L., Šilić, T. & Hundsdörfer, A.K. (2008b) Go east: phylogeographies discordance of mophology, mitochondrial and nuclear genomic markers and rare hybridization. *Journal of Evolutionary Biology*, 21, 527–540.

Fritz, U., Guicking, D., Auer, M., Sommer, R.S., Wink, M. & Hundsdörfer, A.K. (2008a) Diversity of the Southeast Asian leaf turtle genus *Cyclemys*: how many leaves on its tree of life? *Zoologica Scripta*, 37, 367–390.

Fritz, U., Stuckas, H., Vargas-Ramírez, M., Hundsdörfer, A.K., Maran, J. & Päckert, M. (2011) Molecular phylogeny of Central and South American slider turtles: implications for biogeography and systematics (Testudines: Emydidae: *Trachemys*). *Journal of Zoological Systematics and Evolutionary Research*, doi 10.1111/j.1439-0469.2011.00647.x

Fujita, M.K., Engstrom, T.N., Starkey, D.E. & Shaffer, H.B. (2004) Turtle phylogeny: insights from a novel nuclear intron. *Molecular Phylogenetics and Evolution*, 31, 1031–1040.

Gong, S., Shi, H., Mo, Y., Auer, M., Vargas-Ramírez, M., Hundsdörfer, A.K. & Fritz, U. (2009) Phylogeography of the endangered black-breasted leaf turtle (*Geoemyda spengleri*) and conservation implications for other chelonians. *Amphibia-Reptilia*, 30, 57–62.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, 17, 754–755.

Iverson, J.B. (1992) *A revised checklist with distribution maps of the turtles of the world*. Privately printed, Richmond, 363 pp.

Jiang, Y., Nie, L.W., Huang, W.X., Jing, W.X., Wang, L. Liu & Dai, X.T. (2011) Comparison of complete mitochondrial DNA control regions among five Asian freshwater turtle species and their phylogenetic relationships. *Genetics and Molecular Research*, 10, 1545–1557.

Keogh, J.S., Barker, D.G. & Shine, R. (2001) Heavily exploited but poorly known: systematics and biogeography of commercially harvested pythons (*Python curtus* group) in Southeast Asia. *Biological Journal of the Linnean Society*, 73, 113–129.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451–1452.

Louys, J. (2007) Limited effect of the Quaternary's largest super-eruption (Toba) on land mammals from Southeast Asia. *Quaternary Science Reviews*, 26, 3108–3117.

Luo, S.-J., Kim, J.-H., Johnson, W.E., van der Walt, J., Martenson, J., Yuhki, N., Miquelle, D.G., Uphyrkina, O., Goodrich, J.M., Quigley, H.B., Tilson, R., Brady, G., Martelli, P., Subramaniam, V., McDougal, C., Hean, S., Huang, S.-Q., Pan, W.S., Karanth, U., Sunquist, M., Smith, J.L.D. & O'Brien, S. (2004) Phylogeography and genetic ancestry of tigers (*Panthera tigris*). *PLoS Biology*, 2, 2275–2293.

Martin, A.P. & Palumbi, S.R. (1993) Body size, metabolic rate, generation time, and the molecular clock. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 4087–4091.

Nater, A., Nietlisbach, P., Arora, N., van Schaik, C.P., van Noordwijk, M.A., Willems, E.P., Singleton, I., Wich, S.A., Goossens, B., Warren, K.S., Verschoor, E.J., Perwitasari-Farajallah, D., Pamungkas, J. & Krützen, M. (2011) Sex-biased dispersal and volcanic activities shaped phylogeographic patterns of extant orangutans (genus: *Pongo*). *Molecular Biology and Evolution*, 28, 2275–2288.

Parham, J.F., Simison, W.B., Kozak, K.H., Feldman, C.R. & Shi, H. (2001) New Chinese turtles: endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens. *Animal Conservation*, 4, 357–367.

Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14, 817–818.

Praschag, P., Holloway, R., Georges, A., Päckert, M., Hundsdörfer, A.K. & Fritz, U. (2009a) A new subspecies of *Batagur affinis* (Cantor, 1847), one of the world's most critically endangered chelonians (Testudines: Geoemydidae). *Zootaxa*, 2233, 57–68.

Praschag, P., Hundsdörfer, A.K. & Fritz, U. (2007) Phylogeny and taxonomy of endangered South and South-east Asian freshwater turtles elucidated by mtDNA sequence variation (Testudines: Geoemydidae: *Batagur, Callagur, Hardella, Kachuga, Pangshura*). *Zoologica Scripta*, 36, 429–442.

Praschag, P., Hundsdörfer, A.K. & Fritz, U. (2009b) Further specimens and phylogenetic position of the recently described leaf turtle species *Cyclemys gemeli* (Testudines: *Geoemydidae*). *Zootaxa*, 2008, 29–37.

Praschag, P., Sommer, R.S., McCarthy, C., Gemel, R. & Fritz, U. (2008) Naming one of the world's rarest chelonians, the southern *Batagur*. *Zootaxa*, 1758, 61–68.

Praschag, P., Stuckas, H., Päckert, M., Maran, J. & Fritz, U. (2011) Mitochondrial DNA sequences suggest a revised taxonomy of Asian flapshell turtles (*Lissemys* Smith, 1931) and the validity of previously unrecognized taxa (Testudines: Trionychidae). *Vertebrate Zoology*, 61, 147–160.

Rampino, M.R. & Ambrose, S.H. (2000) Volcanic winter in the Garden of Eden: The Toba supereruption and the late Pleistocene human population crash. *In*: McCoy, F.W. & Heiken, G. (Eds), *Volcanic Hazards and Disasters in Human Antiquity*. Geological Society of America Special Papers, Boulder, pp 71–82.

Rehák, I. (2004) Hong Kong turtles, two years after. *Gazella*, 31, 39–48.

Rohlf, F.J. (2005) tpsDig, digitize landmarks and outlines, version 2.04, Stony Brook: Department of Ecology and Evolution, State University of New York.

Russell, A.L., Mendellín, A. & McCracken, F. (2005) Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*). *Molecular Ecology*, 14, 2207–2222.

Samedi & Iskandar, D.T. (2000) Freshwater turtle and tortoise conservation and utilization in Indonesia. *In*: van Dijk, P.P., Stuart, B.L., & Rhodin, A.G.J. (Eds), *Asian Turtle Trade: proceedings of a workshop on conservation and trade of freshwater turtles and tortoises in Asia. Chelonian Research Monographs* 2. Chelonian Research Foundation, Lunenburg, Massachusetts, USA, pp 106–111.

Sheets, H.D. (2003) *IMP*, version 6a, Department of Physics, Canisius College, Buffalo, New York.

Spinks, P.Q., Shaffer, H.B., Iverson, J.B. & McCord, W.P. (2004) Phylogenetic hypotheses for the turtle family Geoemydidae. *Molecular Phylogenetics and Evolution*, 32, 164–182.

StatSoft I (2001) *STATISTICA* (data analysis software system) version 6. Available from www.statsoft.com. (Accessed 24 April 2011).

Stuart, B.L. & Fritz, U. (2008) Historical DNA from museum type specimens clarifies diversity of Asian leaf turtles (*Cyclemys*). *Biological Journal of the Linnean Society*, 94, 131–141.

Stuart, B.L. & Parham, J.F. (2007) Recent hybrid origin of three rare Chinese turtles. *Conservation Genetics*, 8, 169–175.

Stuckas, H. & Fritz, U. (2011) Identity of *Pelodiscus sinensis* revealed by DNA sequences of an approximately 180-year-old type specimen and a taxonomic reappraisal of *Pelodiscus* species (Testudines: Trionychidae). *Journal of Zoological Systematics and Evolutionary Research*, 49, 335–339.

Swofford, D.L. (2002) PAUP*. Phylogenetic analysis using parsimony. Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Thinh, V.N., Moontrick, A.R., Geissmann, T., Li, M., Ziegler, T., Agil, M., Moisson, P., Nadler, T., Walter, L. & Roos, Ch. (2010) Mitochondrial evidence for multiple radiations in the evolutionary history of small apes. *BMC Evolutionary Biology*, 10, 74–87.

Thompson, D.J., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignments aided by quality analysis tool. *Nucleic Acids Research*, 24, 4876–4882.

Vargas-Ramírez, M., Vences, M., Branch, W.R., Daniels, S.R., Glaw, F., Hofmeyr, M.D., Kuchling, G., Maran, J., Papenfuss, T.J., Široký, P., Vieites, D.R. & Fritz, U. (2010) Deep genealogical lineages in the widely distributed African helmeted terrapin: evidence from mitochondrial and nuclear DNA (Testudines: Pelomedusidae: *Pelomedusa subrufa*). *Molecular Phylogenetics and Evolution*, 56, 428–440.

Voris, H.K. (2000) Maps of Pleistocene Sea Levels in South East Asia: Shorelines, River Systems, Time Durations. *Journal of Biogeography*, 27, 1153–1167.

Wiens, J.J., Kuczynski, C.A. & Stephens, P.R. (2010) Discordant mitochondrial and nuclear gene phylogenies in emydid turtles: implications for speciation and conservation. *Biological Journal of the Linnean Society*, 99, 445–461.

Williams, M.A.J., Ambrose, S.H., van der Kaars, S., Ruehlemann, C., Chattopadhyaya, U., Pal, J. & Chauhan, P.R. (2009) Environmental impact of the 73 ka Toba super–eruption in South Asia. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 284, 295–314.

Zelditch, M.L., Swiderski, D.L., Sheets, H.D. & Fink W.L. (2004) *Geometric morphometrics for biologists: A primer*. Elsevier Academic Press, New York and London, 437 pp.

Zhou, Z.H. & Jiang, Z.G. (2008) Characteristics and risk assessment of international trade in tortoises and freshwater turtles in China. *Chelonian Conservation and Biology*, 7, 28–36.

## 9.5 Frynta D., Fraňková M., Čížková B., Skarlandtová H., Galaštoková K., Průšová K., Šmiauer P., Šumbera R. (2011) Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*). *Acta Theriologica*. 56: 289-295.

Litter size is an important component of life history contributing to reproductive success in many animals. Among muroid rodents, spiny mice of the genus *Acomys* are exceptional because they produce large precocial offspring after a long gestation. We analyzed data on 1,809 litters from laboratory colonies of spiny mice from the *cahirinus-dimidiatus* group: *Acomys cahirinus*, *Acomys cilicicus*, *Acomys* sp. (Iran), and *Acomys dimidiatus*. Generalized mixed-effect models revealed that litter size increased with maternal body weight and/or number of immature females present in the family group. Thus, both maternal body reserves and presence of immature descendants demonstrating previous reproductive success enhance further reproduction in this social rodent.

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SHORT COMMUNICATION

# Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*)

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**Abstract** Litter size is an important component of life history contributing to reproductive success in many animals. Among muroid rodents, spiny mice of the genus *Acomys* are exceptional because they produce large precocial offspring after a long gestation. We analyzed data on 1,809 litters from laboratory colonies of spiny mice from the *cahirinus-dimidiatus* group: *Acomys cahirinus*, *Acomys cilicicus*, *Acomys* sp. (Iran), and *Acomys dimidiatus*. Generalized mixed-effect models revealed that litter size increased with maternal body weight and/or number of immature females present in the family group. Thus, both maternal body reserves and presence of immature descendants demonstrating previous reproductive success enhance further reproduction in this social rodent.

**Keywords** *Acomys* · Rodents · Litter size · Maternal investment · Precocial life history

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## Introduction

Within-population variance in litter size is typically large and may be viewed either as an unavoidable consequence of stochastic processes (i.e., combination probabilities of fertilization and/or implantation success and/or embryonic survival) or as a manifestation of individual strategies of females adjusting their reproductive investment according to actual body condition and/or external circumstances (cf. discussion concerning clutch size in birds; Both et al. 1998; Tinbergen and Both 1999; Tinbergen and Sanz 2004).

Taxa producing small litters tend to evolve invariant or nearly invariant litter size (e.g., ungulates, primates, bats; cf. invariant clutch size in some bird and reptile groups; Bennett and Owens 2002; Kratochvíl and Frynta 2006). Otherwise, an increment of even a single newborn affects maternal investment dramatically. It may have considerable fitness consequences for both the mother and offspring, especially in taxa with a precocial developmental strategy and high maternal investment per newborn. This is the case of some rodent species, e.g., guinea pigs (Kasparian et al. 2005) and spiny mice (Kam et al. 2006).

The distribution patterns of developmental time (from conception to eye opening) scaled to maternal body size

among rodent families suggest a high level of evolutionary conservatism. A long ontogenetic development associated with precociality is typical for Hystricomorpha and possibly represents the plesiomorphic character state in rodents (Burda 1989).

Spiny mice of the genus *Acomys* may serve as an example of putatively derived precociality in rodents. This group possesses an exceptional life history among the family Muridae. In contrast to many other muroid rodents, spiny mice produce after an extended gestation period (36–42 days) only small litters consisting of relatively large (the mean litter weight at birth represents 20–25% of maternal weight; Dieterlen 1961) and well-developed precocial newborns (Brunjes 1990). Maternal investment is therefore extremely biased in favor of the prenatal period and consequently is largely determined by litter size itself.

Spiny mice are small, rock-dwelling rodents inhabiting deserts, semideserts, and savannas (i.e., areas with high spatiotemporal variation in resource availability) in Africa and the Middle East. In spite of their earlier systematic placement, they are more related to gerbils than to true murids belonging to the subfamily Murinae, which are represented by rats and house mice (Michaux et al. 2001; Steppan et al. 2004). Spiny mice, even those recently captured in the field, exhibit no behavioral signs of stress under standard laboratory conditions and breed well. Therefore, *A. dimidiatus* or *A. cahirinus* are widely used as an experimental model in both physiological and behavioral studies (e.g., Shafrir 2000; Haim et al. 2006; Pinter-Wollman et al. 2006; Nováková et al. 2008).

Spiny mice are social animals that should be kept in families consisting of an adult male, multiple females, and their descendants (Young 1976), which mimics their wild social system. Their societies are not anonymous, and individual recognition was demonstrated unequivocally (Porter et al. 1986). In groups consisting of related individuals, communal care for the young comprising allosuckling is frequent; however, mothers are able to recognize their own offspring (Porter et al. 1980). Interestingly, male sires participate in parental care and clearly discriminate between own and alien young (Makin and Porter 1984). Our data from laboratory colonies suggest that adolescent males are driven away by the territorial male, while females are tolerated. Occasionally such conflicts may result in apparent social tension within the whole group and immediate suspension of reproduction. In affected groups, mice start to bite each other. As the tails are most vulnerable, this aggression results in frequent tail losses, a phenomenon also reported from natural populations (Shargal et al. 1999).

We analyzed the data on litter size collected in captive colonies of spiny mice belonging to four closely related species/populations of the *cahirinus-dimidiatus* clade (Barome et al. 1998, 2000; Volobouev et al. 2007; Frynta et al. 2010) to determine the effects of maternal life history variables and social composition of the breeding groups on litter size. We tested the hypothesis that litter size is strongly dependent on maternal body size because maternal size and amount of body reserves strongly limit litter size in these rodents with large size of newborns and heavy prenatal maternal investment. We also hypothesized that in this social rodent possessing biparental and communal parental care, presence of additional group members enhances rather than precludes production of larger litters.

## Materials and methods

### Studied animals

Our laboratory colonies of spiny mice were of the following origin: *A. cahirinus*, founder animals were caught in 1995 in the Abu Simbel archeological site, southern Egypt (22° 22′ N, 31° 38′ E); *A. cilicicus*, east of Silifke, southern Turkey (36° 26′ N, 34° 06′ E), obtained in 1993; *A.* sp., Zagros, SW Iran (28° 56′ N, 52° 32′ E), caught in 2002; and *A. dimidiatus*, laboratory strain, Prague Zoo, Czech Republic (the stock imported in the early 1970s from the Bronx Zoo, NY; probably originated from Israel or Sinai). Molecular phylogenetic analysis based on mitochondrial control region sequences in these colonies revealed that all the studied populations/species belong to the *cahirinus-dimidiatus* group of the genus *Acomys*. The former two populations/species belong to the clade of *A. cahirinus* sensu lato inhabiting North Africa and the Eastern Mediterranean (Crete, Cyprus, and the Kilikian coast in Anatolia), while the latter two belong to the *A. dimidiatus* sensu lato clade ranging from Sinai, throughout the Arabian Peninsula, and along the coast of the Gulf of Oman from Iran to Pakistan. The specific/subspecific status of the Iranian population, referred to here as *A.* sp., requires further clarification (Frynta et al. 2010).

The animals were kept in terrariums (60×50×40 cm or 70×60×40 cm) or in rodent plastic cages (VELAZ T4, Czech Republic; 55×32×18 cm) under standard laboratory conditions. Wood shavings were used as bedding material; a clay flowerpot with a lateral opening served as a shelter; and tree branches for climbing and gnawing were provided as environmental enrichment. The light schedule in the animal housing room corresponded to the outdoor light cycle. Food (standard diet for rats and mice, ST1, VELAZ, Czech Republic, occasionally supplemented with a mixture of grains, dry bread, apples, and herb leaves) and water were available ad libitum.

Spiny mice were kept in family groups consisting of two closely related females (full or half siblings), one non-relative male, and their descendants. The groups were

established from founding animals about 3 months of age (maturing age) and then allowed to breed freely for several months. Manipulation of group structure only occurred in the case of male-to-male aggression between the male founder and his mature male offspring (if so, young males were removed). The experimental groups were regularly checked (either daily or every other day); each litter was recorded and sexed immediately after its detection; and the putative mother was identified.

The following life history and social variables were measured: maternal parity (range, 1–11), maternal age in days (range, 52–990 days), postpartum conception (the litter was considered as conceived postpartum when delivered before day 41 after the previous one; categorical variable), number of adult males actually present in the group (aged 90 days or older; range, 1–22), number of adult females (aged 90 days or older; range, 1–20), number of breeding females in the group (i.e., those that already gave birth; range, 1–11), maternal status (first breeding founder, second breeding founder, their daughters; categorical variable), number of immature males (aged under 90 days; range, 0–13), and of immature females (aged under 90 days; range, 0–17). In addition, maternal body weight in grams (range, 25–79.7 g; mean values were 43, 57, 50, and 56 g for *A. cahirinus*, *A. cilicicus*, *A. dimidiatus*, and *A.* sp., respectively) after parturition was included in the particular analyses of individual species. Maternal age and body weight were log-transformed. Litter sex ratio was not included, as the analyses had revealed no consistent relationship of this variable with litter size (Nováková et al. 2010).

Only the records containing a complete set of required explanatory variables were further analyzed. In total, these were 1,809 (1,569 when maternal body weight was also included) litter size records, i.e., 1,037 (968) for *A. cahirinus*, 186 (49) for *A. cilicicus*, 414 (414) for *A. dimidiatus*, and 172 (138) for *A.* sp.

### Statistical analysis

Statistical models were estimated, tested, and visualized using the R statistical package (version 2.11.1). The significance and size of the effects of explanatory variables were estimated using generalized mixed-effect models (GLMM) using package *lme4*, with a quasi-likelihood approach based on the Poisson distribution, and using maternal identity as a random factor. The size of the litter as a response variable excludes, however, zero values, so estimates based on the Poisson distribution could be biased. We have therefore validated the estimates of regression coefficients obtained in GLMM using a generalized linear model (GLM) using zero-truncated ("positive") Poisson distribution, fitted using the package VGAM. Regression coefficients from GLMM that fall into 95% confidence interval of the coefficients estimated using the GLM model were deemed unbiased. This indirect approach was chosen because random maternal effect cannot be fitted in combination with the zero-truncated Poisson distribution, and thus, the inferred type-I errors would underestimate the true ones.

Because the information about maternal body weight was available only for a subset of observations (see above), model selection was performed in parallel on the full dataset, but excluding maternal weight variable, and on the subset of observations with maternal weight values. In either case, the full model was fitted first and then refitted as final only with explanatory variables suggested as significant in the full model. The significance of explanatory variables was estimated separately for both the full and final models using the likelihood-ratio test (LRT), comparing such models with alternative ones, where the tested variable was dropped. Effects of explanatory variables selected into individual models were visualized using the *effects* package.

*Ethical note* The experiments were performed in accordance with Czech law implementing all the corresponding EU regulations and were approved by the Institutional Animal Care and Use Committee.

## Results

We analyzed the effects of ten fixed factors on litter size using GLMM procedures. Initially, we performed separate analyses for each species/population; except for *A. dimidiatus*, we proceeded with both models, including and excluding maternal body weight as a factor. We further provide only significant results of the final reduced models (Table 1). The numbers of immature females and males had significant positive effect on litter size in *A. dimidiatus* ($P$=0.0275, $P$=0.019, respectively); the former factor appeared marginally significant also in *A. cilicicus* ($P$=0.0619). Postpartum conception had positive significant effect in the case of *A. cahirinus* ($P$=0.0391); however, this effect disappeared when maternal body weight was included in the model. Maternal body weight had significant positive effect on litter size both in *A. cahirinus* and *A.* sp. ($P$=0.0016, $P$=0.0102, respectively).

When the data for individual populations were pooled into a single GLMM model, postpartum conception ($P$=0.0124; Fig. 1), number of immature females ($P$=0.0003; Fig. 2), and species ($P$<0.0001; Fig. 3) significantly contributed to litter size. When maternal body weight was included into the model, the effect of this variable appeared

**Table 1** Explanatory variables selected as affecting the litter size in four species/populations of *Acomys*

| | Estimate | Std. error | $\chi^2$ | DF | *P* value |
|---|---|---|---|---|---|
| Models not including maternal body weight | | | | | |
| *A. cahirinus* | | | | | |
| Postpartum conception | 0.0982 | 0.0413 | 4.26 | 1 | 0.0391 |
| *A. cilicicus* | | | | | |
| Number of immature females | 0.0308 | 0.0174 | 3.49 | 1 | 0.0619 |
| *A.* sp. | | | | | |
| Not significant | | | | | |
| Pooled dataset | | | | | |
| Postpartum conception | 0.0859 | 0.0322 | 6.25 | 1 | 0.0124 |
| Number of immature females | 0.0256 | 0.0066 | 13.06 | 1 | 0.0003 |
| Species | 0.0778 | 0.0497 | 81.73 | 3 | <0.0001 |
| Models including maternal body weight | | | | | |
| *A. cahirinus* | | | | | |
| Maternal body weight | 0.4908 | 0.1357 | 9.96 | 1 | 0.0016 |
| *A. cilicicus* | | | | | |
| Not significant | | | | | |
| *A. dimidiatus* | | | | | |
| Number of immature females | 0.0354 | 0.0178 | 4.86 | 1 | 0.0275 |
| Number of immature males | 0.0412 | 0.0194 | 5.50 | 1 | 0.019 |
| *A.* sp. | | | | | |
| Maternal body weight | 1.4382 | 0.4552 | 6.60 | 1 | 0.0102 |
| Pooled dataset | | | | | |
| Maternal body weight | 0.4457 | 0.1196 | 12.25 | 1 | 0.0005 |
| Number of immature females | 0.0200 | 0.0070 | 7.13 | 1 | 0.0076 |
| Species | 0.0941 | 0.0930 | 64.44 | 3 | <0.0001 |

Coefficient estimates affect the log-transformed litter size, so exponential function must be applied to them to see multiplicative effects of the variable. The last three columns ($\chi^2$, DF, and *P* value) summarize the results of variable-wise LRT, displaying respectively the value of the test statistic, degrees of freedom, and estimated probability of type-I error

significant ($P$=0.0005; Fig. 4), while that of postpartum conception disappeared. The effects of number of immature females ($P$=0.0076) and species ($P$<0.0001) remained nearly unchanged. Further testing revealed no need for inclusion of the interaction between species and any of the explanatory variables.

## Discussion

The positive association between litter size and maternal body weight found in the entire dataset as well as in two particular sets (*A.* sp. and *A. cahirinus*) of the breeding records is not surprising in animals exhibiting such a heavy maternal investment in the progeny (Kam et al. 2006). In many other mammals, a positive association between litter size and maternal body size has also been repeatedly reported (e.g., Zejda 1966; Tuomi 1980; Myers and Master 1983 and the references herein). As thoroughly documented in laboratory mice, this close association typical for unselected populations is caused by high correlation between body size and ovulation rate. Nevertheless, this does not restrict a relative independent change in these traits above the population level, as both of the traits are

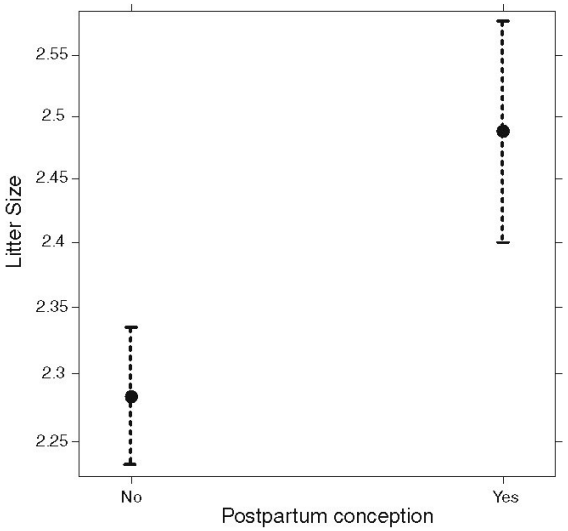


**Fig. 1** The effect of postpartum conception on litter size in laboratory colonies of four species/populations of the genus *Acomys*. Data are given as means and 95% confidence intervals

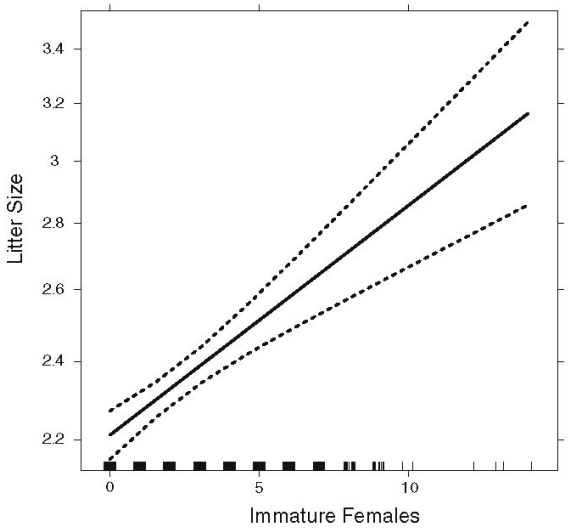


**Fig. 2** The effect of the number of immature females on litter size in four species/populations of the genus *Acomys*. Data are given as means and 95% confidence intervals

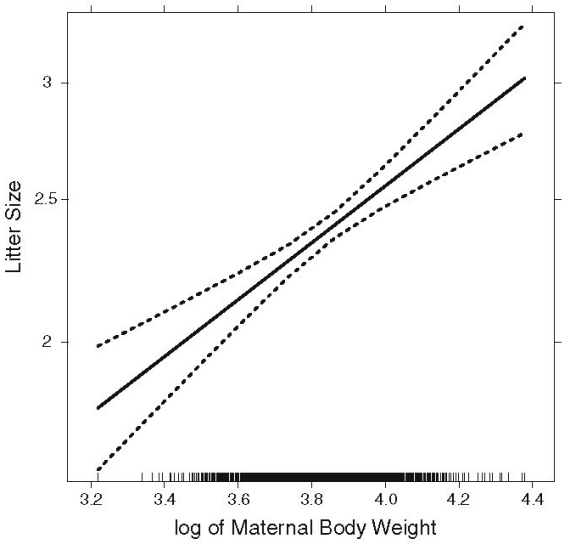


**Fig. 4** The effect of maternal body weight on litter size in four species/populations of the genus *Acomys*. Data are given as means and 95% confidence intervals

determined by specific genes (alleles, QTLs) and thus easily respond to selective forces (for review, see Bünger et al. 2005).

Among other factors we examined, only the number of immature females has consistent effects on litter size both in two particular analyses and the analysis of pooled species/populations. We interpret this finding as the effect of continuous reproduction reflecting positive social settings within the breeding group in social spiny mice. It fits

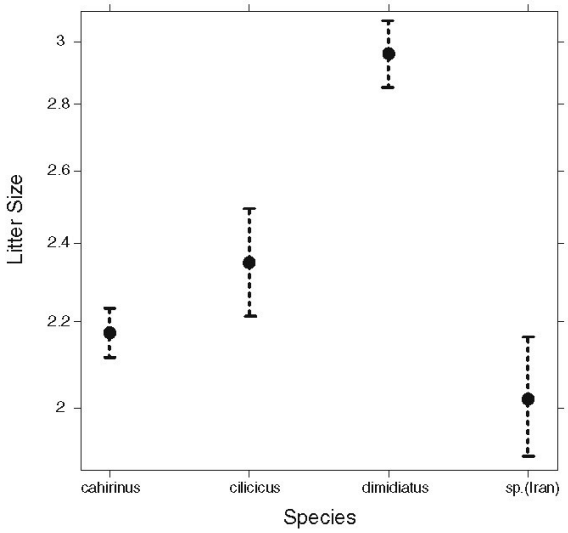


**Fig. 3** Means and 95% confidence intervals for litter size in studied species/populations of the genus *Acomys*: *A. cahirinus*, *A. cilicicus*, *A. dimidiatus*, and *A.* sp. (Iran)

our finding that the levels of stress hormones (fecal glucocorticoid metabolites) differ between the breeding groups in *A. cahirinus* (Nováková et al. 2008). In contrast, Scheibler et al. (2005) reported a negative effect of family size on litter size in Mongolian gerbils (*Meriones unguiculatus*). Therefore, our results do not confirm the intuitive expectation that large size of the family group interferes with reproduction. Regular occurrence of such social phenomena as paternal care (Dieterlen 1962; Makin and Porter 1984), communal nursing (Porter and Doane 1978), and social thermoregulation (note that spiny mice do not build their own burrows and nests) makes large family size advantageous in spiny mice.

The other factors that appeared significant in at least one of our particular analyses of litter size, i.e., postpartum conception and number of immature males, reflect the continuous reproductive activity of the female. Postpartum conception had fairly positive effects on litter size (especially in *A. cahirinus*) when maternal body weight was not controlled; thus, concurrent lactation did not reduce litter size. This may be attributed to the higher body weight of females with postpartum conception that reflects their specific physiological settings and/or presence of body reserves required for continuous reproduction.

The absence of the effect of parity on litter size is somewhat surprising as such a relationship has been frequently reported in rodents (e.g., *Myodes glareolus*, Clarke 1985; Innes and Millar 1990; *M. unguiculatus*, Kai et al. 1995; *Microtus arvalis*, Tkadlec and Krejčová 2001). One may argue that we included parity as a continuous predictor, and the effects may be bimodal. Nevertheless,

detailed inspection of our data did not uncover any markedly shaped relationship. The same is true for maternal age which usually belongs to the most successful predictors of rodent litter size (e.g., in *Apodemus sylvaticus*, Žižková and Frynta 1996; *Peromyscus leucopus*, Havelka and Millar 2004).

In conclusion, variation in litter size within populations of spiny mice kept under laboratory conditions may be partially explained by maternal weight and factors reflecting hospitable social environment of the breeding unit. Nevertheless, manipulative experiments are needed to prove our results based on the correlative approach.

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## References

Barome PO, Monnerot M, Gautun JC (1998) Intrageneric phylogeny of *Acomys* (Rodentia, Muridae) using mitochondrial gene cytochrome b. Mol Phylogenet Evol 9:560–566

Barome PO, Monnerot M, Gautun JC (2000) Phylogeny of the genus *Acomys* (Rodentia, Muridae) based on the cytochrome b mitochondrial gene: implications on taxonomy and phylogeography. Mammalia 64:423–438

Bennett PM, Owens IPF (2002) Evolutionary ecology of birds: life histories, mating systems and extinction. Oxford University Press, Oxford, pp 1–296

Both C, Tinbergen JM, Van Noordwijk AJ (1998) Offspring fitness and individual optimization of clutch size. Proc R Soc Lond B Biol Sci 265:2303–2307

Brunjes PC (1990) The precocial mouse *Acomys cahirinus*. Psychobiology 18:339–350

Bünger L, Lewis RM, Rothschild MF, Blasco A, Renne U, Simm G (2005) Relationships between quantitative and reproductive fitness traits in animals. Philos Trans R Soc Lond B Biol Sci 360:1489–1502

Burda H (1989) Relationship among rodent taxa as indicated by reproductive biology. Z Für Zoologische Systematik Und Evolutionsforschung 27:49–57

Clarke JR (1985) The reproductive biology of the bank vole (*Clethrionomys glareolus*) and the wood mouse (*Apodemus sylvaticus*). Symp Zool Soc Lond 55:33–59

Dieterlen F (1961) Beitrage zur Biologie der Stachelmaus, Acomys cahirinus dimidiatus Cretzschmer. Z Säugetierkunde 26:1–13

Dieterlen F (1962) Geburt und Geburtshilfe bei der Stachelmaus, *Acomys cahirinus*. Z Tierpsychol 19:191–222

Frynta D, Palupčíková K, Bellinvia E, Benda P, Skarlandtová H, Schwarzová L, Modrý D (2010) Phylogenetic relationships within the *cahirinus-dimidiatus* group of the genus *Acomys* (Rodentia: Muridae): new mitochondrial lineages from Sahara, Iran and the Arabian Peninsula. Zootaxa 2660:46–56

Haim A, Alma A, Neuman A (2006) Body mass is a thermoregulatory adaptation of diurnal rodents to the desert environment. J Therm Biol 31:168–171

Havelka MA, Millar JS (2004) Maternal age drives seasonal variation in litter size of *Peromyscus leucopus*. J Mammal 85:940–947

Innes DGL, Millar JS (1990) Numbers of litters, litter size and survival in two species of microtines at two elevations. Holarctic Ecol 13:207–216

Kai O, Sakemi K, Suzuki Y, Sonoda Y, Imai K (1995) Effects of age at first-pairing on the reproductive performance of Mongolian gerbils (*Meriones unguiculatus*). Exp Anim 44:307–313

Kam M, Khokhlova IS, Degen AA (2006) Partitioning of metabolizable energy intake in sucking altricial and precocial rodent pups. J Zool 269:502–505

Kasparian K, Geissler E, Trillmich F (2005) Optimal offspring size in a small mammal: an exception to the tradeoff invariant life-history rule. Oikos 111:271–278

Kratochvíl L, Frynta D (2006) Egg shape and size allometry in geckos (Squamata: Gekkota), lizards with contrasting eggshell structure: why lay spherical eggs? J Zool Syst Evol Res 44:217–222

Makin JW, Porter RH (1984) Paternal behavior in the spiny mouse (*Acomys cahirinus*). Behav Neural Biol 41:135–151

Michaux J, Reyes A, Catzeflis F (2001) Evolutionary history of the most speciose mammals: molecular phylogeny of muroid rodents. Mol Biol Evol 18:2017–2031

Myers P, Master LL (1983) Reproduction by *Peromyscus maniculatus*: size and compromise. J Mammal 64:1–18

Nováková M, Palme R, Kutalová H, Janský L, Frynta D (2008) The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*). Physiol Behav 95:187–193

Nováková M, Vašáková B, Kutalová H, Galeštoková K, Průšová K, Šmilauer P, Šumbera R, Frynta D (2010) Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: *Acomys*). Behav Ecol Sociobiol 64:371–379

Pinter-Wollman N, Dayan T, Eilam D, Kronfeld-Schor N (2006) Can aggression be the force driving temporal separation between competing common and golden spiny mice? J Mammal 87:48–53

Porter RH, Doane HM (1978) Studies of maternal behavior in spiny mice (*Acomys cahirinus*). Z Tierpsychol 47:225–235

Porter RH, Cavallaro SA, Moore JD (1980) Developmental parameters of mother-offspring interactions in *Acomys cahirinus*. Z Tierpsychol 53:153–170

Porter RH, Matochik JA, Makin JW (1986) Discrimination between full-sibling spiny mice. (*Acomys cahirinus*) by olfactory signatures. Anim Behav 34:1182–1188

Scheibler E, Weinandy R, Gattermann R (2005) Social factors affecting litters in families of Mongolian gerbils, *Meriones unguiculatus*. Folia Zool 54:61–68

Shafrir E (2000) Overnutrition in spiny mice (*Acomys cahirinus*): beta-cell expansion leading to rupture and overt diabetes on fat-rich diet and protective energy-wasting elevation in thyroid hormone on sucrose-rich diet. Diabetes Metab Res Rev 16:94–105

Shargal E, Rath-Wolfson L, Kronfeld N, Dayan T (1999) Ecological and histological aspects of tail loss in spiny mice (Rodentia: Muridae, *Acomys*) with a review of its occurrence in rodents. J Zool 249:187–193

Steppan SJ, Adkins RM, Anderson J (2004) Phylogeny and divergence-date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. Syst Biol 53:533–553

Tinbergen JM, Both C (1999) Is clutch size individually optimized? Behav Ecol 10:504–509

Tinbergen JM, Sanz JJ (2004) Strong evidence for selection for larger brood size in a great tit population. Behav Ecol 15:525–533

Tkadlec E, Krejčová P (2001) Age-specific effect of parity on litter size in the common vole (*Microtus arvalis*). J Mammal 82:545–550

Tuomi J (1980) Mammalian reproductive strategies: a generalised relation of litter size to body size. Oecologia 45:39–44

Volobouev V, Auffray JC, Debat V, Denys C, Gautun JC, Tranier M (2007) Species delimitation in the *Acomys cahirinus-dimidiatus* complex (Rodentia, Muridae) inferred from chromosomal and morphological analyses. Biol J Linn Soc 91:203–214

Young DAB (1976) Breeding and fertility of the Egyptian spiny mouse, *Acomys cahirinus*: effect of different environments. Lab Anim 10:15–24

Zejda J (1966) Litter size in *Clethrionomys glareolus* Schreber, 1780. Zoologické Listy 15:193–206

Žižková M, Frynta D (1996) Reproduction in *Apodemus sylvaticus* (Rodentia: Muridae) in captivity. Acta Soc Zool Bohem 60:83–93

## 9.6 Nováková M., Vašáková B., Kutalová H., Galaštoková K., Průšová K., Šmilauer P., Šumbera R., Frynta D. (2010) Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: *Acomys*). *Behavioral Ecology and Sociobiology*. 64: 371-379.

Abstract Spiny mice of the genus *Acomys* (Muridae) represent a very suitable mammalian model for studying factors influencing the secondary sex ratio (SSR). The maternal effort in these rodents is extremely biased in favour of the prenatal period and, therefore, maternal manipulation of the SSR is potentially more advantageous. We studied the SSR in four populations/species of spiny mice kept in family groups consisting of two closely related females, one non-relative male and their descendants. The groups were established from founding animals aged about 3 months (maturing age) and were allowed to breed freely for several months. Each litter was sexed after birth, and relevant data were thoroughly recorded. Altogether, data were collected on 1684 litters: 189 of Acomys sp. from Iran, 203 of *A. cilicicus*, 875 of *A. cahirinus*, and 417 of *A. dimidiatus*. We recorded the sex of 4048 newborns of which 1995 were males and 2053 were females. The overal sex ratio was close to 1:1 (49.2%). Generalized linear mixed models and/or generalized linear models were constructed to evaluate the effect of four life history and eight social variables on the sex ratio. No consistent effects of these variables on the sex ratio were found and, interestingly, none of the variables associated with maternal life history had any effect on the sex ratio. Three factors associated with group composition (i.e. the number of immature males, the number of immature females and the number of breeding females) did have significant effects on the sex ratio, but these effects were not consistent across the studied species. In conclusion, our evaluation of this large dataset revealed that the sex ratio in spiny mice is surprisingly stable.

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ORIGINAL PAPER

# Secondary sex ratios do not support maternal manipulation: extensive data from laboratory colonies of spiny mice (Muridae: *Acomys*)

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**Abstract** Spiny mice of the genus *Acomys* (Muridae) represent a very suitable mammalian model for studying factors influencing the secondary sex ratio (SSR). The maternal effort in these rodents is extremely biased in favour of the prenatal period and, therefore, maternal manipulation of the SSR is potentially more advantageous. We studied the SSR in four populations/species of spiny mice kept in family groups consisting of two closely related females, one non-relative male and their descendants. The groups were established from founding animals aged about 3 months (maturing age) and were allowed to breed freely for several months. Each litter was sexed after birth, and relevant data were thoroughly recorded. Altogether, data were collected on 1684 litters: 189 of *Acomys sp.* from Iran, 203 of *A. cilicicus*, 875 of *A. cahirinus*, and 417 of *A. dimidiatus*. We recorded the sex of 4048 newborns of which 1995 were males and 2053 were females. The overall sex ratio was close to 1:1 (49.2%). Generalized linear mixed models and/or generalized linear models were constructed to evaluate the effect of four life history and eight social variables on the sex ratio. No consistent effects of these variables on the sex ratio were found and, interestingly, none of the variables associated with maternal life history had any effect on the sex ratio. Three factors associated with group composition (i.e. the number of immature males, the number of immature females and the number of breeding females) did have significant effects on the sex ratio, but these effects were not consistent across the studied species. In conclusion, our evaluation of this large dataset revealed that the sex ratio in spiny mice is surprisingly stable.

**Keywords** Parental effort · Rodents · Sex allocation · Sex ratio · Social behaviour

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## Introduction

The secondary sex ratios (SSRs) of newborn mammals have attracted enormous research effort since Darwin (1871). There are several theoretical backgrounds for maternal manipulation that would result in a biased sex ratio of the progeny. These include the Fisherian theory, which assumes the allocation of equal investment to male and female progeny (Fisher 1930), the model of local resource competition, which expects SSRs to be biased in favour of the dispersing sex (Clark 1978) and the maternal condition hypothesis, which predicts a higher proportion of males under favourable conditions and, conversely, a higher proportion of females under unfavourable conditions (Trivers and Willard 1973; Carranza 2002; Cameron and

Linklater 2002; for a review, see Cameron 2004). Current theoretical studies combine the above models as well as other selective forces into complex mathematical models for predicting the sex ratios (e.g. Wade et al. 2003; Wild and West 2007).

Empirical results support the view that SSR is usually close to equality; nevertheless, significant deviations from the 1:1 ratio have been repeatedly reported (Austad and Sunquist 1986; Gosling 1986; Labov et al. 1986; Huck et al. 1990; Perret and Colas 1997; Creel et al. 1998; Côté and Festa-Bianchet 2001; Cameron 2004; Sheldon and West 2004; Kaňková et al. 2006). These deviations have been attributed either to the above-mentioned ultimate causes or to proximate mechanisms that are by definition not mutually exclusive. Of the proximate mechanisms, the most promising are (1) the level of circulating steroid hormones (James 1996, 1998, 2004, 2006; Grant 2007), (2) developmental asynchrony of sexes in blastocyst growth (Krackow 1995, 1997; Krackow and Burgoyne 1998; Krackow et al. 2003) and (3) circulating glucose level (Gutiérrez-Adán et al. 2001; Cameron 2004; Kimura et al. 2005; Cameron et al. 2008; Helle et al. 2008).

In recent years, the evidence for the adaptive maternal SSR adjustment model has been questioned both on empirical (see below) and theoretical grounds (Krackow 2002). Festa-Bianchet (1996) accentuated the high frequency of contradictory results and also the selective publication success of papers presenting positive results. In this respect, he shared the scepticism of an earlier paper of Clutton-Brock and Iason (1986). A similar conclusion is also supported by a thorough review by Cockburn et al. (2002). Interestingly, an empirical study analysing extensive datasets from wild savannah baboons (*Papio cynocephalus*) has clearly demonstrated that significant results are a product of stochastic biases that arise in small samples (Silk et al. 2005). The only effects on mammalian SSR clearly supported by recent reviews are those of maternal nutritional status around the time of conception (Cameron 2004; Rosenfeld and Roberts 2004; Sheldon and West 2004).

Among mammals, rodents are a suitable model for studying sex ratios and influencing factors, since they may be easily kept under laboratory conditions, thereby providing an opportunity to gather sufficient sample sizes required for relevant statistical analysis (for review, see Sikes 2007). Classical laboratory rodents, such as mice (*Mus musculus*; Krackow and Hoeck 1989; Krackow and Gruber 1990; Kaňková et al. 2007), rats (*Rattus norvegicus*; Bird and Contreras 1986), golden hamsters (*Mesocricetus auratus*; Labov et al. 1986, Huck et al. 1990) and guinea pigs (*Cavia porcellus*; Peaker and Taylor 1996) are traditional models for empirical studies. Nevertheless, SSRs have been studied in many other species as well, such as coypus (*Myocastor coypus*; Gosling 1986), Mongolian gerbils (*Meriones unguiculatus*; Clark et al. 1991), wood rats (*Neotoma floridana*; McClure 1981), wood mice (*Apodemus sylvaticus*; Frynta and Žižková 1994) and voles (*Microtus agrestis*, *M. oeconomus*; Hansson 1987; Ims 1994). Unfortunately, most of the published studies rely solely on correlations between sex ratio and parameters of maternal life history. Experimental studies have mostly focussed on the manipulation of food intake and food quality (e.g. Huck et al. 1986; Wright et al. 1988; Koskela et al. 2004; Rosenfeld et al. 2003; Cameron et al. 2008; Fountain et al. 2008). The effects of true social factors, such as dominance (golden hamsters: Pratt and Lisk 1989), population density (voles: *Microtus townsendii*, Lambin 1994; *M. oeconomus*, Aars et al. 1995), group size (house mice: Wright et al. 1988) and/or group composition (marmots (*Marmota flaviventris*): Armitage 1987, Mongolian gerbils: Scheibler et al. 2005), on sex ratios have been addressed less frequently (but see Cameron 2004 for review in other mammals). Although social factors have complex consequences that are usually difficult to interpret on a proximate level, they cannot be ignored. The considerable variation in densities and social circumstances that affects breeding females of most rodent species even under natural conditions has to be taken into account.

In the study reported here, we focussed on the sex ratio in spiny mice of the genus *Acomys* and its relationships to social and life history variables. Spiny mice are social animals, and under laboratory conditions they should be kept in families consisting of an adult male, multiple females and their descendants (Young 1976), which mimics their wild social system. Their societies are not anonymous, and individual recognition has been demonstrated unequivocally (Porter et al. 1986). In groups consisting of related individuals, communal care for the young comprising allosuckling is frequent; however, mothers are able to recognize their own offspring (Porter et al. 1980). In addition to the ease of keeping and breeding spiny mice under laboratory conditions, their newborns, unlike those of many other rodents, can be easily and reliably sexed according to external characters. Moreover, spiny mice are likely to be predisposed to maternal manipulation of the sex ratio for the following three reasons.

(1) In contrast to many other muroid rodents, after an extended gestation period (36–42 days, which is nearly twice as long as that for laboratory mice), spiny mice produce only small litters (most frequently consisting of two or three newborns, range1–7; Frynta et al. unpublished results) consisting of relatively large and well-developed newborns (Brunjes 1990). Maternal effort is therefore high in the prenatal period compared with that in many other muroid rodents. Thus, we consider that maternal manipulation of the sex ratio

prior to parturition is more advantageous than sex-selective parental infanticide of newborns.

(2) The large body size of newborns relative to maternal body size (the mean litter weight at birth represents 20–25% of maternal weight; Dieterlen 1961) facilitates the potential effect of litter size on offspring quality. Resources available per individual offspring are considerably reduced even by the increment of a single newborn. Small litters thus may be predisposed for the production of the sex exhibiting a more closer relationship between consumed maternal resources and fitness prospects (Trivers and Willard 1973). Nevertheless, the assumptions of the Trivers–Willard hypothesis (Hewison and Gaillard 1999; Carranza 2002; Cameron and Linklater 2002; Blanchard et al. 2005) predicting an association between parameters related to maternal condition and allocation of maternal investment to the sexes have not been empirically tested in spiny mice yet. Two of these three principal assumptions (mothers in better condition can produce weanlings in better condition; adult males benefit more from a good condition than adult females) are most probably satisfied in spiny mice due to high maternal investment and strong male-male competition, while the validity of the third one (correlation between weanling and adult condition) is likely, but remains unproved. Spiny mice live in environments with high spatiotemporal variation in resource availability (semideserts, seasonal savannas and woodlands). Under natural conditions, the reproductive value of the offspring produced in good and bad conditions may differ considerably, and thus alternative reproductive strategies that switch according to actual ecological and nutritional conditions may evolve.

(3) Spiny mice are social rodents in which social circumstances may also contribute to fitness. The reproductive fate of rodent female may be determined by the presence or absence of maternal kin in the neighbourhood (e.g. Lambin and Yoccoz 1998). Both sexes, but especially males, may be limited by the presence of older cohort of the same sex. Our data from laboratory colonies suggest that adolescent males are driven away by the territorial male while females are tolerated. Local resource competition between the mother and female offspring can, therefore, be a reasonable expectation. The number of females within a family may indicate the expected cost of bearing additional daughters. Consequently, we hypothesized positive association between the number of females in the family group and the sex ratio. Conversely, an excess of juvenile and/or adolescent males in the environment may reduce the reproductive prospect of additional male offspring. Thus, a negative association between the number of immature males and sex ratio should be expected.

Spiny mice are, therefore, a suitable model for critically testing the predictions of the sex ratio theory. We have analysed the data on SSR collected in captive colonies of spiny mice belonging to four closely related species. The aim of our study was to assess (1) deviations from the one to one and/or Fisherian ratios, (2) effects of life history variables and (3) social composition of the breeding groups on SSR.

## Material and methods

Spiny mice of the genus *Acomys* are small rock-dwelling rodents inhabiting Africa and Middle East. Despite their earlier systematic placement, spiny mice are more related to gerbils than to true murids belonging to the subfamily Murinae, which are represented by rats and house mice (Steppan et al. 2004).

Our laboratory colonies of spiny mice were of the following origin: *Acomys cahirinus*, Abu Simbel archaeological site, southern Egypt (22° 22′ N, 31° 38′ E); *A. cilicicus*, east of Silifke, southern Turkey (36° 26′ N, 34° 06′ E); *Acomys. sp.*, Zagros, southwestern Iran (28° 56′ N, 52° 32′ E); *A. dimidiatus*, laboratory strain, Prague zoo, probably from Israel or Sinai. Phylogenetic analysis of mitochondrial control region sequences in these colonies revealed that all of the studied populations/species belong to the *cahirinus/dimidiatus* group. The former two populations/species belong to the clade of *A. cahirinus* sensu lato inhabiting North Africa and the eastern Mediterranean region (Crete, Cyprus, Kilikian coast in Anatolia), while the latter two belong to the *A. dimidiatus* sensu lato clade ranging from Sinai, throughout the Arabian Peninsula and along the coast of the Gulf of Oman from Iran to Pakistan. The specific/subspecific status of the Iranian population, referred to here as *Acomys sp.*, needs further clarification (Frynta and Průšová unpublished results).

The animals were kept in terrariums (60×50×40 cm or 70×60×40 cm) or in rodent standard plastic cages (VELAZ T4 , 55×32×18 cm) under standard laboratory conditions. Wood shavings were used as bedding material, a clay flowerpot with a lateral opening served as a shelter and tree branches for climbing and gnawing were provided as environmental enrichment. Food (standard diet for rats and mice ST1; VELAZ, Czech Republic, supplemented with a mixture of grains, dry bread, apples and herb leaves) and water were available ad libitum.

The spiny mice were kept in family groups consisting of two closely related females (full sisters or uterine sisters), one non-relative male and their descendants. The groups were established from founding animals about 3 months of age (maturing age) and then allowed to breed freely for several months. Manipulation of group structure only occurred in the case of male-to-male aggression between the male founder and his mature male offspring (if so, young males were removed). The experimental groups were regularly checked (either daily or every other day), and each litter was sexed immediately after its detection. Compared to many other rodents, sexing in the spiny mice does not rely solely on ambiguous anogenital distances, as female nipples are clearly visible, even in newborns. Thus, the sexing error approaches zero in these animals. The putative mother was identified, and other circumstances (see below) were thoroughly recorded.

The recorded data included the sex ratio itself (expressed as the proportion of males in the litter), maternal life history variables, such as (1) parity, (2) age in days, (3) postpartum estrus (the litter was considered as conceived postpartum when delivered before day 41 after the previous one) and (4) litter size, and social variables, such as (5) time from the founding of the group (in months), (6) litter order (from the group perspective), (7) number of adult males actually present in the group (aged ≥90 days), (8) number of adult females (see 7), (9) number of breeding females (i.e. those that already gave birth) in the group—coded as the presence of either one or more than one breeding female in the group and further referred to as breeding females, (10) maternal status (first breeding founder, second breeding founder, their daughters), (11) number of immature males and (12) number of immature females (aged ≤90 days for both sexes). It should be noted that maternal body weight was assessed but not included because it was missing in an additional 342 cases. Nevertheless, the statistical models referred below were not substantially affected by the inclusion of maternal body weight, and this factor remained non-significant.

### Statistical analysis

We estimated generalized linear mixed models (GLMM) and/or generalized linear models (GLM) in which litter sex ratio was treated as a dependent variable with a binomial distribution and the logit link function was adopted. As the models require complete sets of explanatory variables, all litters with at least one missing value were excluded ($n$=212). Therefore, the numbers of litters and newborns used for the computation of the overall sex ratios exceed those included in GLMM analyses.

We first computed GLMMs in which maternal identity was included as a random factor to avoid pseudoreplications (Krackow and Tkadlec 2001). The significance of this random effect was tested using the log-likelihood ratio test based on $\chi^2$ distribution, and the effect was found to be non-significant. This allowed us to further use the simpler GLMs instead of GLMMs.

As no effect of any factor associated with maternal identity (i.e. maternal parity, age, postpartum estrus and status) was found to be significant in either the complete GLMM analysis or in any separate GLMM analysis of data concerning individual species, we removed these variables from subsequent GLMs. We then incorporated all remaining explanatory variables into main effects GLMs computed separately for each species and pooled the data. Finally, we computed GLM allowing interactions between species and explanatory variables. The above full models were then reduced to variables with $P<0.1$ (see Results).

The size of the effects is presented either graphically and/or as the percentage point difference in sex ratio from the nominal value of 50% due to a unit change of the predictor (CPU). The calculations were performed using the R statistical package ® development core team 2005). Traditional chi-square tests were also conducted to test deviations of the observed sex ratios from the expected equality. Although this approach is theoretically less appropriate than the above-mentioned one and may inflate the significance, it is more intuitive and allows for the inclusion of all records.

## Results

We recorded the sex of 4048 newborns of which 1995 were males and 2053 were females. Thus, the overall sex ratio was very close to 1:1 (49.2%, $\chi^2$=0.831, $P$=0.36). Among the studied species, only *Acomys sp.* from Iran exhibited a significant deviation from the balanced sex ratio (42.5% males; Table 1).

*Generalized linear mixed models* The initial full GLMM evaluating the effect of all examined factors—i.e. four maternal life history (maternal parity, age, postpartum estrus and litter size) and eight social variables (time from the founding of the group, litter order, number of adult males, number of adult females, number of breeding females, maternal status, number of immature males, number of immature females)—revealed significant effects of species (*A. cilicicus* CPU=9.02, *A. cahirinus* CPU=7.89, *A. dimidiatus* CPU=9.55) and litter size (CPU=-1.89) on the sex ratio. Table 2 presents details on the statistics. The effect of the number of immature males approached the chosen α level of 0.05 (CPU=0.75; $F_{(1,963)}$=3.21, $P$=0.0734).

**Table 1** Secondary sex ratio in study population categorized according to species

| Species | Number of litters | Males | Females | Total number of newborns | Sex ratio (%) |
|---|---|---|---|---|---|
| *A. cahirinus* | 875 | 988 | 979 | 1967 | 50.2 |
| *A. cilicicus* | 203 | 241 | 241 | 482 | 50.0 |
| *A. dimidiatus* | 417 | 612 | 625 | 1237 | 49.5 |
| *Acomys sp.* | 189 | 154 | 208 | 362 | 42.5* |
| Total | 1684 | 1995 | 2053 | 4048 | 49.2 |

*$P<0.05$

**Table 2** Results of analyses using the GLMM and GLM models for testing the secondary sex ratio in four species/populations of the genus *Acomys*

| Model | CPU | LCI | UCI | *F* | *df* | Residual *df* | *P* |
|---|---|---|---|---|---|---|---|
| GLMM | | | | | | | |
| Species | | | | 3.18 | 3 | 487 | 0.0238 |
| *A. cilicicus* | 9.02 | 1.41 | 16.22 | | | | |
| *A. cahirinus* | 7.89 | 1.63 | 13.92 | | | | |
| *A. dimidiatus* | 9.55 | 2.95 | 15.81 | | | | |
| Litter size | −1.89 | −3.53 | −0.24 | 5.19 | 1 | 963 | 0.0229 |
| Main effect GLM | | | | | | | |
| Immature males | 0.81 | 0.13 | 1.49 | 6.49 | 1 | 1679 | 0.0109 |
| Litter size | -1.61 | −3.15 | −0.06 | 4.15 | 1 | 1675 | 0.0415 |
| GLM with interactions | | | | | | | |
| Immature females × species | | | | 9.34 | 4 | 1646 | <0.0001 |
| Immature females × *A. cilicicus* | -4.64 | −6.67 | −2.60 | | | | |
| Immature females × *A. cahirinus* | 0.95 | −0.16 | 2.05 | | | | |
| Immature females × *A. dimidiatus* | -2.02 | −3.63 | −0.41 | | | | |
| Immature females × *Acomys sp.* | 3.95 | 0.84 | 7.03 | | | | |
| Immature males × species | | | | 6.73 | 3 | 1654 | 0.0002 |
| Immature males × *A. cilicicus* | 8.88 | 4.43 | 13.20 | | | | |
| Immature males × *A. cahirinus* | 1.08 | −2.75 | 4.90 | | | | |
| Immature males × *A. dimidiatus* | 2.22 | −1.86 | 6.26 | | | | |
| Breeding females × species | | | | 2.96 | 4 | 1650 | 0.0184 |
| Breeding females × *A. cilicicus* | 1.08 | −11.09 | 13.13 | | | | |
| Breeding females × *A. cahirinus* | -9.73 | −16.40 | −2.69 | | | | |
| Breeding females × *A. dimidiatus* | 11.22 | 3.05 | 18.80 | | | | |
| Breeding females × *Acomys sp.* | -6.73 | −18.83 | 6.23 | | | | |
| Immature males | -1.19 | −4.85 | 2.49 | 6.55 | 1 | 1661 | 0.0105 |
| Separate GLMs | | | | | | | |
| *A. cilicicus* | | | | | | | |
| Immature males | 7.46 | 5.07 | 9.82 | 20.57 | 1 | 201 | <0.0001 |
| Immature females | -4.93 | −6.90 | −2.94 | 26.62 | 1 | 200 | <0.0001 |
| *A. cahirinus* | | | | | | | |
| Breeding females | −10.21 | -16.73 | −3.32 | 5.81 | 1 | 867 | 0.0159 |
| *A. dimidiatus* | | | | | | | |
| Immature females | −1.89 | -3.39 | −0.38 | 6.05 | 1 | 414 | 0.0139 |
| Breeding females | 12.19 | 4.31 | 19.47 | 5.92 | 1 | 415 | 0.0150 |
| *Acomys sp.* | | | | | | | |
| Immature females | 2.70 | 0.06 | 5.33 | 4.04 | 1 | 187 | 0.0444 |

Only significant effects for reduced models are shown

CPU, percentage point difference in sex ratio from the nominal value of 50%, due to a unit change of the predictor; LCI, lower bound of 95% confidence interval (CI); UCI, upper bound of 95% CI; GLMM, generalized linear mixed models; GLM, generalized linear models

*Main-effects GLM* We then excluded the factors associated with maternal identity and performed the GLM analysis. The effect of the number of immature males reached significance ($F_{(1,1661)}$=6.55, $P$=0.0105), while the significance of effects of the species ($F_{(3,1658)}$=2.56, $P$=0.0530) and litter size ($F_{(1,1655)}$=3.23, $P$=0.0722) decreased. When the model was further reduced to include only these three factors, the analysis revealed significant effects of the number of immature males (CPU=0.81) and litter size (CPU=-1.61), but not of the species.

*Generalized linear model allowing interactions with species* When the interactions between the factors included in the original GLM and the species identity were allowed, three of the factors appeared to be significant. The final model (including effects with $P$<0.1; see Table 2) revealed significant effects of the number of immature females × species interaction (Fig. 1), the number of immature males × species interaction (Fig. 2), the number of immature males (CPU=−1.19) and the number of breeding females × species interaction. The effects of the species ($F_{(3,1658)}$=2.56, $P$=0.0530) and litter size ($F_{(1,1657)}$=3.27, $P$=0.0707) dropped below the significance level.

*Separate GLMs for each species* We also performed separate analyses for each species. Only the results of the final reduced models that revealed the following significant factors are provided: in *A. cahirinus* the number of breeding females (CPU=−10.21); in *A. cilicicus*, the number of immature males (CPU=7.46) and the number of immature females (CPU=-4−93); in *A. dimidiatus*, the number of breeding females (CPU=12.19) and the number of immature females (CPU= −1.89); in *Acomys sp.*, the number of immature females (CPU=2.70). For detailed statistics see Table 2.

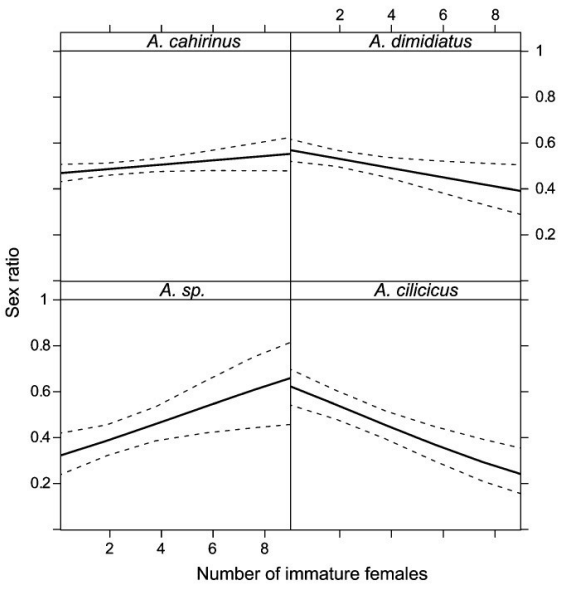


**Fig. 1** The predictions of the generalized linear model (GLM) for the number of immature females and species interaction

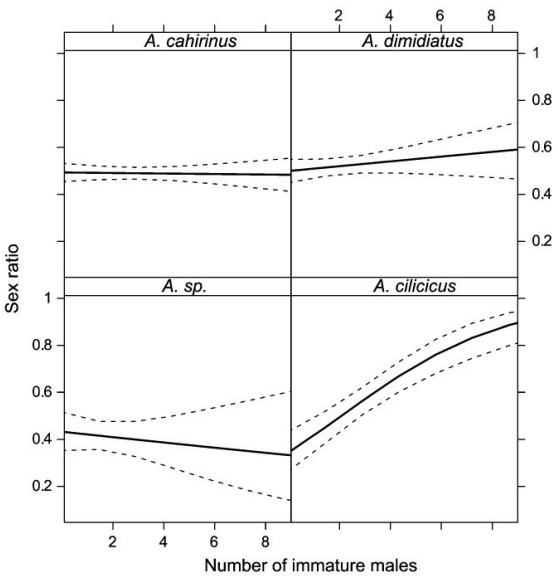


**Fig. 2** The predictions of the GLM for the number of immature males and species interaction

In *A. cahirinus*, the species represented by the largest dataset, we also calculated partial GLMs, including those for only litters consisting of two and three newborns (i.e. most common litter sizes), respectively. This analysis was performed to avoid the possible interaction of the effects of differential cost by sex and by litter size. No significant effect was revealed by these partial analyses.

## Discussion

The SSRs found in our dataset were very close to parity in three of the four populations/species studied. Interestingly, the only population exhibiting a slightly female-biased sex ratio was represented by the smallest sample size. Overall, these results were unsurprising, as balanced ratios at birth in other mammals have been frequently reported (Clutton-Brock and Iason 1986). Nevertheless, this phenomenon cannot be viewed as an unavoidable consequence of chromosomal sex determination. Although mammalian sex ratios are primarily determined by the sperm carrying the sex chromosomes and, consequently, are not biased to any large extent at

the time of conception, episodes of sex-specific selective mortality of zygotes and embryos could lead to considerable deviations from parity, especially during early ontogeny (Kirby et al. 1967; Milki et al. 2003). These processes provide a good chance for maternal manipulation of the SSR (compare Grant and Irvin 2009). Therefore, the evolutionary maintenance of unbiased sex ratios requires the presence of a specific stabilizing mechanism, as assumed, for example, by Fisher (1930) who expected natural selection to favour an equal allocation of investment in male and female progeny. In any case, the demonstration of unbiased sex ratios does not mean a falsification of the hypothesis of maternal sex ratio adjustment (Wild and West 2007).

Despite our extensive datasets, we detected only a few significant and consistent effects on sex ratios in spiny mice. Surprisingly, the final models included only three variables, all of which were associated with the composition of the group: the number of breeding females, the number of immature males and the number of immature females. Thus, if any factor plays a role in sex ratio adjustment in these species, it has probably something to do with the actual social environment. This hypothesis corresponds quite well with the recent finding that individual families of spiny mice differ in levels of faecal cortisol metabolites (Nováková et al. 2008). Conversely, a significant interaction with species is revealed in all of the three variables included in our final models. The same factors (e.g. number of immature females; Fig. 1) showed even opposite effects in separate analyses of particular species/populations of spiny mice. In other words, the detected effects are not consistent across the studied species. Although information on the ecology of spiny mice under natural conditions is scarce, these species have fairly comparable requirements, and we can provide no straightforward explanation for the observed inconsistency. Therefore, these results need to be interpreted with caution.

None of the remaining nine explanatory variables appeared to be significant. Our results provide some support for the assumption that the effects of most factors of mammalian SSRs tend to be only small and biologically unimportant, while significant effects are mostly associated with small sample sizes and publication bias (e.g. Festa-Bianchet 1996; Cockburn et al. 2002; Silk et al. 2005, but see Cameron 2004).

Factors such as glucose level in the circulating blood around the time of conception (Cameron et al. 2008) and its correlates (such as fat content in diet; Rosenfeld et al. 2003; Rosenfeld and Roberts 2004) are currently considered to be promising proximate mechanisms of maternal SR manipulation. Not one of our variables provides a direct measure of maternal condition or metabolic status. It can be reasonably expected that some of the examined factors, such as postpartum conception, age and, possibly, social status, are related to maternal condition; however, we have no data on maternal glucose or fat levels, which are difficult to collect in large samples. Thus, covert effects of these variables cannot be excluded.

As there are both good theoretical reasons for maternal manipulation of the offspring sex ratios and data from reliable studies demonstrating the influence of various factors on sex ratios (see Introduction), we avoid drawing over-generalized conclusions from our particular study and instead focus on the peculiarities of the biology of the studied species.

True laboratory animals were selected in order to maximize the reproductive efforts for many generations. However, spiny mice are originally savanna and/or desert dwellers living in unpredictable or seasonal environments. They are therefore likely to be able to regulate their reproduction in response to actual resource availability (e.g. rainfall: Sicard and Fuminier 1996) and the corresponding prospect of the reproductive event. We may only speculate that since spiny mice as wild and more K-selected species (remember their large-sized precocial newborns) strictly avoid breeding whenever they perceive the environmental or social conditions as not fully favourable, there may be reduced variance in body condition and, consequently, no reason for maternal manipulation and/or any other maternal effect on the sex ratio of the progeny.

In conclusion, we found no consistent effects of the studied factors on the sex ratio in spiny mice. Although our correlation approach to the sex ratios has many inherent limitations, it still represents the only easy approach to obtain sufficient datasets from non-domestic mammals. Our results are not interpretable in terms of the most popular sex ratio theories (e.g. the Trivers–Willard hypothesis and/or local resource competition hypothesis). We found fairly balanced SSRs, and suspect that only some factors associated with group composition affect this trait in spiny mice.

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**Ethical note** Any harm to experimental animals was avoided. The experiments were performed in accordance with Czech law implementing all corresponding EU regulations and were approved by the Institutional Animal Care and Use Committee.

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## References


Aars J, Andressen HP, Ims RA (1995) Root voles: litter sex ratio variation in fragmented habitat. J Anim Ecol 64:459–472

Armitage KB (1987) Do female yellow-bellied marmots adjust the sex ratios of their offspring? Am Nat 129:501–519

Austad SN, Sunquist ME (1986) Sex-ratio manipulation in the common opossum. Nature 324:58–60

Bird E, Contreras RJ (1986) Maternal dietary sodium chloride levels affect the sex ratio in rat litters. Physiol Behav 36:307–310

Blanchard P, Festa-Bianchet M, Gaillard JM, Jorgenson JT (2005) Maternal condition and offspring sex ratio in polygynous ungulates: a case study of bighorn sheep. Behav Ecol 16:274–279

Brunjes PC (1990) The precocial mouse, *Acomys cahirinus*. Psychobiology 18:339–350

Cameron EZ (2004) Facultative adjustment of mammalian sex ratios in support of the Trivers-Willard hypothesis: evidence for a mechanism. Proc R Soc Lond B Biol Sci 271:1723–1728

Cameron EZ, Linklater WL (2002) Sex bias in studies of sex bias: the value of daughters to mothers in poor condition. Anim Behav 63: F5–F8

Cameron EZ, Lemons P, Bateman PW, Bennett NC (2008) Experimental alteration of litter sex ratios in a mammal. Proc Roy Soc B 275:323–327

Carranza J (2002) What did Trivers and Willard really predict? Anim Behav 63:F1–F3

Clark AB (1978) Sex ratio and local resource competition in a Prosimian primate. Science 201:163–165

Clark MM, Galef BG, Saal FSV (1991) Nonrandom sex composition of gerbil, mouse, and hamster litters before and after birth. Dev Psychobiol 24:81–90

Clutton-Brock TH, Iason GR (1986) Sex ratio variation in mammals. Q Rev Biol 61:339–374

Cockburn A, Legge S, Double MC (2002) Sex ratios in birds and mammals: can the hypotheses be disentangled? In: Hardy ICW (ed) Sex ratios: concepts and research methods. Cambridge, Cambridge University Press, pp 266–286

Côté SD, Festa-Bianchet M (2001) Offspring sex ratio in relation to maternal age and social rank in mountain goats (*Oreamnos americanus*). Behav Ecol Sociobiol 49:260–265

Creel S, Creel NM, Monfort SL (1998) Birth order, estrogens and sex-ratio adaption in African wild dogs (*Lycaon pictus*). Anim Reprod Sci 53:315–320

Darwin C (1871) The descent of man, and selection in relation to sex. John Murray, London

Dieterlen F (1961) Beitrage zur Biologie der Stachelmaus. *Acomys cahirinus dimidiatus* Cretzschmer. Z Säugetierkunde 26:1–13

Festa-Bianchet SA (1996) Offspring sex ratio studie of mammals: does publication depend upon the duality of the research or the direction of the results? Ecoscience 3:42–44

Fisher RA (1930) The genetical theory of natural selection. Clarendon, Oxford

Fountain ED, Mao J, Whyte JJ, Mueller KE, Ellersieck MR, Will MJ, Roberts RM, MacDonald R, Rosenfeld CS (2008) Effects of diets enriched in omega-3 and omega-6 polyunsaturated fatty acids on offspring sex-ratio and maternal behavior in mice. Biol Reprod 78:211–217

Frynta D, Žižková M (1994) Sex ratio in *Apodemus sylvaticus* (Rodentia: Muridae): a comparison of field and laboratory data. Acta Soc Zool Boh 57:183–192

Gosling LM (1986) Selective abortion of entire litters in the coypu: adaptive control of offspring production in relation to quality and sex. Am Nat 127:772–795

Grant VJ (2007) Could maternal testosterone levels govern mammalian sex ratio deviations? J Theor Biol 246:708–719

Grant VJ, Irvin RJ (2009) A simple model for adaptive variation in the sex ratios of mammalian offspring. J Theor Biol 258:38–42

Gutiérrez-Adan A, Granados J, Pintado B, De La Fuente J (2001) Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. Reprod Fertil Dev 13:361–365

Hansson L (1987) Vole sex ratios: the importance of mating systems and maternal condition. Oikos 49:161–164

Helle S, Laaksonen T, Adamsson A, Paranko J, Huitu O (2008) Female field voles with high testosterone and glucose levels produce male-biased litters. Anim Behav 75:1031–1039

Hewison AJM, Gaillard JM (1999) Successful sons or advantaged daughters? The Trivers-Willard model and sex-biased maternal investment in ungulates. Trends Ecol Evol 14:229–234

Huck UW, Labov JB, Lisk RD (1986) Food restricting young hamsters (*Mesocricetus auratus*) affects sex-ratio and growth of subsequent offspring. Biol Reprod 35:592–598

Huck UW, Seger J, Lisk RD (1990) Litter sex ratios in the golden hamsters vary with time of mating and litter size and are not binomially distributed. Behav Ecol Sociobiol 26:99–109

Ims RA (1994) Litter sex ratio variation in laboratory of two geographically distinct strains of the root vole *Microtus oeconomus*. Ecography 17:141–146

James WH (1996) Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels at the time of conception. J Theor Biol 180:271–286

James WH (1998) Hypotheses on mammalian sex ratio variation at birth. J Theor Biol 192:113–116

James WH (2004) Hypotheses on how selection for some traits in rodents led to correlated responses in offspring sex ratios. J Theor Biol 228:1–6

James WH (2006) Possible constraints on adaptive variation in sex ratio at birth in humans and other primates. J Theor Biol 238:383–394

Kaňková Š, Šulc J, Nouzová K, Fajfrlík K, Frynta D, Flegr J (2006) Women infected with parasite *Toxoplasma* have more sons. Naturwissenschaften 94:122–127

Kaňková Š, Kodym P, Frynta D, Vavřínová R, Kuběna A, Flegr J (2007) Influence of latent toxoplasmosis on secondary sex index in mice. Parasitology 134:1709–1717

Kimura K, Spate LD, Green MP, Roberts RM (2005) Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Mol Reprod Dev 72:207–207

Kirby DRS, McWhirter KG, Teitelbaum MS, Darlington CD (1967) A possible immunological influence on sex ratio. Lancet 1:139–140

Koskela E, Huitu O, Koivula M, Korpimaki E, Mappes T (2004) Sex-biased maternal investment in voles: importance of environmental conditions. Proc R Soc Lond B Biol Sci 271:1385–1391

Krackow S (1995) The developmental asynchrony hypothesis for sex ratio manipulation. J Theor Biol 176:273–280

Krackow S (1997) Further evaluation of the developmental asynchrony hypothesis of the sex ratio variation. Appl Anim Behav Sci 51:243–250

Krackow S (2002) Why parental sex ratio manipulation is rare in higher vertebrates (invited article). Ethology 108:1041–1056

Krackow S, Burgoyne PS (1998) Timing of mating, developmental asynchrony and the sex ratio in mice. Physiol Behav 63:81–84

Krackow S, Gruber F (1990) Sex ratio and litter size in relation to parity and mode of conception in three inbred strains of mice. Lab Anim 24:345–352

Krackow S, Hoeck HN (1989) Sex ratio manipulation, maternal investment and behaviour during concurrent pregnancy and lactation in house mice. Anim Behav 37:177–186

Krackow S, Tkadlec E (2001) Analysis of brood sex ratios: implications of offspring clustering. Behav Ecol Sociobiol 50:293–301

Krackow S, Schmidt TA, Elepfandt A (2003) Sexual growth dimorphism affects birth sex ratio in house mice. Proc R Soc Lond B Biol Sci 270:943–947

Labov JB, Huck UW, Vaswani P, Lisk RD (1986) Sex ratio manipulation and decreased growth of male offspring of undernourished golden hamsters (*Mesocricetus auratus*). Behav Ecol Sociobiol 18:241–249

Lambin X (1994) Sex ratio variation in relation to female philopatry in Townsend's voles. J Anim Ecol 63:945–953

Lambin X, Yoccoz NG (1998) The impact of population kin-structure on nestling survival in Townsend's voles, *Microtus townsendii*. J Anim Ecol 67:1–16

McClure PA (1981) Sex-biased litter reduction in food-restricted wood rats (*Neotoma floridana*). Science 211:1058–1060

Milki AA, Jun SH, Hinckley MD, Westphal LW, Giudice LC, Behr B (2003) Comparison of the sex ratio with blastocyst transfer and cleavage stage transfer. J Assist Reprod Genet 20:323–326

Nováková M, Palme R, Kutalová H, Janský L, Frynta D (2008) The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*). Physiol Behav 95:187–193

Peaker M, Taylor E (1996) Sex ratio and litter size in the guinea-pig. J Reprod Fertil 108:63–67

Perret M, Colas S (1997) Manipulation of sex ratio at birth and maternal investment in female mouse lemurs (*Microcebus murinus*), Primates. Appl Anim Behav Sci 51:275–283

Porter RH, Cavallaro SA, Moore JD (1980) Developmental parameters of mother-offspring interactions in *Acomys cahirinus*. Z Tierpsychol 53:153–170

Porter RH, Matochik JA, Makin JW (1986) Discrimination between full-sibling spiny mice. (*Acomys cahirinus*) by olfactory signatures. Anim Behav 34:1182–1188

Pratt NC, Lisk RD (1989) Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). J Reprod Fertil 87:763–769

R Development Core Team (2005) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available at: www.R-project.org

Rosenfeld CS, Roberts RM (2004) Maternal diet and other factors affecting offspring sex ratio: a review. Biol Reprod 71:1063–1070

Rosenfeld CS, Grimm K, Livingston K, Brokman A, Lamberson WE, Roberts RM (2003) Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. Proc Natl Acad Sci USA 100:4628–4632

Scheibler E, Weinandy R, Gattermann R (2005) Social factors affecting litters in families of Mongolian gerbils, *Meriones unguiculatus*. Folia Zool 54:61–68

Sheldon BC, West SA (2004) Maternal Dominance, maternal condition, and offspring sex ratio in ungulate mammals. Am Nat 163:40–54

Sicard B, Fuminier F (1996) Environmental cues and seasonal breeding patterns in Sahelian rodents. Mammalia 60:667–675

Sikes RS (2007) Facultative sex ratio adjustment. In: Sherman PW, Wolff JO (eds) Rodent societies: an ecological & evolutionary perspective. The University Chicago Press, Chicago, pp 129–138

Silk JB, Willoughby, Brown (2005) Maternal rank and local resource competition do not predict birth sex ratios in wild baboons. Proc R Soc Lond B Biol Sci 272:859–864

Steppan SJ, Adkins RM, Anderson J (2004) Phylogeny and divergence-date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. Syst Biol 53:533–553

Trivers RL, Willard DE (1973) Natural selection of parental ability to vary the sex ratio of offspring. Science 179:90–92

Wade MJ, Shuster SM, Demuth JP (2003) Sexual selection favors female-biased sex ratios: the balance between the opposing forces of sex-ratio selection and sexual selection. Am Nat 162:403–414

Wild G, West SA (2007) A sex allocation theory for vertebrates: combining local resource competition and condition-dependent allocation. Am Nat 170:E112–E128

Wright SL, Crawford CB, Anderson JL (1988) Allocation of reproductive effort in *Mus domesticus*: responses of offspring sex ratio and quality to social density and food availability. Behav Ecol Sociobiol 23:357–365

Young DAB (1976) Breeding and fertility of the Egyptian spiny mouse, *Acomys cahirinus*: effect of different environments. Lab Anim 10:15–24