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Využití genetické variability pro účely konzervační biologie: příkladové studie populací v lidské péči a přírodě

Dizertační práce

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Prohlášení:

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Abstrakt

V této dizertační práci jsem se věnovala převážně tématu ochrany vzácných druhů a to ve třech projektech realizovaných ve spolupráci se ZOO Praha. Další dva dílčí projekty, které jsou součástí této dizertační práce, se věnují tématu analýzy genetické diverzity v přírodě a vznikly ve spolupráci s institucemi v Indonésii a Arménii. Všechny tyto projekty jsem řešila pomocí obdobné laboratorní a výpočetní metodiky, ačkoliv každý projekt řesil odlišné problémy. Ve stěžejním, a asi i nejdůležitějším projektu jsem zabývala scinky rodu *Tiliqua* žijícími na Molukách, Nové Guinei (Irian Jaya) a přilehlých ostrovech. Analýzou mitochondriálních a jaderných znaků jsem řešila jejich vzájemné fylogenetické a populační vztahy. Druhá práce byla zaměřená na studium *ex situ* populace *Mauremys annamensis* v Evropských chovech, určovala jsem haplotypovou diverzitu a detekovala F1 hybridy v populaci. Ve třetí práci jsem zkoumala genetickou variabilitu pro potřeby organizace *ex situ* chovů *Orlitia borneensis* u jedinců chovaných v Evropských zoologických zahradách. Čtvrtá práce se zabývala výzkumem genetické variability populace *Chilabothrus angulifer* v Evropských chovech, zabývala se otázkou dlouhodobého přežití této populace. V poslední páté práci jsem za použití genetických metod identifikovala druhy, sekvenční a haplotypou diverzitu sympatrických druhů z rodu *Apodemus* (*Sylvaemus*) v Arménii.

Ve všech pracích používám hlavně mtDNA, která byla ještě v minulém tisíciletí používána jako univerzální marker. Na použitých příkladech ukazuji, že mtDNA je stále využitelná zpravidla v kombinaci s dalšími znaky (v mém případě sekvencemi vybraných jaderných genů). Ačkoliv jsem použila podobné molekulární a analytické postupy, každá z dílčích studií si vzhledem k odlišné povaze problému a zjištěné genetické variability, vyžádala výrazně odlišnou interpretaci výsledků. Proto je tato práce i příkladem využití rozdílných interpretačních schémat.

Abstract

In this dissertation thesis, I focused mainly on the topic of endangered species conservation in three projects, which were realized in cooperation with Prague ZOO. The other two subprojects, which are part of this dissertation, deal with the topic of genetic diversity analysis in nature and were realized in cooperation with institutions in Indonesia and Armenia. In all these projects I used a similar laboratory and computational methodology, although each project addressed different problems. In the main and probably the most important project, I studied the skinks of genus *Tiliqua* living in the Moluccas, New Guinea (Irian Jaya) and adjacent islands. I analyzed mitochondrial and nuclear markers to reveal the phylogenetic and population relationships. In the second project, I focused on the study of *ex situ* population of *Mauremys annamensis* in European captive breeding. The goal was to determine the haplotype diversity and detect the F1 hybrids in population. In the third project, I examined the genetic variability of *Orlitia borneensis* in European zoological gardens for the purposes of *ex situ* breeding program organization. The fourth project is focused on examination of genetic variability of *Chilabothrus angulifer* population, which is kept in European breeding. I studied possible long-term survival of this population. In the last fifth project, I used the genetic methods to identify the species, examine the sequence and haplotype diversity of sympatric species from genus *Apodemus* (*Sylvaemus*) in Armenia.

In all projects, I mainly use the mtDNA, which was considered as a universal marker in the last millennium. In the projects was confirmed, that using of mtDNA is still useful, especially in the combination with other markers (in my works with nuclear genes sequences). Although I used similar molecular and analytical approaches, each of the sub-studies interprets the results in a significantly different way, considering the diversity of studied problems and genetic variability. Therefore, this thesis is an example of the use of different interpretation schemes.

Obsah

1. Úvod do problematiky a cíle práce

V této dizertační práci se věnuji převážně genetickým aspektům tématu ochrany vzácných druhů. Je to výzkumný program, který se poprvé prosadil v roce 1982 (Schonewald-Cox et al. 1982, pro přehled viz Avise 2014). Od té doby jeho význam progresivně rostl a řada jeho výsledků je rutinně uplatňována v druhové ochraně (Frankham et al. 2002, 2010). Dle Frankhama (2010) je ochranářská genetika užitou vědou, která naráží na postatný rozpor mezi tím, co je technologicky možné a tím, co je v reálné situaci z finančních a praktických důvodů skutečně proveditelné. Pro *ex situ* projekty se alespoň omezené zapojení genetických přístupů stalo nezbytností (Witzenberger & Hochkirch 2011).

Tři z pěti dílčích projektů této disertace jsou založeny na projektech, které vznikly ve spolupráci se ZOO Praha. Výzkumné cíle vznikly na základě požadavků a možností této instituce. Pro potřebu *ex situ* projektů mezinárodně koordinovaných pracovníky zoo, které zároveň zajistily potřebný genetické vzorky z evropských zoologických zahrad. Tyto projekty se týkaly dvou kriticky ohrožených druhů, želv annámských (*Mauremys annamensis*), orlícií bornejských (*Orlitia borneensis*), jako i ohroženého druhu hroznýšovce kubánského (*Chilabothrus angulifer*). Zbylé dva projekty, týkající se scinků rodu *Tiliqua* a myšic rodu *Apodemus*, vyplynuly ze spolupráce s institucemi v Indonésii a Arménii. Jsou zaměřeny spíše na analýzu genetické diversity v přírodě. V prvním případě mohou výsledky posloužit zejména vymezení ochranářských jednotek (conservation units) druhů, které byly v minulosti do Evropy často ve velkých počtech importovány, ale *ex situ* programy i z důvodu nejasné taxonomie a populační struktury zatím nevznikly.

1.1. *Ex situ* versus *in situ* přístupy

Současný koncept doporučuje koordinovaně využívat, jak *ex situ*, tak i *in situ* přístupy. Jsou totiž komplementární. Doporučuje se je kombinovat dle konkrétních potřeb ochrany jednotlivých druhů (IUCN/SSC, 2014). Pojmem ex situ máme na mysli záchranné chovy nebo zvířata chovaná v zoologických zahradách či jiných chovech. Za tímto účelem jsou vedené plemenné knihy, pomocí kterých je pravidelně sledován vývoj populací chovaných v lidské péči. Každá plemenná kniha obsahuje kromě aktualizovaného přehledu jednotlivých chovů i řadu genetických či populačních analýz daného druhu (či alespoň podkladů pro takové

analýzy), díky kterým můžeme snáze vyhodnocovat životaschopnost populace a lépe naplánovat i případné změny ve složení chovů. Ohrožené druhy jsou v lidské péči chovány tak, aby byla zachována co největší genetická diverzita a minimalizováno příbuzenské křížení (Ralls & Balou, 1986; Pelletier et al. 2009; Frankham et al. 2010). *Ex situ* záchranné chovy mohou být užitečné zejména v případech, kdy je populace daného druhu v přírodě oslabena, ať už náhlou událostí (infekční onemocnění, přírodní katastrofy, či jiné) nebo setrvalým poklesem počtu jedinců, a není schopná přežití ve svém přirozeném prostředí (Bowling & Ryder 1987, Boyd et al. 1988, Wakefield et al. 2002). Pokud počet jedinců v populaci výrazně poklesne, klesne i genetická diverzita v rámci populace a může se začít projevovat inbrední deprese. V takovém případě mohou jedinci držení ex situ pomoci obohatit genofond populace v přírodě (Johnson et al. 2010). Tok genů mezi přírodními a ex situ populacemi je ovšem zapotřebí regulovat. Známe totiž řadu příkladů, kdy kromě benefitu může introgrese z chovů vytvořit i problém v podobě šíření nežádoucích alel. Taková situace byla popsána například u populace divokých kachen *Anas platyrhynchos* (Söderquist et al. 2017).

1.2. Proč jsem použila hlavně mitochondriální geny?

Příkladové studie, obsažené v této dizertační práci byly publikovány v širokém rozpětí mezi lety 2012 a 2021 (disertační projekt začal v roce 2011), podstatná část laboratorních výsledků vznikla ještě v době před rozvojem genomických metod, přesněji řečeno před tím, než se tyto techniky staly běžně dostupnými v našem prostředí (míním tím i dostupnost cenovou v relaci k objemu prostředků grantů, ze kterých byl výzkum financován). Mimo jiné i z toho důvodu nejsou genomické metody zahrnuty do řešení stanovených cílů. Ve světovém měřítku se ovšem hlasy po zavedení genomických přístupů začaly ozývat opakovaně v roce 2010 (Allendorf et al. 2010, Ouborg et al. 2010) a v podobě spíše teoretických deklarací dokonce i o něco dříve (Kohn et al. 2006, Primmer 2009).

Pokusím se však ukázat, že analýza mtDNA je pro některé třídy případů doposud vhodným praktickým nástrojem pro studium genetické variability *ex situ* a *in situ* populací i pro další ochranářská doporučení. Analýza mtDNA nabízí některé výrazné výhody. U obratlovců se mtDNA primárně dědí po mateřské linii, potomci obou pohlaví dědí mitochondrie od matky (Giles et al. 1980, Birky 2001, Rokas et al. 2003, Stewart & Chinnery 2015). Během embryogeneze je mitochondrie obsažená ve spermii buď zničena nebo inaktivována (Cummins et al. 1997, Cummins et al. 1998). Ovšem i přes popsaný

mechanismus dědičnosti mtDNA po mateřské linii, byly popsány případy, kdy došlo k částečné dědičnosti mtDNA z otcovy strany u slepic (Alexander et al. 2015), u myší a ovcí (Shitara et al. 1998), nebo dokonce i u lidí (Schwartz & Vissing 2002, Bromham et al. 2003, Luo et al. 2018). Ovšem tyto případy jsou extrémně vzácné. Ve většině případů mtDNA předávána bez rekombinace (Barr et al. 2005). Další přednost byla popsána již v práci od Brown et al. 1979, která přišla s objevem překvapivě vysoké evoluční rychlosti mtDNA – zhruba 2% sekvenční divergence za milion let u vyšších primátů.

1.3. Cíle práce

Tato dizertační práce zahrnuje pět příkladových studií, které používají obdobnou laboratorní i výpočetní metodiku k řešení odlišných problémů. Na jedné straně tak připomínají univerzální použitelnost analýzy sekvencí mitochondriálních genů. Na druhou stranu, poukazují na třídy případů, kdy se sekvence mitochondriálních genů i v současnosti mohou uplatnit, buď samostatně nebo v kombinaci s jinými daty (v mém případě sekvencemi vybraných jaderných genů či morfometrií). Každá z těchto tříd případů však vyžaduje úplně jinou interpretaci získaných výsledků. Odlišnost těchto interpretačních schémat pak dokládá, že zdánlivě rutinní analytické postupy jsou jen prvním krokem pro vhodné vyřešení konkrétních otázek, na které měly výzkumné projekty zahrnuté v této v disertační práci odpovědět.

Navíc k výše uvedeným cílům spíš koncepční povahy, má tato práce i celou řadu dílčích cílů vyplývajících z toho, že podnětem k jednotlivým projektům byly také potřeby praktické ochrany zkoumaných druhů. Hlavní z nich jsou:

1) Vymezení druhů, populací (ochranářských jednotek) a jejich vzájemných fylogenetických a populačních vztahů mezi scinky z rodu *Tiliqua* žijícími na Molukách, Nové Guinei (Irian Jaya) a přilehlých ostrovech.
2) Detekování F1 hybridů a určení haplotypové diverzity *ex situ* populace *Mauremys annamensis* v Evropských chovech.
3) Je *ex situ* populace *Orlitia borneensis* v Evropských chovech geneticky homogenní?
4) Je genetická variabilita *ex situ* populace *Chilabothrus angulifer* v Evropských chovech dostatečná pro dlouhodobé přežití populace? A není až příliš velká?

5) Identifikace druhů, sekvenční a haplotypové diverzity sympatrických druhů z rodu *Apodemus* (*Sylvaemus*) v Arménii.

2. Současný stav poznání

2.1. Vlastnosti mtDNA

Mitochondriální genom je prstencovitá molekula DNA (deoxyribonukleová kyselina), která se nachází v matrix semiautonomní organely – mitochondrie. Je tvořena dvěma řetězci (Zou et al. 2020). Vnitřní řetězec se označován lehký (L, light strand), vnější řetězec je označován těžký (H, heavy strand), protože obsahuje více guaninu, díky kterému má vyšší molární hmotnost (Yan et al. 2019). Toto označení se promítá do označení existujících primerů pro amplifikaci mtDNA markerů. Mitochondriální DNA můžeme najít i mimo mitochondrii – jako jaderno-mitochondriální sekvence (NUMTs) v jaderném genomu. NUMTs vznikly zabudováním části genetické informace mitochondrií do jaderného genomu (Stewart a Chinnery 2021). Obvykle se z nich v další evoluci stávají nukleární pseudogeny. Mohou ovlivňovat spolehlivé určení mitochondriálních haplotypů (Sturk-Andreaggi et al. 2022). Například v lidském genomu, podle Dayama et al. 2014, jsou NUMTs celkem recentního původu, většina z nich vznikla v uplynulých 100 000 letech. Také vznikají s vyšší četností na konktrétních pozicích mitochondriálního genomu – v oblasti 12,612–13,105 a 16,390–16,527 (Sturk-Andreaggi et al. 2022). Navíc většina NUMTs byla zastoupena s nízkou četností, což naznačuje, že proces vzniku stále probíná (Hazkani-Covo et al. 2010, Dayama et al. 2014). Každopádně je nutné s touto možností během analýzy mtDNA počítat. Alespoň částečně jsou odhalitelné pomocí porovnáním forward a reverse sekvence.

Mitochondriální DNA má vyšší mutační rychlost než jaderná. Nejspíše je to způsobeno absencí histonů kolem mtDNA, které by ji chránili (Bogenhagen 2012). Kromě toho mtDNA nemá vyvinuté opravné procesy, které jsou v buněčném jádře (Kennedy et al. 2013). U vyšších primátů byla pozorována evoluční rychlosti mtDNA zhruba 2% sekvenční divergence za milion let (Brown et al. 1979). Vzniklé mutace rozlišujeme podle frekvence jejich zastoupení v mitochondrii na heteroplazmické, které se vyskytují pouze v některých molekulách mtDNA v mitochondrii nebo homoplazmické, které jsou zastoupeny ve všech molekulách mtDNA v dané mitochondrii (Van Den Amaele et al. 2020).

Další nespornou předností mtDNA je, že u většiny živočichů se mtDNA dědí výhradně po mateřské linii, potomci obou pohlaví dědí mitochondrie od matky (Dawid & Blackler 1972; Hutchinson et al. 1974; Hayashi et al. 1978; Avise et al. 1979; Francisco et al. 1979; Giles et

al. 1980). Navíc je ve většině případů předávána bez rekombinace (Barr et al. 2005). Tyto vlastnosti na jednu stranu umožňují získat informaci o předcích z mateřské linie, což je výhodou při studii v zajetí chovaných potomků, jejichž rodič pocházel z přírody. Na druhou stranu nám ovšem analýza mtDNA nesděluje žádné informace o druhém rodiči, tedy není možné pouze na základě analýzy mtDNA odhalit hybridizaci či introgresi.

2.2. Obecné problémy *ex situ* ochrany

Dnešní vedení *ex situ* chovů naráží i na některé obecné problémy, se kterými se můžeme setkat. Nejčastějším z nich se budu věnovat v této kapitole.

1) Optimalizování počtu ochranářských jednotek (angl. conservation units) je významné jak pro *ex situ*, tak *in situ* přístupy. Zejména v případě *ex situ* projektů je stěžejní vyřešit, zda populace chovaná v zajetí je dostatečně geneticky variabilní, aby byla dlouhodobě udržitelná bez dramatického vlivu driftu a inbreedingu. Ukazuje se, že počet nepříbuzných zakladatelských jedinců musí být přinejmenším větší než 15 a velikost populace větší než 100 rozmnožujících se jedinců (Witzenberger & Hochkirch 2011). Na druhé straně je třeba z ochranářských jednotek vyloučit jedince hybridního původu (mezidruhové a mezi conservation units).

2) Pořízení nových jedinců z přírody je s ohledem na současné právní prostředí i vysoký formální stupeň ochrany v domovských zemích často zhola nemožné. Řada zemí exportní povolení jednoduše nevydává vůbec, jindy byrokratické bariéry i respektované instituce typu zoologických zahrad od dovozu odradí. Jediné populace, s kterými lze reálně pracovat jsou jedinci ze zoologických zahrad a soukromých chovů. Tato zvířata byla buď legálně dovezena v minulosti (v souladu s tehdy platnou legislativou) nebo pocházejí z konfiskátů. Jejich přesný původ je zpravidla nejasný, v prvním případě protože reprezentují již několikátou generaci potomků zakladatelských jedinců a rodokmen není vždy přesně znám, ve druhém případě díky nespolupráci původních importérů.

3) Velikosti populací držených v zoologických zahradách jsou extrémně malé (Marešová & Frynta 2008, Frynta et al. 2010a, Frynta et al. 2013), průměrně je

v zoologických zahradách celého světa sečteno dohromady chováno jen několik jedinců (v případě plazů je medián 12 jedinců od jednoho druhu – vypočteno jen z druhů skutečně chovaných alespoň v jedné zoo, Frynta et al. 2010b). Jen zanedbatelný počet druhů je tedy chován v počtech, které představují alespoň krátkodobou perspektivu z hlediska rizika vymření z důvodů demografických nebo negativních efektů genetického driftu a inbreedingu (O´Grady et al. 2006, Boakes et al. 2007, Hoeck et al. 2015).

4) *Ex situ* populace vznikly často z velmi malého počtu jedinců, mnohdy navíc příbuzných nebo špatně zvoleného složení (Brockman 1989). Simulace vývoje genetické variability ukazují, že pokud bylo na počátku více než cca 20 nepříbuzných genomů a následně došlo k prudké populační expanzi, nevede efekt hrdla lahve k fatálním důsledkům. Skutečně se podařilo založit přírodní populace jen z několika málo jedinců (kůň převalského - Bowling a Ryder 1987, Boyd et al. 1988, Wakefield et al. 2002). Populace byly ale mnohdy po generace drženy v malém počtu a tak došlo k významnému ochuzení počtu alel a snížení heterozygotnosti. Tyto procesy jsou nevratné, nedojde-li k novým importům. Ohrožení *ex situ* populací vlivem inbrední deprese je tedy ve většině případů naprosto zásadní (Hedrick et al. 1997).

5) Ne vždy je dostatečně vyřešena taxonomická příslušnost jedinců žijících v lidské péči. S tím souvisí potřeba vymezení taxonů a ochranářských jednotek. Zde se potkává nedostatečná znalost genetické variability v přírodě i lidské péči s koncepčními nejasnostmi (např. koncept druhu, definice conservation unit) a lidsky pochopitelnou snahou o vymezení a pojmenování co největšího počtu taxonů a populačních jednotek (Emerson & Wallis 1994, Hebert et al. 2004). Vysoký počet ochranářských jednotek sice účinně brání možnému outbreedingu, ale vede do opačné pasti fragmentace populací a inbreedingu. Náklady na oddělené držení a ochranu většího počtu jednotek tím neúnosně rostou. Tím spíše je třeba najít optimální kompromis podložený o relevantní analýzy

6) Mezidruhová hybridizace a introgrese mohou představovat nebezpečí genetické eroze pro populace v přírodě i lidské péči (Avise 2004). Detekce hybridů je tedy významným přínosem pro ochranu některých ohrožených druhů. Z opačného pohledu, občas můžou být hybridi chybně prohlášeni za vzácný druh, který je třeba chránit (Stuart & Parham 2007).

7) Genetická adaptace na podmínky v lidské péči. V lidské péči působí odlišné selekční tlaky a genetická adaptace a domestikace je tedy nevyhnutelná. Počet generací, po které by měly být populace drženy v lidské péči, by měl být co nejmenší (Williams & Hoffman 2009). To vše směřuje k potřebě integrovat *ex situ* a *in situ* přístupy do jednotné strategie.

8) Z veterinárních důvodů je potřeba jen opatrně přistupovat k žádostem o reintrodukce do přírody. Bez rizika rozšíření nežádoucích patogenů z chovů v lidské péči jsou introdukce tam, kde divoká populace v daném území již neexistuje (*Equus przewalskii* - Rüegg et al. 2006) nebo není bez takového zásahu životaschopná (*Puma concolor* - Johnson et al. 2010). Naopak v rámci přírodní populace mohou být rozšířeny určité patogeny, se kterými se jedinci z ex situ chovů nesetkávali a potom je potřeba přistupovat ke sloučení ex situ a in situ populací velmi opatrně (*Bison bonasus* - Cabaj et al. 2021).

2.3. Co je třeba vyřešit při formulaci otázek a interpretaci výsledků?

V následujícím přehledu se zaměřím na problémy, se kterými jsem se při práci na disertaci přímo setkala a které jsem v jednotlivých studiích řešila:

1) Hlavním problémem pro studie založené primárně na mtDNA je možný nesoulad mezi výsledky založenými na mitochondriálních a jaderných genech. Obecněji řečeno to, že evoluce jednotlivých genů nejsou vzájemně kongruentní a proto evoluce žádného z nich přesně nekopíruje evoluci druhu (gene tree nerovná se species tree). Musíme brát v úvahu procesy jako lineage sorting (Reich et al. 2010; Huerta-Sánchez et al. 2014; Mailund et al. 2014; Guerzoni & McLysaght 2016; Peyrégne et al. 2017; Skov et al. 2020; Schaefer et al. 2021; Teixeira et al. 2021). Část z těchto problémů odpadá při evoluci probíhající, jako sled vzácných kolonizačních událostí na nevelkých, ale dobře a dlouhodobě isolovaných ostrovech. Tam lze očekávat, že kolonizační historii popíše fylogeneze mtDNA věrně. Lze tedy snadno ohlídat, aby byly Conservation units důsledně monofyletické (holofyletické). Na druhou stranu i zde je obtížné odhadnout kolik ochranářských jednotek vymezit. mtDNA divergence je sice možno použít jako proxi pro divergenci genomu a predikci možné přítomnosti postzygoických RIM, výrazná outbrední deprese se však nemusí vytvořit ani po několika miliónech let isolace (Jančúchová-Lásková et al. 2015). V praxi bývají proto ochranářské jednotky i taxony na úrovni druhu či poddruhu vymezovány pragmaticky tak,

aby každý ostrov s výhledem na dlouhodobou isolaci byl považován za samostatnou jednotku i když je sekvenční divergence zanedbatelná. Možný teoretický argument opravňující k tomuto postupu představuje model zrychlené geografické speciace vedoucí prostřednictvím driftu, efektu hrdla lahve (bottle necking), efektu zakladatele (founder effect) a intensivního výběru (selekce) k závažným změnám morfologie, fyziologie a chování v některých případech velmi rychle (např. u ostrovních hroznýšů u pobřeží středoamerického kontinentu: Boback & Siefferman 2010; Card et al. 2016).

Vhodných, dlouhodobě isolovaných ostrovních systémů však na zemi není nekonečně. Velmi dobrým příkladem jsou Velké Antily, které se skládají z kontinentálních fragmentů navzájem od sebe isolovaných již po desítky miliónů let (i když právě největší z těchto ostrovů, tj. Kuba a Hispaniola, vznikly nedávnou kolizí čtyř respektive dvou takových fragmentů). Práce studující fylogenezi charizmatických antilských leguánů z rodu *Cyclura* naznačují, že kolonizace jednotlivých ostrovů byla skutečně jednosměrná v převládajícím směru mořských proudů (Malone et al 2000, Frynta et al. 2010c). Téměř stejný vzorec najdeme i v případě hroznýšovců z rodu *Chilabothrus* (Reynolds et al. 2013)*,* kam patří i *C. angulifer*, který je hlavním tématem jedné z prací zařazených do této disertace (Rehák et al. 2021). Na téměř opačném konci zeměkoule najdeme Wallaceu. Území mezi Weberovou a Lydekkerovou linií, odkud pocházel náš materiál scinků z rodu *Tiliqua.* Jsou to ostrovy zároveň dlouhodobě existující a nikdy nespojené s oblastí Sahulu a Velkých Sund (Voris 2000). V této oblasti byl až dosud prokázán jen omezený počet kolonizací pozemními obratlovci. Mohli jsme proto důvodně předpokládat, že fylogenetický vzor získaný z mitochondriálních genů bude nejspíše souladný se zbytkem genomu (Frynta et al. 2021).

2) Další situace, která může nastat, je, pokud empiricky zjistíme, že sekvenční divergence mtDNA je velmi nízká. V takovém případě lze předběžně dovodit, že by zkoumané OTU (angl. Operational taxonomic unit) , populace či jedinci mohly tvořit jedinou ochranářskou jednotku. Samozřejmě předběžně, protože je třeba vyloučit případy zásadního nesouladu mezi vzory variability jaderného a mitochondriálního genomu (Toews & Brelsford 2012). Varující jsou v tomto smyslu případy introgrese mitochondriálního genomu (Peters et al. 2007; Macholán et al. 2011; Ďureje et al. 2012). K fixaci mtDNA může dojít i pod vlivem silné selekce (Elson et al. 2004). Proto je doporučeníhodné výsledky mitochondriálních

markerů potvrdit i jadernými markery. V našem případě jsme ve studii *Orlitia borneensis* (Palupčíková et al. 2012) analyzovali kromě mtDNA markerů i jaderný marker R35. S podobným přístupem kombinujícím analýzu mtDNA a jaderné DNA se setkáváme v mnoha publikovaných pracích z nedávných let, které studují jiné druhy plazů (Kornilios et al. 2020, Zhao et al. 2020, Campillo-García et al. 2021). V naší práci o *Orlitia borneensis* jsme identifikovali tři haploskupiny, které se od sebe geneticky odlišovaly jen velmi málo a nebyly podpořeny rozdíly v jaderném genu. Neměli jsme tedy žádný dostatečný důvod opravňující navrhnout rozdělení populace na dílčí jednotky.

Existuje ovšem celá řada prací, kdy autoři doporučují rozdělit zkoumaný druh na několik ochranářských jednotek, i když pozorovaná sekvenční divergence je zcela minimální. Příkladem mohou být populace *Theropithecus gelada* v Etiopii, kde pomocí mtDNA markerů bylo identifikováno pět haploskupin (Zinner et al. 2018), které se ale od sebe příliš geneticky nelišily (k jejich rozdělení došlo před 0.67 až 0.43 miliony let). Vzhledem k nepříliš objasněným fylogenetickým vztahům mezi těmito skupinami bylo doporučeno další zkoumání k tomu, které by potvrdilo vhodnost chovat tento druh ve třech ochranářských jednotkách (severní, centrální a jižní populace zvlášť) (Shotake et al. 2016). Důvodem pro takové rozhodnutí je, že haploskupiny byly jasně geograficky vymezené. Ovšem i tak je takové rozhodnutí problematické, protože hlavním ohrožením ohrožených druhů je spíše inbreeding než outbreeding (Frankham 2010).

3) Třetím případem, se kterým jsem se během vypracovávání dizertační práce setkala, je vysoká sekvenční divergence přítomná v populacích evidentně dlouhodobě oddělených geograficky či postzygotickými RIM. A tedy tvořících dobré druhy dle většiny konceptů druhu, ovšem určitelné podle jiných než molekulárních znaků jen obtížně. V takovém případě je mtDNA ideálním nástrojem a levným pro druhovou identifikaci. Z prací zahrnutých v této disertaci je to případ rozlišení mezi čtyřmi příbuznými druhy rodu *Apodemus* žijícími v Zakavkazí (Balasanyan et al. 2018).

Pro řešení těchto cílů se nejčastěji používal barcoding, tj. krátké (600 – 800 bp) sekvence obvykle COI (Moritz 1994). Původně byl určen pro získání prvního vhledu do počtu druhů u dosud nepříliš prozkoumaných skupin. Dnes se používá spíše na doplnění informací u naopak dobře prozkoumaných druhů, kde známe celý kontext z jiných třeba genomických přístupů (Krishnamurthy a Francis 2012, Ahmed 2022). Místo aby se používala na druhy,

které potřebují více prozkoumat. Přes počáteční naděje, které doufaly v jednoduchou a univerzální metodu pro identifikaci druhů, se rozšířila povědomost o značných limitacích tohoto přístupu. Při použití pouze DNA barcodes dat bez dalšího kontextu, získáme pouze zlomek informace nezbytné pro správné vyhodnocení conservation units (Rubinoff 2006). Řešením problémů je využívat i jiné mitochondriální geny, jaderné markery, multilokusové a dnes také genomické přístupy.

4) Dalším případem byla situace, kdy jsme se setkali s velmi hlubokou sekvenční divergencí mezi haploskupinami v rámci jednoho druhu s nepřerušeným areálem a zachovanou schopností plodného křížení. Tato divergence byla natolik hluboká, že odpovídala divergenci běžné mezi druhy v daném taxonu. V takovém případě bychom měli zpozornět. Pokud by šlo o dlouhodobě propojené a přitom ne extrémně početné populace, mělo by již dávno dojít ke koalescenci. Ta probíhá za počet generací řádově odpovídající efektivní velikosti populace (Kimura a Crow 1963). V populaci se budou nacházet pouze jedinci, které mají stejného matrilineárního předchůdce (Avise 2000). Pokud v přírodní populaci nedošlo ke splynutí původně izolovaných populací, ale dlouhodobě v ní přetrvává více mitochondriálních linií, může to být způsobeno několika důvody. Například mohla být populace dlouhodobě extrémně početná nebo dojít nedávno k významnému poklesu její velikosti. Kolísání početnosti totiž významně ovlivňuje počet generací, za které dojde ke koalescenci.

Pokud tedy v dlouhodobě izolované populaci doposud nedošlo ke koalescenci, většinou se jedná o případy, které jsou obvykle důsledkem hybridizace resp. splynutí populací v minulosti dlouhodobě či alespoň periodicky oddělených např. geografickou bariérou. Viz komplexní historie chřestýšů (Schield et al. 2015, 2017, 2018, 2019). V našem případě jsem se setkali s podobnou situací v případě *C.angulifer* (Rehák et al. in print) ale také u vietnamských želv druhu *Mauremys* annamensis (Somerová et al. 2015).

5) Pomocí analýzy mtDNA můžeme identifikovat vhodné jedince pro koordinaci chovů i mezi potomky původních zakladatelů. V případě, že zakladatelé již nežijí a část rodokmenů prvních generací již chybí, maternálně děděná nerekombinující mtDNA nám poskytuje možný

zdroj informací. Počet haplotypů (haplotype diversity) mitochondriální DNA je relevantním odhadem minimálního počtu maternálních zakladatelů. Příkladem taková práce je třeba studie *ex situ* populace *Chelonoidis elephantopus* (Russello et al. 2010), u jejichž jedinců byl původ neznámý. Potvrzení, že je genetická variabilita zakladatelů *ex situ* populace dostatečná je primárním motivem pro vznik práce Rehák et al. in print. Navíc, v případě matrilokálních druhů, jako jsou hadi, kde dispergujícím pohlavím jsou zpravidla samci, poskytuje maternálně děděný marker dokonce lepší predikci pro budoucí hledání geografického původu zakladatelských jedinců než jiné markery (Avise 1995).

6) Analýzou mtDNA je také možná rychlá detekce prvních generací mezidruhových hybridů mezi zakladateli *ex situ* populace a vyjasnění možného komplexního původu druhu, který byl v přírodě téměř úplně vyhuben.

Obecně je hybridizace v *ex situ* populacích nežádoucí. Ovšem současná tendence k taxonomickému dělení (splitting), za kterým následuje i někdy nadměrné vytváření dalších ochranářských jednotek, vede k závažným problémům. Tato tendence je především důsledkem odklonu od konceptu biologického druhu (Mayr 1942) a příklonu k jiným definicím druhu, především genetickému (genetic species concept, Baker a Bradley 2006). Geneticky variabilní a zjevně životaschopná populace zahrnující více jednotek je posléze prohlášena za hybridní a její podstatná část vyřazena ze záchranných programů. Příkladem mohou být i extrémně vzácná a v přírodě téměř vyhubená zvířata jako orangutani. Všichni jedinci orangutanů *Pongo pygmaeus* z Bornea a *Pongo abelii* ze Sumatry byly původně v *ex situ* chovech drženi jako jedna populace dohromady. Ale později se analýzou DNA zjistilo, že se jedná o dva odlišné druhy (Zhi et al. 1996).

Někdy je ale outbreeding spojen s výraznými postzygotickými reprodukčně isolačními mechanismy a vyřazení hybridů z chovu je velmi důležité. Příkladem je pozorovaná neplodnost u samců z rodu *Madoqua* chovaných v zajetí, která byla důsledkem nevhodného křížení kryptických chromozomálních linií (Ryder 1989). Hybridizace se tak stává postrachem záchovných programů (Witzenberger a Hochkirch 2011). Ne vždy je ovšem jisté, že je zcela nepřirozeným jevem. Naopak v posledních desetiletích máme k disposici množství příkladů dokládajících, že je hybridizace a introgrese jsou významnou součástí evoluční historie

mnoha druhů, včetně člověka (Huerta-Sánchez et al. 2014, Racimo et al. 2015, Dannemann et al. 2016, Mondal et al. 2019).

Některé taxony jsou nesmírně náchylné na hybridizaci morfologicky nepodobných a geneticky vzdálených forem. Želvy z čeledi Geoemydidae jsou v tomto ohledu extrémním příkladem (Galgon a Fritz 2002, Fritz a Mendau 2002, Fritz et al. 2004, Schilde et al. 2004, Spinks et al. 2004, Stuart a Parham 2004, Buskirk et al. 2005, Shi et al. 2008). Známe i četné příklady záměrného křížení těchto druhů na asijských komerčních farmách (Parham et al. 2001, Stuart a Parham 2004). U vietnamských želv z rodu *Mauremys* je hybrididní historie dosti pravděpodobná, ex situ populace totiž pochází právě z farem, protože byla v přírodě téměř zcela vyhubena (Fong et al. 2019). Naše práce (Somerová et al. 2015) měla za cíl přispět k vytipování možných hybridů i rozřadit zakladatelské jedince do již dříve identifikovaných haploskupin (Fong et al. 2007).

3. Přehled použitých laboratorních postupů

3.1. Způsoby odběru a zpracování vzorků

K izolaci DNA lze použít rozmanitý genetický materiál, který musí obsahovat buněčná jádra nebo mitochondrie s nedegradovanou DNA. Tento materiál můžeme získat několika způsoby. Destrukční způsob získává DNA odběrem tkání potřebných na genetické analýzy, kvůli kterým je živočich usmrcen. Nedestrukčním způsobem (invazivním) se odebírá vzorek tkáně (ideálně takové, při jejímž odběru nedojde ke zranění zvířete, například drápy), stěry bukální sliznice nebo vzorek krve z odchyceného živočicha. Poslední způsob získání DNA je neinvazivní, kdy jako genetický materiál využijeme zdroj DNA, který živočich po sobě zanechal, například trus, chlupy nebo svlečky. Díky tomuto způsobu není potřeba živočicha odchytávat ani s ním manipulovat.

V příkladových studiích jsme se snažili o co nejšetrnější způsob odběru DNA u všech žijících jedinců. Ve většině případů jsme se snažili získat DNA odběrem stěru bukální sliznice (*Chilabothrus angulifer*, komplex druhů *Tiliqua scincoides-gigas*). Tento způsob odběru je však zcela nemožné použít u želv, vzhledem k tomu, že želva v případě, kdy se cítí ohrožena, schová svojí hlavu do krunýře. Proto jsme u želv (*Mauremys annamensis*, *Orlitia borneensis*) přistoupili k jinému způsobu odběru vzorků DNA. A sice se nám jako vhodný zdroj DNA osvědčily drápy, u kterých sice byla nutná delší doba rozpouštění v proteináze, ale výtěžnost izolace DNA byla překvapivě velká. Ve studiích jsme analyzovali i uhynulé jedince, kde jsme použili jako zdroj DNA kousek prstu nebo špičku ocasu (*Apodemus*). Všechny odebrané vzorky DNA jsme uchovávali v čistém ethanolu. DNA jsme izolovali ve většině případů pomocí komerčně dodávaných kitů DNAeasy Tissue Kit (Qiagej, Hilden, Germany) nebo dříve izolované vzorky jsme zpracovávali pomocí NucleoSpin Tissue kit (Macherey-Nagel). Pokud bychom chtěli snížit náklady, můžeme izolaci provést například metodu využívající enzym proteinázu K a organická rozpouštědla (fenol a chloroform). Úspěšnost izolace DNA jsme ověřovali pomocí měření koncentrace na spektrometru.

3.2. Polymerázová řetězová reakce

Polymerázová řetězová reakce (polymerase chain reaction, PCR) umožňuje in vitro amplifikovat požadovaný specifický úsek DNA v neomezeném množství (Mullis et al. 1986, Mullis & Faloona 1987). K amplifikaci stačí velmi malé množství DNA (teoreticky stačí jedna molekula). PCR probíhá ve třech krocích – denaturace, nasedání primerů a extenze. Roztok pro PCR obsahuje DNA templát, směs všech čtyř nukleotidů dNTP, dvojici primerů, Taq polymerázu, komerčně dodávaný pufr, MgCl2 a kvalitní redestilovanou vodu.

Princip PCR je velmi jednoduchý, ale bohužel praxe tolik jednoduchá nebývá. Vzhledem k tomu, že v praxi vzájemně působí velké množství faktorů, které mohou ovlivnit finální výsledek. Zpravidla je nutné empiricky si ověřit nastavení hodnot jednotlivých parametrů (koncentrace iontů, teplotní řežim a další). Klíčovým pro úspěšnost PCR je správné vybrání primerů. Před samotnou PCR jsem si udělala průzkum literatury a DNA databází, jaké geny se zkoumaly u nejbližších příbuzných druhů a jaké primery se v daných studiích používaly. Zpravidla jsem se snažila najít blízce příbuzný druh, u kterého se zkoumaly mtDNA markery, které by se hodily pro námi plánované analýzy u jednotlivých příkladových studií. Poté jsem vyzkoušela gradientovou PCR s rozpětím teplot nasedání s vybranými primery dostupnými v literatuře na několika vzorcích. V žádné z příkladových studií se mi nestalo, že by PCR nefungovala kompletně u všech vzorků. Stávalo se, že byla v rámci jedné práce různá teplota nasedání pro stejné primery (*Apodemus*, *Orlitia borneensis*, *Tiliqua scincoides-gigas* komplex). V jednom případě jsme přistoupili k navržení vnitřních primerů, protože se nám nepodařilo podmínky PCR optimalizovat pro všechny studované vzorky (*Apodemus*). Primery jsem navrhli podle sekvencí vzorků, u kterých byla PCR úspěšná.

3.3. Sangerova metoda sekvenace

Frederick Sanger a kolektiv publikovali v roce 1977 metodu, pomocí které je možná analýza delšího úseku DNA. Sangerova (enzymatická) metoda („chain termination method“, „dideoxy method“) je založena na přidání dideoxynukleozidtriforsfátů (ddNTP) do PCR reakční směsy. ddNTP jsou analogy normálních deoxynukleozidtriforsfátů, ale chybí jim OH-skupina na 3‘-uhlíku deoxyribózy. Pokud se tyto ddNTP naváží na matrici zkoumané DNA, je díky chybějící OH-skupině nemožné navázat další nukleotid a dojde k terminaci PCR. Touto metodou je možné sekvenovat pouze ssDNA.

Značné zjednodušení a zrychlení přinášejí automatické sekvenátory, které jsou v dnešní době díky jejich rychlosti pro sekvenaci využívány výhradně. Většina těchto sekvenátorů je založena na metodě termálního cyklického sekvencování. Syntéza DNA probíhá v normálním termocykleru a principiálně je shodná s asymetrickou PRC s jedním primerem. Používá se fluorescenční značení ddNTP místo radioaktivního. Každý ddNTP má odlišnou barvu fluorescenčního značení a tím pádem již není nutné provádět sekvenaci za použití čtyř vzorků, ale reakce může probíhat pouze v jediném. Produkty jsou rozděleny během kapilární elektroforézy, během které zároveň probíhá detekce pomocí stacionárního laserového detektoru.

Ve všech příkladových studiích obsažených v této práci jsme využili Sangerovu metodu sekvenování pomocí automatických sekvenátorů. Vzhledem k tomu, že touto metodou je možné sekvenovat pouze ssDNA, vzorky jsme sekvenovali dvakrát pomocí každého z primerů, abychom měli spolehlivější sekvenační data.

4. Použité metody analýzy sekvenačních dat

Dle současných znalostí můžeme sekvenační data vyhodnocovat velkým počtem způsobů s ohledem na cíle analýzy.

Do souboru námi získaných sekvenačních dat použitých k vyhodnocení, můžeme přidat i data volně dostupná na některém ze světových úložišť. Existují tři nukleotidové databáze, které jsou veřejně dostuné a paralelně udržované v rámci International Nucleotide Sequence Database Collaboration ve Spojených státech, Evropě a Japonsku.

Nejznámější a nejpoužívanější databází je GenBank (Sayers et al. 2021), které je provozovaná v NCBI (National Center for Biotechnology Information, Bethesdam Maryland, USA), její webová adresa je: https://www.ncbi.nlm.nih.gov/genbank/. Tuto databázi jsem hojně využívala jak při přípravě metodiky našich studií, kdy jsme si dělala review dostupných sekvencí. Ale využívala jsme ji také i poté ke stažení sekvencí, které jsem přidávala do analýz společně s námi osekvenovanými vzorky. Ať už jako outgroups nebo k doplnění našich vzorků.

Velikost databáze se zvětšuje každým rokem. K srpnu 2020 obsahovala databáze GenBank více než 9.89 triliónů nahraných párů bazí z více jak 2.1 biliónů sekvencí od 478 000 formálně popsaných druhů (Sayers et al. 2021). Kromě databáze samotné jsem využívala i nástroj BLAST, který NCBI spravuje. BLAST je jeden z velmi užitečných nástrojů. Pomocí tohoto nástroje jsme schopní si u získané sekvence ověřit, kterému organismu patří.
V příkladových studiích jsme měli celkem konkrétní představu o tom, jaký organismuszkoumáme. Ale i tak jsem získané sekvence ověřovala pomocí BLAST. Abychom se ujistila, že během odebírání nebo zpracování vzorku nedošlo k jeho kontaminaci a nedopatřením jsem neosekvenovala jiný organismus, než bylo zamýšleno. Program našel sekvence s největší shodou, které byly v databázi dostupné, a pomohl mi s identifikací druhů.

K seřazení sekvencí se asi nejvíce používá některý z rodiny programů Clustal, například ClustaX nebo ClustaW (Thompson et al. 1997, Chenna et al. 2003), který má rozšířené grafické rozhraní a jako vstupní soubory dokáže zpracovat řadu formátů – Clustal, NEXUS, PHYLIP, FASTA a jiné. ClustalX využívá progresivní seřazování. Tyto programy jsem využívala i já pro seřazení sekvencí ve všech příkladových studiích ať už samostatně nebo jako součást jiných programů, například BioEdit (Hall 1999).

4.1. Fylogenetické stromy

Rekonstrukce fylogeneze je v podstatě odhad skutečné evoluční historie na základě informace, která je obsažená v získaných datech. Naše údaje jsou většinou omezené a jednotlivé znaky mohou poskytovat protichůdné informace, snažíme se o získání co nejlepšího odhadu. Tedy snažíme se najít fylogenetický strom, který se co nejvíce blíží skutečné fylogenezi. Výsledný strom je pouze hypotéza, kterou můžeme porovnávat s jinými hypotézami. Máme dvě možnosti, jak konstruovat fylogenetické stromy.

První možností rekonstrukce fylogeneze jsou metody založené na stanovení kritéria optimálnosti. Tyto metody mají dva kroky. V prvním kroku se definuje kritérium, podle kterého je ke každému stromu přiřazena určitá hodnota (skóre). Toto skóre se poté používá k vzájemnému porovnávání stromů. V druhém kroku se pomocí specifického algoritmu vypočítá hodnota funkce znázorňující kritérium optimálnosti a hledá se strom s nejlepší hodnotou této funkce. Evoluční předpoklady jsou tedy v prvním kroku a matematické řešení je v kroku druhém. Metody optimálnosti můžeme využít pro analýzu dat ve formě jednotlivých znaků, například pro metody maximální úspornosti (MP), maximální věrohodnosti (ML) nebo bayesovská analýza (BA). Ale můžeme pomocí metod optimálnosti hodnotit i data distanční, například metodou minimální evoluce. Vzhledem k tomu, že metody posuzují často velké množství alternativních stromů, může dosažení konečného výsledku trvat někdy i výrazně déle. Fylogenetický strom je výsledkem fylogenetické analýzy. V přiložených pracích jsem použila pouze zakořeněné stromy (rooted tree). K jejich výpočtu jsem používala výše zmíněné metody - maximální úspornosti (Maximum parsimony, MP), maximální věrohodnosti (Maximum likelihood, ML) nebo bayesovskou analýzu (BA). Výpočty jsem prováděla v programech PAUP* 4.0b10 (Swofford 2002), MrBayes v3.2.6 (Huelsenbeck et al. 2001; Ronquist et al 2012).

Další možností jsou algoritmické metody viz níže.

4.2. Haplotypové sítě

Haplotypové sítě jsou jiné grafické znázornění nezakořeněných stromů. K jejich výpočtu jsem používala algoritmické metody, které kombinují konstrukci stromu a definici „nejlepší“ fylogenie do jediného algoritmu. Jejich výpočet není díky své jednoduchosti náročný na čas.

Haplotypové sítě jsou dobrými ukazateli genetické vzdálenosti. Díky tvaru a topologii můžeme ohadnout minulé události v populaci jako bottle neck nebo recentní expanze (tzv. star pattern). K výpočtu haplotypových sítí v této dizertační práci jsem používala programy NETWORK (verze 4.6.0.0, 4.6.1.2, 4.6.1.4) a PopART (Leigh & Bryant 2015). Ve všech případech jsem použila k výpočtu Median-joining algoritmus.

4.3. Divergence p-distance

Pozorovanou distanci neboli p-distanci lze vypočítat pomocí vydělení počtu pozic, ve kterých se dvě sekvence od sebe liší, celkovým počtem nukleotidů. Bohužel tento výpočet může podhodnocovat skutečnou míru divergence mezi sekvencemi. Nezohledňuje totiž opakované substituce na stejné pozici. I přes toto omezení jsme p-distance použili u všech příkladových studií k porovnání s distancemi v rámci příbuzných taxonů.

Zároveň nám divergence mitochondriálních genů může poukázat na schopnost hybridizace mezi analyzovanými jedinci. Například mezi druhy v řádu šupinatých je pozorovaná hybridizace až do 18-21% divergence mezi sekvencemi cyt b (Jančúchová-Lásková et al. 2015a), výsledkem hybridizace byli alespoň částečně plodní potomci (Jančúchová-Lásková et al. 2015b). Stejně tak v čeledi Geoemydidae byly dokumentovány hybridizace i mezi velmi vzdálenými druhy patřícím do různým rodů (Parham et al. 2001; Stuart and Parham 2007; Fong a Chen 2010). Díky p-distanci jsme tedy mohli zohlednit i uskutečnitelnost hybridizace v rámci zkoumaných skupin ve vzorových studiích a tím pádem dát relevantnější doporučení ohledně chovatelského záměru.

4.4. Demografické charakterisky a Bayesssian skyline plot

Pomocí programu DnaSP v5 5.10.01 (Librado & Rozas 2009) jsem u studií počítala následující parametry: haplotypovou diverzitu (*h*), počet segregačních míst (S),

nukleotidovou diverzitu (π), Tajima's D, Fu & Li's F*, Fu & Li's D* a Fu's FS testy. Podle Russell et al. 2005 lze pomocí těchto údajů odhadnout, zda populace je konstantní velikosti (vysoká hodnota *h* a současně nízká hodnota π) nebo zda populace prošla nedávnou expanzí (nízká hodnota π a naopak vysoká hodnota *h*). K odhadu populační dynamiky jsme take používali Bayesian skyline plot, který jsme počítali pomocí program Beast (Drummond & Rambaut 2007).

4.5. Metody vymezování počtu druhů

Při vymezování počtu druhů obecně platí, že méně škody napáchá, pokud je vymezeno méně druhů nežli více (Carstens et al. 2013). Respektive vymezení jednotek, které nereprezentují správné evoluční linie, může napáchat nevratné škody v péči o *in situ* i *ex situ* populace. Je tedy vhodné vymezovat druhy pomocí širší škály analýz a důvěřovat takovým vymezením druhů, která jsou shodná napříč všemi použitými analýzami. Pokud nám různé analýzy dávají různá druhová vymezení, obvykle to značí nesoulad schopnosti detekovat kryptické druhy v rámci jednoho nebo více zvolených přístupů a mohlo by nám to naznačit, že předpoklady takových přístupů byly porušeny a tudíž se pro analýzu daného souboru dat nehodí. Ve studiích, které jsou součástí této dizertační práce, jsme se spoléhali převážne na vymezení druhů pomocí přístupu Bayesianské statistiky a Markov Chain Monte Carlo výpočtů, které jsou vhodné pro menší data sety (Zhang et al. 2013). Každopádně přístupy pro vymezení druhů jsou obecně dost problematické, jak již bylo shrnuto v dostupné literatuře (Carstens et al. 2013, De Queiroz 2007, Leliaert et al. 2014, Lim et al. 2012).

5. Přehled výsledků jednotlivých prací a jejich interpretace

5.1. Vymezení druhů, populací (ochranářských jednotek) a jejich vzájemných fylogenetických a populačních vztahů mezi scinky z rodu *Tiliqua* žijícími na Molukách, Nové Guinei (Irian Jaya) a přilehlých ostrovech.

Oblast Nové Guiney a přilehlých ostrovů Wallacey je na rozhraní Pacifické a Australské tektonické desky. Současná podoba Nové Guiney vznikla poměrně nedávno z několika samostatných ostrovů zhruba před pěti milióny let (Toussaint et al. 2014). Svojí rozlohou je Nová Guinea druhým největším ostrovem na světě, je rozdělena centrálním pohořím. Toto pohoří působí jako přirozená bariéra, která způsobilo rozděluje populace mnoha druhů obratlovců na severní a jižní linie (Pigram 1987; Dumbacher a Fleischer 2001; Rawlings a Donnellan 2003) a současně zastavilo to genů mezi těmito liniemi (Hall 2002). Během glaciálních maxim v Pleitocénu došlo k opakovanému poklesu hladiny moří a vznikly pevninské mosty, které spojily i v současné době oddělené ostrovy (Voris 2000). Nová Guinea byla připojena k Sahulskému šelfu a některé z přilehlých ostrovů s ní (Biak, Aru). Oproti tomu ostrovy mezi Wallaceovou linií a Lydekkerovou linií zůstaly trvale izolované i v době glaciálních maxim (Molucké ostrovy). I přes toto oddělení došlo k osídlení některých ostrovů jak už s Australského regionu tak i z Asijského (Koch 2012). Oblast mezi Weberovou a Lydekkerovou linií osídlila převážně herpetofauna z Nové Guiney (De Lang 2011).

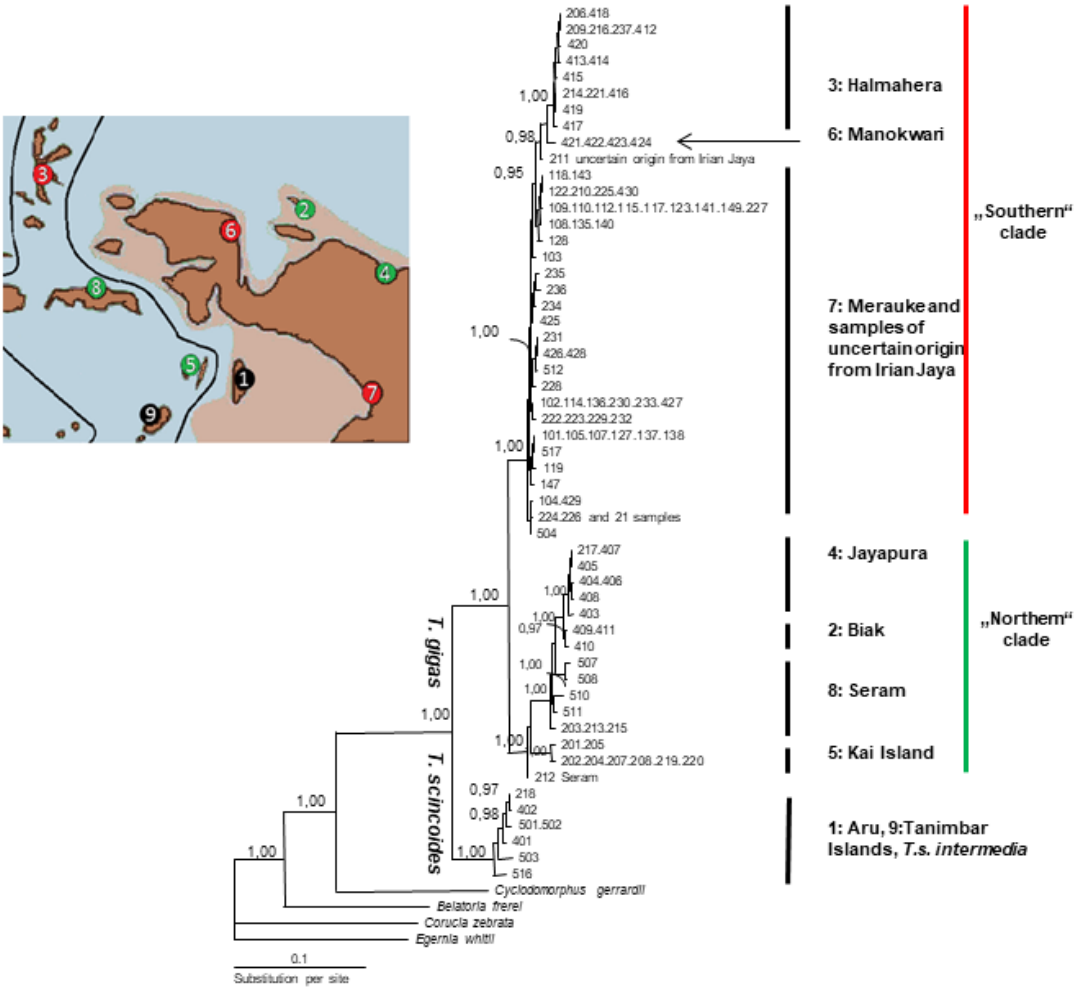
Obr. 1: *Tiliqua gigas* z lokality Merauke, Nová Guinea (foto Andran Abramjan)

Druhy z rodu *Tiliqua* (Scincomorpha: Lygosomoidea Mittleman, 1952), které žijí v Austrálii, jsou poměrně dobře prostudované. Ale druhy tohoto rodu na Nové Guinee a ostrovech Wallacey byly popsány pouze na základě morfologických znaků (Shea 1992, 2000; Hitz and Hauschild 2000; Noel 2014), ale doposud nebyly publikovány žádné práce studující genetické znaky u těchto populací. Námi studovanou oblast obývají podle informací z dostupné literatury dva druhy – *T. scincoides* a *T. gigas*. V rámci každého z obou druhů byly popsány tři poddruhy. *T. gigas gigas* (Schneider, 1801) který byl nalezen na centrálních Moluckých ostrovech (Morotai, Ternate, Halmahera, Ambon, Seram), Irian Jaya (včetně ostrovů Biak, Japen a Doom). Poddruh *T. gigas evanescens* Shea 2000 byl popsán na jihovýchodě Nové Guiney, ale podle Hitz a Hauschild (2000) se tento poddruh nachází i v blízkosti Merauke a na ostrovech u východního a severovýchodního pobřeží Nové Guiney. Výskyt *T. gigas keyensis* Oudemans, 1894 byl popsán na ostrovech Kai a Aru (Shea 2000; Hitz a Hauschild 2000). Na ostrově Tanimbar a Babarských ostrovech byl popsán poddruh *T. scincoides chimaerea* Shea, 2000. Zbývající dva poddruhy *T. scincoides - T. s. scincoides* (White, 1790) a *T. s. intermedia* Shea, 2000 jsou rozšířeny na jihovýchodě, východě a severu Autrálie (Unverzagt 2004; Cogger 2000).

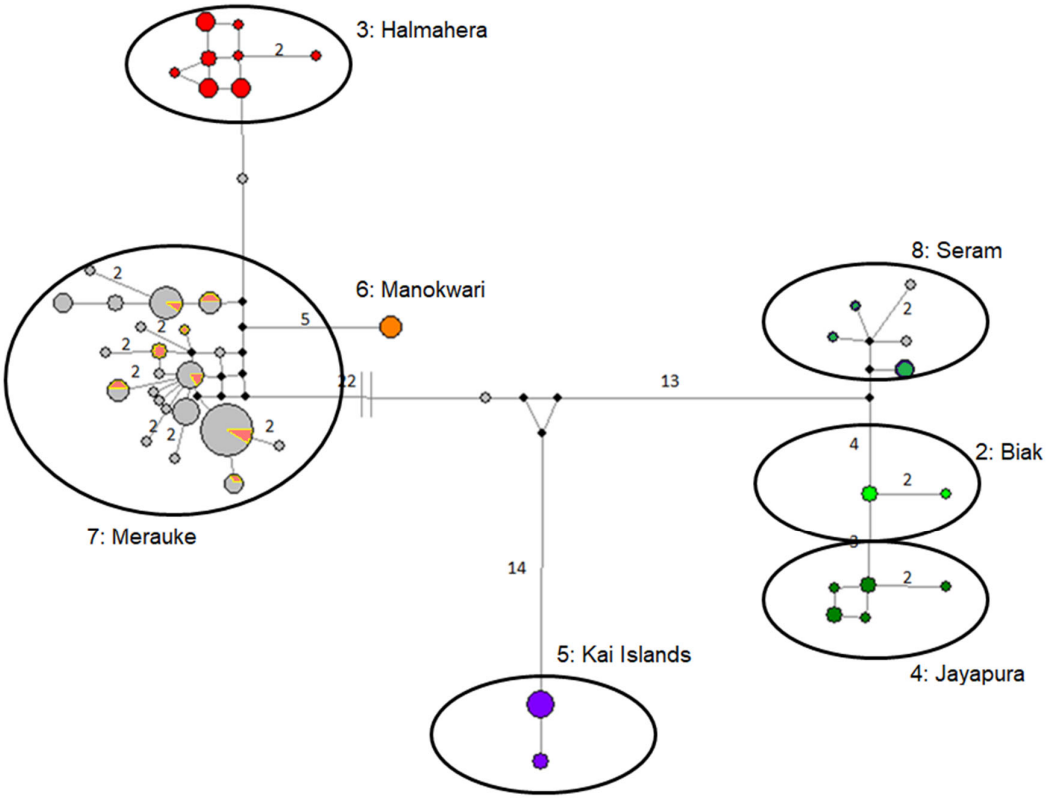
Vzhledem k historii studované geografické oblasti by šlo předpokládat, že kolonizace ostrovů probíhala pouze ojedinělými náhodnými událostmi a zpětný tok genů v populaci byl minimální, pokud vůbec nějaký byl. Z toho důvodu by měla být analýza mtDNA příhodně zvoleným způsobem řešení otázky fylogeneze studovaných druhů z rodu *Tiliqua*. Ale protože Melanézie je lidmi dlouhodobě silně osídlená oblast s poměrně intenzivním tradičním obchodem mezi ostrovy, nelze úplně vyloučit, možnost introdukce lidmi.

V článku, který je součástí této dizertační práce, jsme zkoumali 128 jedinců z rodu *Tiliqua*. Sekvenovali jsme mitochondriální ND4, 12rRNA a jaderný gen cmos. Díky fylogenetické analýze jsme objevili dvě skupiny v rámci zkoumaných jedinců. Tyto dvě skupiny korespondují s druhy *Tiliqua scincoides* a *Tiliqua gigas*. Jako první jsme dokumentovali přítomnost Tiliqua scincoides na ostrově Aru, popsali její blízký genetický vztah k populaci *Tiliqua scincoides chimaerea* z ostrova Tanimbar. Ostatní zkoumaní jedinci patřili do druhu *Tiliqua gigas*. Avšak i v rámci jedinců patřících do druhu *T. gigas* jsme objevili distinktní phylogenetickou strukturu, která je v souladu s geografickým původem jedinců. V populaci *T. gigas* jsme identifikovali dva klády, které podporují rozdělení populací na severní

a jižní, jež bylo popsáno i u jiných druhů obývající danou oblast (Donnellan a Aplin 1989; Harvey et al. 2000; Rawlings a Donnellan 2003; Austin et al. 2010). Do jižního kládu byli rozděleni jedinci z jižní Nové Guinee, Bird's Head poloostrova a ostrova Halmahera. Jedinci z Halmahery tvořili oddělenou větev uvnitř tohoto kládu. V severním kládu se nacházeli jedinci ze severu Nové Guiney a překvapivě také jedinci z ostrovů Kai a Seram, které přiléhají k jihu. Rozdělení severního kládu podporovalo dříve popsaný výskyt *T. gigas keyensis* poddruhu na ostrovech Kai (Shea 2000; Hitz a Hauschild 2000). Pomocí kontrukce haplotypové sítě mitochondriálního ND4 genu jsme znázornili výrazné odlišení jednotlivých skupin populací.



Obr. 2: BA fylogenetický strom kontruovaný za použití kombinovaného alignmentu ND4, 12S rRNA a cmos o celkové délce 1644 bp, jsou zobrazeny pouze posteriorní pravděpodobnosti alespoň 0.95 a vyšší (Frynta et al. 2021)



Obr. 3: Haplotypová síť vypočítaná z alignmentu ND4 o délce 825 bp pomocí Median-Joining algoritmu. Šedá barva označuje vzorky s nejistou lokalitou původu, odstíny červené jižní klád, odstíny zelené severní klád.

Poznámka: Tato studie je nejdůležitější prací mojí dizertace – podílela jsem se na sběru genetického materiálu, veškerou experimentální práci jsem navrhla a vypracovala zcela samostatně, prováděla jsem všechny výpočty a analýzy, podílelala jsem se významnou měrou na sepisování odborného článku. Jsem uvedena jako korespondenční autor, zajišťovala jsem předložení článku a nezbytné revize.

5.2. Detekování F1 hybridů a určení haplotypové diverzity ex situ populace *Mauremys annamensis* v Evropských chovech.

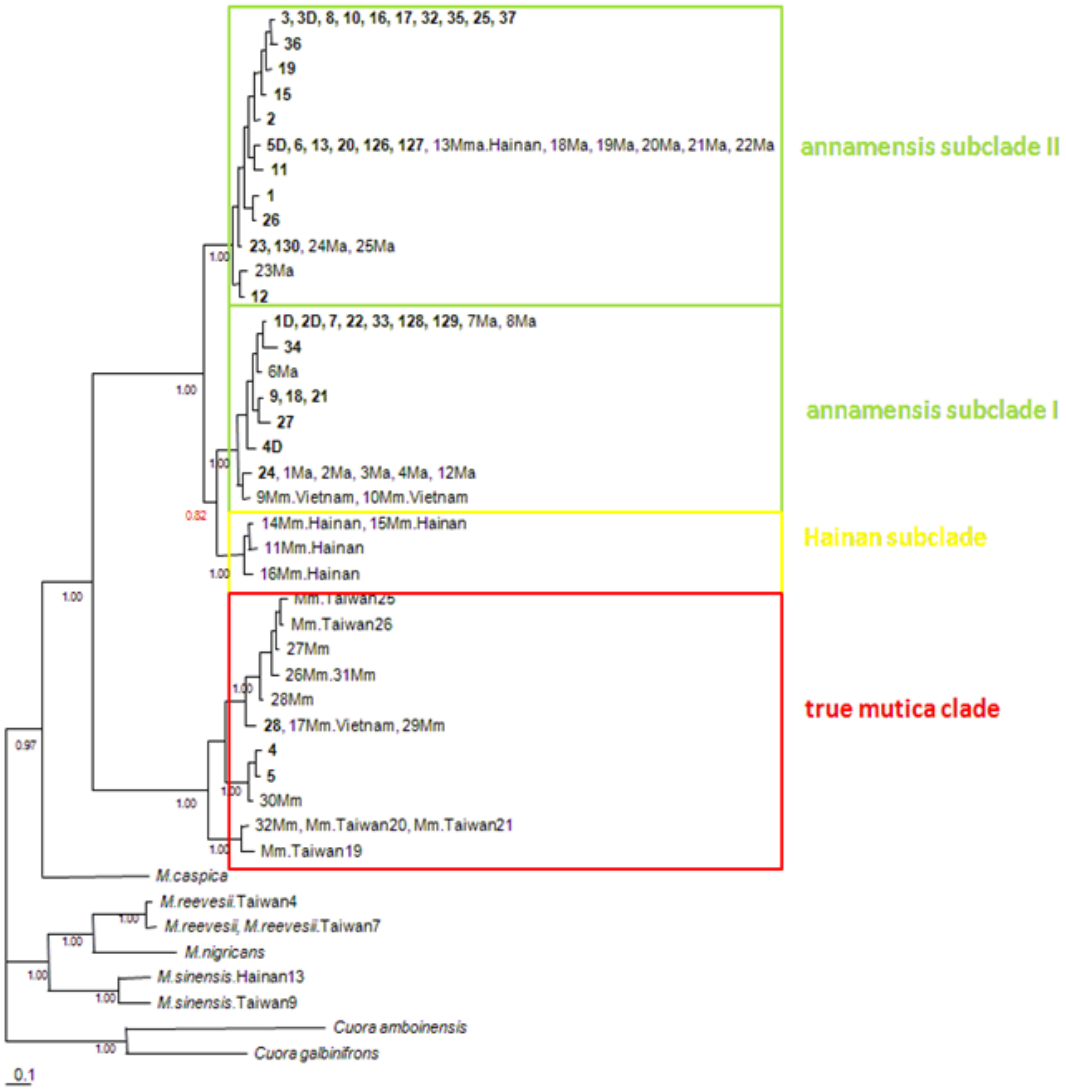
Želví populace v Asii čelí v současné době vyhynutí v důsledku ničení jejich přirozeného prostředí výskytu a vysoké poptávky po želvím mase na čínském trhu (Van Dijk et al. 2000, Le

et al. 2004, Cheung & Dudgeon 2006, Stanford et al. 2019). Populace druhu *Mauremys annamensis* (Annam leaf turtle) z čeledi Geoemydidae byla poměrně dlouhou dobu považována za vyhynulou v přírodě, ale v tomto tisíciletí bylo popsáno několik fragmentovaných populací v přírodě středního Vietnamu (Le et al. 2004, Parham et al. 2006, Fong et al. 2019). Dnes patří *M. annamensis* mezi 25 nejohroženějších druhů želv (Fong et al. 2019, Standford et al. 2019), je vedená jako kriticky ohrožená dle IUCN (McCormack et al. 2020). Z toho důvodu jsou jedinci chovaní v zajetí nesmírně důležití. *Ex situ* populace jsou chované v Evropských zoologických zahradách, USA a Vietnamu. Tyto populace jsou nesmírně důležité pro reintrodukční programy. Správné vedení chovu je tedy naprosto nezbytné. Možnost odchovu v *ex situ* populacích byla zdokumentována případy úspěšného rozmnožení tohoto druhu v lidské péči (Velenský 2006, Raffel & Meier 2013).

Vzhledem k tomu, že v čeledi Geoemydidae je pozorovaná celkem běžná hybridizace a to i mezi vzdálenými druhy (Galgon & Fritz 2002, Fritz & Mendau 2002, Fritz et al. 2004, Schilde et al. 2004, Spinks et al. 2004, Stuart & Parham 2004, Buskirk et al. 2005, Shi et al. 2008), může to ohrozit správné vedení *ex situ* chovů. Nejbližším příbuzným druhem je *M. mutica* (Barth et al. 2004, Feldman & Parham 2004, Spinks et al. 2004), s kterou *M. annamensis* běžně hybridizuje (Fong et al. 2007). Proto je nezbytně nutné pomocí genetických analýz jedince hybridního původu v ex situ chovech odhalit a z dalších chovů je vyloučit.

Ve studii jsme zkoumali 39 jedinců *M. annamensis* a 4 jedince *Mauremys mutica* z Evropských zoologických zahrad a dalších Evropských chovů. Jedince *M. mutica* jsme přiřadili z důvodu možného srovnání s *M. annamensis.* U všech jedinců jsme sekvenovali mitochondriální ND4 gen a jaderný R35 intron. Tyto markery byly použity k fylogenetickým analýzám v již publikovaných pracích (Stuart & Parham 2004; Fujita et al. 2004, Fong et al. 2007). Pomocí analýzy jaderného markeru R35 bylo možné jednoznačně odlišit M. annamensis od *M. mutica*. Data získaná analýzou mitochondriálního markeru ND4 potvrdila výskyt dvou linií v rámci druhu *M. annamensis*, které byly popsány v dříve publikovaných pracích (Fong et al. 2007; Fong 2008). Analýzou obou markerů jsme potvrdili, že v rámci námi zkoumaných jedinců identifikovaných jako *M. annamensis* není žádný hybridní jedinec první filiální generace. Pomocí použitých metod bylo nemožné vyloučit přítomnost hybridních jedinců vyšších generací, ale vzhledem ke generační době zkoumaného druhu, věku jedinců a

jejich původu, jsme dospěli k závěru, že přítomnost takových hybridů je značně nepravděpodobná.



Obr. 4: BA fylogenetický strom konstruovaný z 817 bp dlouhého alignmentu ND4, tučným písmem jsou označeny vzorky sekvenovány v Somerová et a 2015. Pokud byla známá lokalita původu, je napsaná přímo u označení větví. (Mm = *Mauremys mutica*, Ma = *Mauremys annamensis*)

Poznámka: Práce o *M. annamensis* je druhá nejdůležitější, k tématu jsem se v teoretické rovinně dostala již v dřívějším průběhu studia. Podílela jsem se jak na odběru genetického materiálu, návrhu a provedení laboratorní práce, výpočetním vyhodnocení

výsledků, tak i na sepsání publikace. Tento článek jsem jako první autor předkládala k posouzení do oborného časopisu a zajistila nezbytné opravy v průběhu recenzního řízení.

5.3. Je *ex situ* populace *Orlitia borneensis* v Evropských chovech geneticky homogenní?

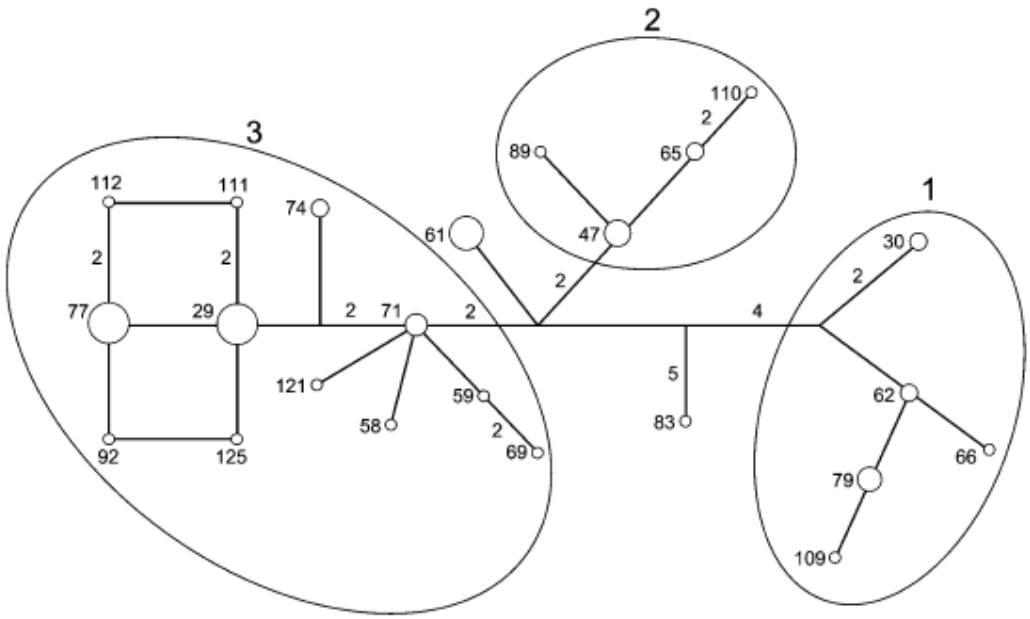
Orlitia borneensis (Malayan Giant Turtle) je málo prozkoumaný druh želvy také z čeledi Geoemydidae žijící na ostrovech Borneu, Sumatře a Malajském poloostrově (Iverson 1992). Velké Sundy a Malajský poloostrov jsou od sebe odděleny pouze mělkým mořem. Během glaciálních maxim před 250, 150 a 17 tisíci lety klesla hladina moře natolik, že toto mělké moře ustoupilo a vytvořily se pevninské šelfy, které ostrovy a poloostrov spojily (Voris 2000). Tyto šelfy umožnily migraci druhů mezi ostrovy a vzhledem k přítomnosti řek na nich, mohla i *O. borneensis* využít tuto příležitost.

Obr. 5: *Orlitia borneensis* (foto Daniel Frynta)

Orlitia borneensis stejně jako výše zmíněná *M. annamensis* patří k ohroženým druhům díky želví krizi v Asii (Cheung & Dudgeon 2006, Zhou & Jiang 2008, Chen et al. 2009). Tato sladkovodní želva je také vedena jako kriticky ohrožený druh, dle hodnocení IUCN uděláném v roce 2018 (Horne et al. 2020) – v době, kdy vznikala naše publikace, byl tento druh vedený ještě jako ohrožený (Palupčíková et al. 2012). Export těchto želv je dnes zakázaný, ale i přes to byla v roce 2002 v Hong Kongu zkonfiskována zásilka obsahující mimo jiné i tato zvířata. Zvířata byla rozdělena do Evropských a Amerických zoologických zahrad. A díky těmto zvířatům bylo možné rozeběhnout projekty na plánování *ex situ* záchranných

chovů. Vzhledem k tomu, že jsou zakladatelé nejistého geografického původu a k časté a dokumentované hybridizaci v čeledi Geoemydidae (Parham et al. 2001, Stuart & Parham 2007, Fong & Chen 2010), byl genetický a morfologický průzkum všech jedinců v záchranných chovech nezbytný.

Cílem naší práce byl prvotní pohled na genetickou a fenotypovou variabilitu jedinců *O. borneensis* chovaných v Evropských chovech. U všech 61 zkoumaných jedinců jsme sekvenovali mitochondriální cyt b a jaderný R35 gen. Analýzou sekvencí cyt b jsme identifikovali 23 haplotypů, v rámci těchto haplotypů byla divergence menší než 1.5 %, fylogenetická struktura těchto haplotypů byla málo podpořená. Nízkou genetickou divergenci podpořila i analýza jaderného R35 genu. Vzhledem k relativně nízké mitochondriální divergenci jsme doporučili zkoumanou populaci *O. borneensis* chovat jako jednu konzervační jednotku.



Obr. 6: Hyplotypová síť konstruovaná z alignmentu cyt b o délce 1119 bp (číselně jsou u větví znázorněny pouze mutační kroky vyšší než 1) pomocí Median-Joining algoritmu.

Poznámka: Tématu výzkumu genetické variability *O. borneensis* jsem se věnovala již v dřívější fázi studia. Během mého doktorského studia se nám podařilo získat genetický materiál od všech dostupných jedinců v Evropských chovech. Jako druhý autor jsem podílela

na návrhu a provedení laboratorní práce, výsledných výpočtech a sepisování samotného článku.

5.4. Je genetická variabilita *ex situ* populace *Chilabothrus angulifer* v Evropských chovech dostatečná pro dlouhodobé přežití populace?

Chilabothrus angulifer je druh hroznýšovce žijící výlučně na ostrově Kuba a přilehlých ostrovech. Obecně je terestrická fauna na Velkých Antilách charakteristická vysokou diverzitou a endemismem. To je způsobeno biogeografickou historií Karibské oblasti (Hedges et al. 2019). I přes trvalé oddělení ostrovů od Amerického kontinentu Karibským mořem, byly tyto ostrovy opakovaně osídlovány druhy z pevniny (Ali 2012). Každopádně osídlování přes moře probíhalo pomocí ojedinělých událostí které byly extrémně vzácné. Tím pádem dnešní druhová rozmanitost je výsledek následné speciace několika málo linií, kterým se na Velké Antily podařilo dostat.

Obr. 7: *Chilabothrus angulifer* (foto Daniel Frynta)

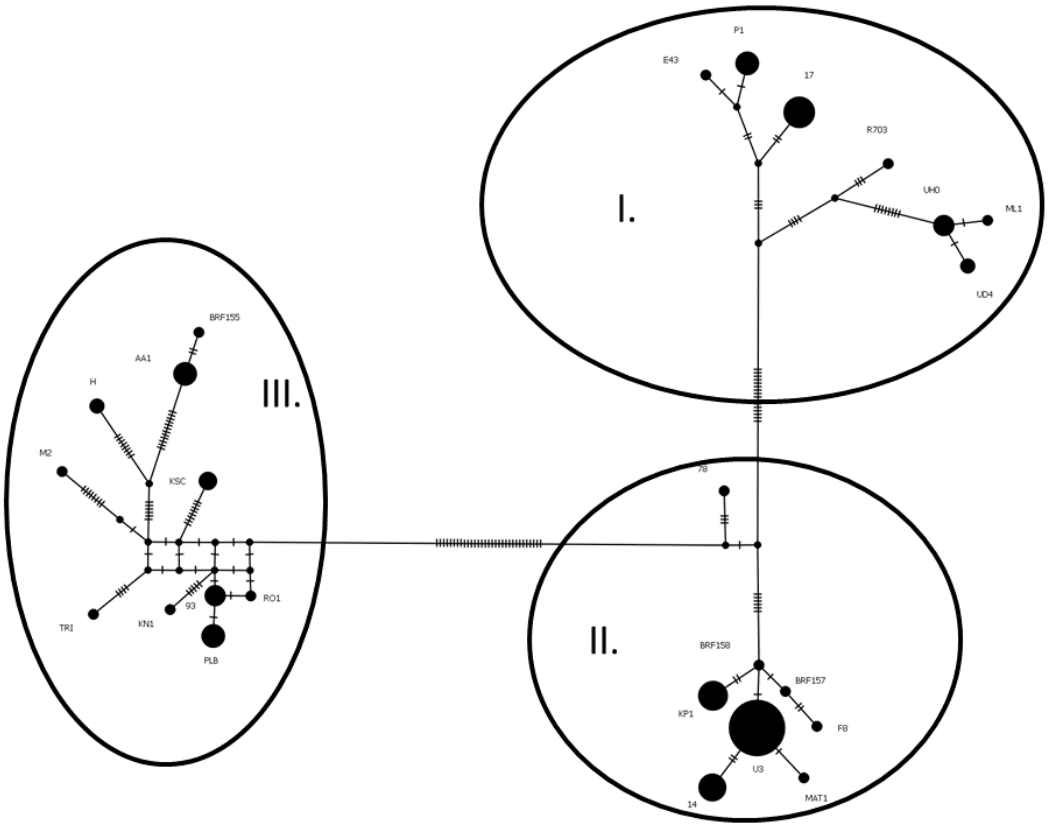
Zatímco rozmanitost druhů savců byla opakovaně ovlivněná nedávnými vymíráními v důsledku lidské činnosti a značně se zredukovala. Populace plazů z řádu šupinatých (Squamata) byly ovlivněny v mnohem menší míře a jsou tedy lepším modelem pro fylogenetické a populační studie endemismu v Karibské oblasti. Studie endemických větví anolisů (*Anolis* sensu lato, Losos 2009, Cádiz et al. 2018) a leguánů z rodu *Cyclura* (Malone et

al. 2000) jednoznačně ukazují korelaci mezi geografickou izolací a divergencí. Na Kubě je velmi dobře zdokumentovány oblasati výskytu jednotlivých druhů plazů (Glor et al. 2004, Rodríguez Schettino et al. 2010, Rodríguez Schettino et al. 2013). Ostrov lze rozlišit na čtyři hlavní zoogeografické oblasti – západ, střed, Camagüey-Maniabón a východ (Estrada & Ruibal 1999). Obzvlášť východní a západní oblast má velmi složitý pattern a dokonce i v rámci těchto endemických oblastní byly identifikovány další (Murray & Clother 2019).

Rod *Angulifer* patří mezi první kolonizující druhy na Antilách, oblast osídlil z Jižní Ameriky (Tolson 1987, Reynolds et al. 2014) a dnes je identifikováno 14 druhů v rámci tohoto rodu (Pyrron et al. 2014, Reynolds & Henderson 2018, Landestoy et al. 2021). Fylogenetické vztahy uvnitř rodu byly opakovaně studovány (Burbrink 2004, Reynolds et al. 2013, Reynolds et al. 2014), *C. angulifer* je sesterský druh ke všem ostatním druhům uvnitř rodu *Angulifer*. Odhad času oddělení *C. angulifer* od všech ostatních druhů z rodu *Chilabothrus* je 21.7 (16.9 – 26.0) Mye (Reynolds et al. 2013), tedy se jedná o velmi vzdálenou linii.

V této studii jsme zkoumali genetickou variabilitu populace kubánského hroznýšovce *Chilabothrus angulifer* v Evropských chovech. Analyzovali jsme 96 jedinců *C. angulifer* a jednoho jedince *Chilabothrus inornatus*. K analýze jsme použili sekvence mitochondriálních cyt b a ND4 genů. V populaci *C. angulifer* jsme analýzou cyt b identifikovali 25 distinktních haplotypů s výjimečnou diverzitou. V rámci druhu *C. angulifer* jsme objevili tři výrazně vymezené haploskupiny. Díky známému časovému odhadu oddělení *C. angulifer* od všech ostatních druhů z rodu *Chilabothrus*, jsme mohli spočítat i čas oddělení těchto tří haploskupin. Námi odhadovaný čas oddělení byl 3.57 a 2.26 Mye, což naznačuje, že mezi jedním popsaným druhem *C. angulifer* jsou tři evoluční linie, jejichž distance jsou větší než u některých jiných druhů v rodu *Chilabothrus*. Tento fakt naznačuje, že hroznýšovec kubánský by mohl představovat ve skutečnosti minimálně dva kryptické druhy nebo poddruhy.

Každopádně po zvážení současného stavu *ex situ* chovů, námi získaných fylogeografických údajů a velikosti lokality výskytu jsme doporučili chovat celou *ex situ* populaci *C. angulifer* jako jednu jednotku. V přírodě



Obr. 8: Hapotypová síť konstruovaná z 1059 bp dlouhého cyt b alignmentu, který obsahoval všech 96 vzorků. K výpočtu byl použit algoritmus Median-joining.

Poznámka: K tomuto projektu jsem se dostala až ve finální fazi, poté co byl rozpracovaný projekt dlouhodobě odložený. Samotná laboratorní práce se prováděla zhruba před dvaceti až deseti lety, byly udělány i předběžné analýzy, ale samotná publikace již nebyla sepsána. Díky potřebě data co nejdříve publikovat, hrozilo, že toto téma bude publikovat jiný tým a tak jsem byla k tomuto projektu přizvána. Protože jsem se nepodílela na sběru materiálu ani laboratorní práci, jsem oprávněně v pořadí až třetím z autorů. Re-analyzovala jsem dříve získaná data a vyhodnotila výsledky (s výjimkou časové kalibrace), také jsem se podílela na sepsání části textu, především metodiky a výsledků.

5.5. Identifikace druhů, sekvenční a haplotypové diverzity sympatrických druhů z rodu *Apodemus* (Sylvaemus) v Arménii.

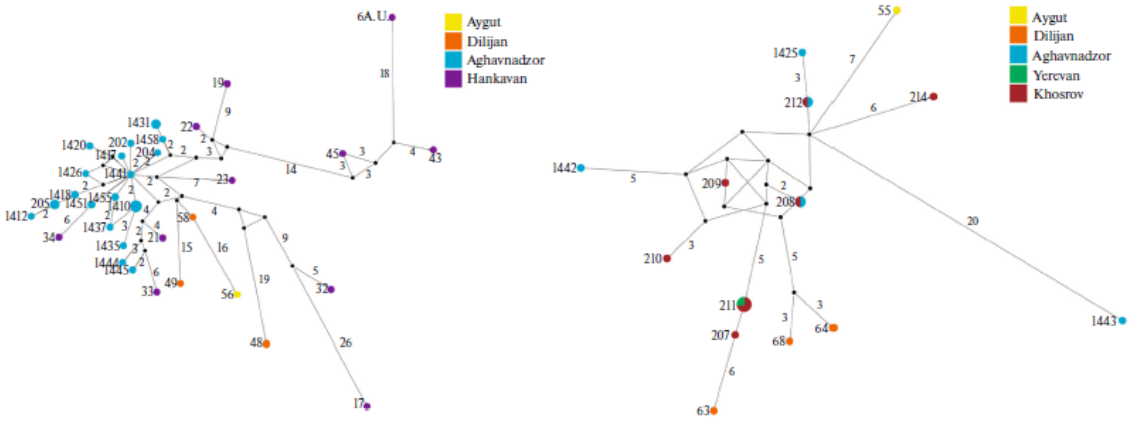
Myšice rodu *Apodemus* Kaup, 1929 patří k nejběžnějších hlodavcům v Eurasii (Musser 1996, Orlov et al. 1996), mají schopnosti obývat široké spektrum habitatů. V publikovaných

studiích bylo popsáno, že druhy *Apodemus* žijící v regionu Středního východu jsou si morfologicky velmi podobné (Musser 1996; Frynta et al. 2001; Macholán et al. 2001; Michaux et al. 2002; Frynta et al. 2006), což znemožňuje jejich správné určení bez využití molekulárních dat. V naší práci jsme zkoumali genetickou variabilitu populace myšic dvou druhů – *Apodemus uralensis* a *A. witherbyi* žijící na území Arménské republiky. Ačkoliv je toto území relativně malé rozlohy, můžeme zde najít různé druhy habitatů – od aridních subtropických polopouští po horské oblasti s nadmořskou výškou více jak 4000 m nebo smíšené lesy. Oblast má bohatou geologickou historii a biodiverzita je ovlivněna mnoha procesy včetně klimatických změn během dob ledových (Hewitt 1999, Ort et al. 2002, Seddon et al. 2002, Haynes et al. 2003).

Obr. 9: *Apodemus witherbyi* (foto Daniel Frynta)

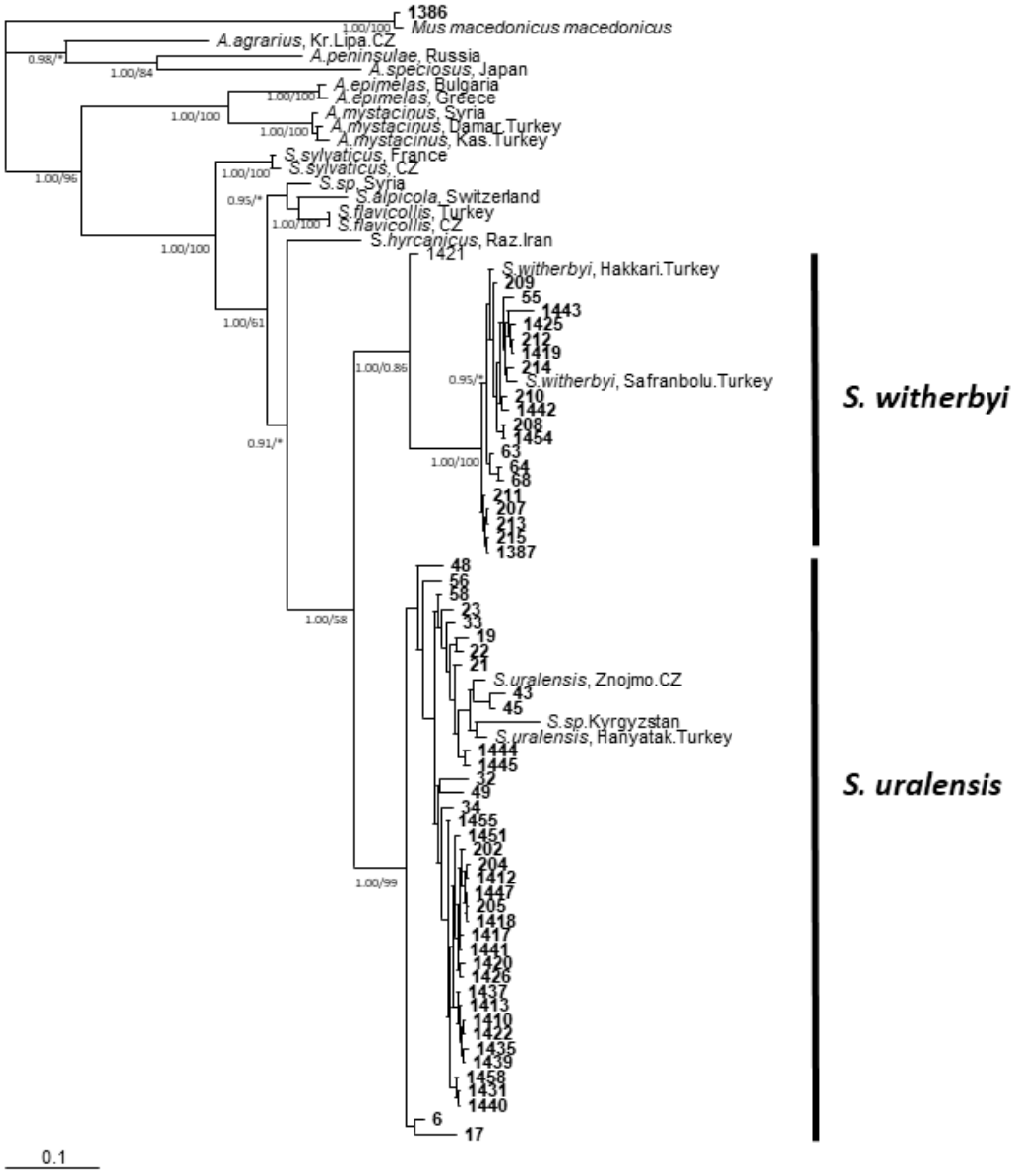
Vzhledem k vysoké morfologické a ekologické podobnosti napříč druhy rodu *Apodemus*, je identifikování druhů opravdu obtížné. V oblasti středního východu je situace ještě více zkomplikovaná díky chybějícímu taxonomickému určení druhů z této oblasti. V oblastech sousedících s Armenií byl zaznamenán výskyt pěti druhů z rodu *Apodemus* (Macholán et al. 2001, Bellinvia 2004, Frynta et al. 2006, Suzuki et al. 2008) – *A. uralensis*

(Pallas, 1811), *A. witherbyi* (Thomas, 1902), *A. flavicolis* (Michaux et al. 2004), *A. hyrcanicus* (Vorontsov et al. 1992) and *A. mystacinus* (Michaux et al. 2005, Darvish et al. 2015).



Obr. 10: Haplotypové sítě konstruované metodou Median-joining z alignmentů D-Loop, vlevo je síť obsahující všechny vzorky *A. uralensis*, vpravo je šíť obsahující vzorky *A. witherbyi*. Lokality původu jsou označené barevně.

Abychom mohli určit druhovou příslušnost zkoumaných 58 jedinců patřících do druhů *Apodemus uralensis* a *Apodemus witherbyi*, sekvenovali jsme fragment mitochondriální DNA obsahující kontrolní region „d-loop“ a přilehlé tRNA geny. Zkoumaní jedinci pocházeli ze šesti oblastí v Arménii. Pomocí fylogenetických analýz jsme potvrdili přítomnost dvou distinktních linií, které korespondují s popsanými druhy *A. uralensis* a *A. witherbyi*. Ve většině zkoumaných lokalit se vyskytovaly oba druhy, což naznačuje jejich sympatrický výskyt. Mezi studovanými jedinci jsme objevili velkou genetickou variabilitu. V rámci populace 38 jedinců *A. uralensis* bylo identifikováno 32 haplotypů a nejvyšší p-distance 4.91 %. V rámci populace 19 jedinců *A. witherbyi* bylo identifikováno unikátních 14 haplotypů s nejvyšší p-distanci 2.37%.



Obr. 11: Bayesianský fylogenetický strom kontruovaný z alignmentu D-Loop, u větví jsou doplněny posteriorní pravděpodobnosti z maximum parsimony stromu.

Poznámka: Tato práce nebyla původně plánovanou součástí mého doktorského studia. Ale díky příležitosti zahraniční spolupráce s institucí v Arménii jsem byla pověřena vedením laboratorní praxe Valentiny Balasanyan během jejího studijního pobytu v České republice. V průběhu jejího vedení jsme získaly dostatek laboratorních výsledků, tato data jsem zanalyzovala a poté jsem se podílela na sepsání publikace i na předkládání k posouzení.

Závěr

Tato práce je založená na pěti studiích. V první práci z jsme pomocí kombinace mitochondriálního a jaderného znaku dokázali vymezit druhy, populace a jejich vzájemné fylogenetické a populačních vztahy mezi scinky z rodu *Tiliqua* žijícími na Molukách, Nové Guinei (Irian Jaya) a přilehlých ostrovech. Studie byla limitována počtem jedinců, které jsme získali přímo z Moluckých ostrovů a Nove Guinei, resp. dostupností materiálu. Výsledky poukazují na jasný vzor variability, i když některé zvláštnosti nemají žádnou paralelu u jiných skupin. Zatímco rozdělení severu a jihu Nové Guinee je zřetelné a zcela v souladu s fylogeografickou historií jiných taxonů. Tak afinita populací ze Ceramu a souostroví Key k populacím na severu Nové Guinee neodpovídá současné vzájemné geografické poloze těchto oblastí.

Ve druhé přiložené práci jsme za použití kombinace mitochondiálního a jaderného znaku detekovali F1 hybridy, určili jsme haplotypovou diverzitu *ex situ* populace *Mauremys annamensis* v Evropských chovech. Každopádně použití pouze těchto dvou znaků nedokáže zcela odhalit starší částečné introgrese v genomu studované populace, například přítomnost starších hybridních událostí. Navíc jsme během analyzování jaderného znaku zjistili možnou heterozygotnost u R35 intronu *M. annamensis*. Abychom heterozygotnost potvrdili nebo vyvrátili, museli bychom použít klonovací techniky. Potvrdili jsme výskyt dvou vzdálených mitochondriálních linií v rámci druhu *M. annamensis*. Protože tento druh již v přírodě prakticky vyhynul, nejde již zjistit, zda původní výskyt těchto linií byl allopatrický.

Ve třetí studii jsem zkoumali genetickou variabilitu populace *Orlitia borneensis* chované v Evropských chovech. Použili jsme kombinaci mitochondriálního a jaderného znaku. V populaci jsme odhalili poměrně vysokou haplotypovou diverzitu a současně relativně nízkou nukleotidovou diverzitu (méně než 1.5 % u cytochromu b). Z čehož můžeme soudit, že populace je dostatečně geneticky variabilní a dlouhodobý chov je u ní možný. Zároveň jsme doporučili chovat celou populaci jako jednu ochranářskou jednotku, i když nemůžeme vzhledem k absenci původu studovaných jedinců vyloučit možný geografický vzor této variability. Je potřeba ovšem mít na paměti, že generační doba u takto velkých želv je velice dlouhá a pozorovaná divergence může být starší, než by tomu bylo u jiných taxonů.

Ve čtvrté práci jsme zkoumali genetickou variabilitu *ex situ* populace *Chilabothrus angulifer* v Evropských chovech, použili jsme mitochondriální znaky cyt b a ND4. Podařilo se nám zjistit, že genetická variabilita je velmi výrazná, dostatečná pro dlouhodobé udržení této populace v lidské péči. Hlavním problémem bylo, že uvnitř jediného ostrova se udržely tři hluboce odělené linie. To naznačuje, že populace musely být buď extrémně početné nebo částečně izolované. Na základě použitých metod nejde zjistit, zda máme před sebou druhy ve stavu zrodu a nebo jediný druh s komplexní historií izolací a introgresí.

V poslední páté studii jsme identifikovali druhy, sekvenční a haplotypovou diverzitu sympatrických druhů z rodu *Apodemus* (*Sylvaemus*) v Arménii. Ze čtyř podobných druhů se nám molekulárně podařilo prokázat jenom dva – *A. uralensis* a *A. wiitherbyi*. Sekvenční i haplotypová variabilita byla v rámci obou druhů vysoká, což je v souladu s komplexitou prostředí a biogeografickým významem Zakavkazského prostoru.

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8.1.

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Barbora Somerová is the last and corresponding author.

Genetic variation of blue-tongue skinks of the genus Tiliqua *(Squamata: Scincidae) from New Guinea and Wallacea*

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REVIEW

Genetic variation of blue-tongue skinks of the genus *Tiliqua* (Squamata: Scincidae) from New Guinea and Wallacea

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Abstract

Populations of blue-tongue skinks inhabiting eastern Wallacea and New Guinea are traditionally assigned to either *Tiliqua gigas*, which is endemic to this area, or to *Tiliqua scincoides* that extends its range from the Australian continent. Despite a wide morphological variation among local populations, genetic data from non-Australian populations were almost absent. We examined 128 specimens and sequenced mitochondrial ND4, 12rRNA and nuclear cmos genes. A phylogenetic analysis revealed the presence of two main clades corresponding to species *Tiliqua scincoides* and *Tiliqua gigas*. We provide the first report of *Tiliqua scincoides* from the Aru Island and confirm that it is genetically related to the Tanimbar populations reported as *Tiliqua scincoides chimaerea*. The other samples belonged to *Tiliqua gigas*, which also shows a distinct phylogenetic structure congruent with the geographic origin of the samples. The main split conforms to the north-south pattern of genetic variation, which was also reported in other animal species in New Guinea. (1) Samples from the northern coast of Irian Jaya, Seram, and Kai Islands belong to a distinct clade, which further splits supporting the recognition of the *Tiliqua gigas keyensis* subspecies. (2) Samples from the Bird's Head and southern coast of the Irian Jaya and Halmahera Island form the other clade within *Tiliqua gigas*, in which the Halmahera samples formed a shallow, but clearly distinct branch. The haplotype network analysis of mitochondrial ND4 gene in *Tiliqua gigas* samples suggests a strong differentiation among major population groups.

Keywords Wallacea · New Guinea · Australia · Scincidae · mtDNA · Cmos

Introduction

The fauna inhabiting New Guinea and adjacent islands of the Sahul shelf and eastern Wallacea has been extensively studied since pioneering studies of Wallace (1869). In recent years, our understanding of biogeography and history of animal populations in this area has been considerably improved by molecular phylogenetics (Rawlings et al. 2004). In this paper, we focus on blue-tongue skinks of the genus *Tiliqua* Gray, 1825.

Geological history and formation of the second largest island in the world, New Guinea, is surprisingly complex. New Guinea is situated at the boundary of the Pacific and Australian plates, its present shape is relatively young and contains a landmass of heterogeneous origin. Considerable sea transgressions and regressions were associated with rapid tectonic processes in this area during the past 30 Ma (Hall and Holloway 1998). Prior to the late Miocene, New Guinea was divided into several solitary islands. Formation of the New Guinea Highlands was dated to about five million years before present (Toussaint et al. 2014). On New Guinea, we can find both lowlands and a 1300 km long and up to 150 km wide central highland chain with peaks as high as 4700 m above the sea level. This chain caused an isolation of southern and northern populations of lowland vertebrate taxa (Pigram and Davies 1987; Dumbacher and Fleischer 2001; Rawlings and

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Donnellan 2003) and stopped the genetic drift between them (Hall 2002).

During the late Pleistocene glaciations and deglaciations resulted in the rise and drop of the sea level. These processes have considerably affected the landmass configuration. During glacial maxims, particularly the periods of 17,000, 150,000, and 250,000 years before present, the sea level was lower than nowadays and large areas of exposed land were created (Voris 2000). The areas temporarily connected with the Asian and Australian continent by land bridges during the glacial maxims are further referred to as the Sunda and Sahul shelfs, respectively.

In contrast to the islands on the Sahul shelf (including New Guinea), where immigration of animal species from Australia was enabled thanks to the land bridges, the islands of Wallacea were not connected to the Sahul nor Sunda shelf and their highly endemic fauna relies almost exclusively on overseas migration. Wallacea is delimited from the Oriental zoogeographic region by the Wallace's Line and from the Australian zoogeographic region by the Lydekker's Line which corresponds to the margins of the Sahul shelf (Whitten et al. 1987). The third biogeographic line, the Weber's Line, delimits the eastern part of Wallacea consisting namely of Morotai, Halmahera, Ternate, Buru, Seram, Ambon, Kai, Tanimbar and Babar islands (Fig. 1). These lines delineate the limited options of species migration from Australia to New Guinea and adjacent islands of eastern Wallacea (Wilson and Heinsohn 2007).

Despite the marine barrier some reptile species managed to cross this area and migrated in both directions, from the Oriental to Australian region and vice versa (Koch 2012). Migrations from the Asian region are very well documented – agamas of the genus *Physignathus* (Hugall et al. 2008), species of the genus *Varanus* (Ziegler et al. 2007), pythons (Rawlings and Donnellan 2003), or even some skinks (Reeder 2003, Skinner 2007; Honda et al. 2000). Herpetofauna at the eastern side of the Weber's Line includes mainly species from New Guinea, which succeeded to cross the Lydekker's Line (De Lang 2011). But generally, animals have crossed these lines only exceptionally, which leads to extreme endemism in this area.

In this study, we focused on genetic variation of selected blue-tongue skinks of the genus *Tiliqua* (Scincomorpha: Lygosomoidea Mittleman, 1952) inhabiting Irian Jaya and adjacent islands of the eastern Wallacea and Sahul shelf. The genus belongs to the subfamily Egerniinae of the Scincidae family sensu *lato* (Uetz et al. 2019). The subfamily contains nine extant genera and 62 species (Uetz et al. 2019). Egerniinae are endemics of the Australian zoogeographic province. The genus *Tiliqua* has radiated in Australia and five of seven currently recognized *Tiliqua* species are still confined

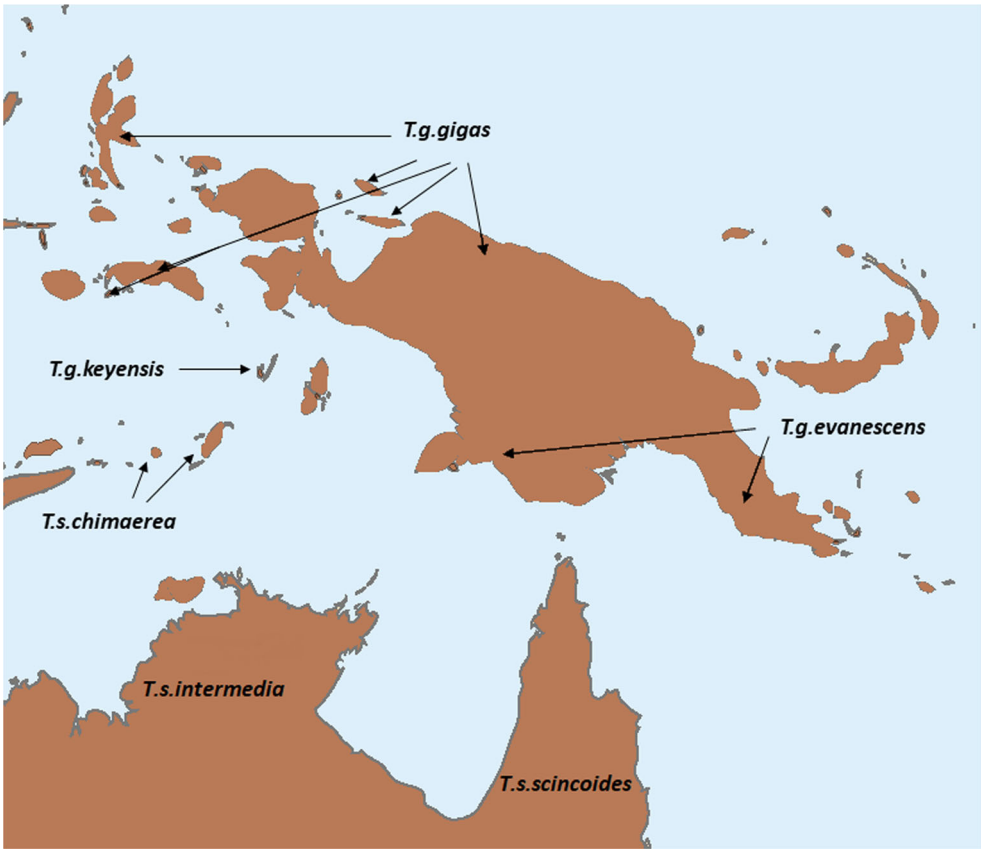

Fig. 1 Map of Sunda and Sahul containing the locality of *T. scincoides* and *T. gigas* subspecies as described in Shea (1992, 2000), Hitz and Hauschild (2000) and Noel (2014)

to this continent. Only *T. gigas* (Schneider, 1801) is distributed exclusively in the areas north of the Torres Strait, but *T. scincoides* (White, 1790) also extends its range from continental Australia northwards (Fig. 1). According to available molecular phylogeny, these two are sister species (Pyron et al. 2013). No molecular data, except the barcode (Chambers et al. 2016), are available for *Tiliqua multifasciata* (Sternfeld, 1919) distributed in the northern, north-western, and central parts of Australia (Cogger 2000). Previous phylogeographic studies include *T. nigrolutea* (Quoy and Gaimard, 1824), *T. occipitalis* (Peters, 1863), and *T. adelaidensis* (Peters, 1863) inhabiting the southern parts of Australia and *T. rugosa* (Gray, 1825) with a wider distribution range than *T. adelaidensis*, but without any habitat in the extreme north of Australia close to New Guinea (Cogger 2000). *T. gigas* - *T. scincoides* clade is more related to *T. nigrolutea* and *T. occipitalis* than to *T. rugosa* and *T. adelaidensis* (Gardner et al. 2008a, b; Pyron et al. 2013).

Systematics of *Tiliqua* populations in New Guinea and adjacent islands was thoroughly elaborated based on morphological characters (Shea 1992, 2000; Hitz and Hauschild 2000; Noel 2014) (see Fig. 1). Populations inhabiting the northern and central parts of Maluku Islands (i.e., Morotai, Ternate, Halmahera, Ambon and Seram), Irian Jaya (including Biak, Japen, Doom islands) were assigned to *T. g. gigas* (Schneider, 1801). Shea (2000) described a subspecies *T. g. evanescens* from the south-western part of Papua New Guinea (terra typica: lowlands and adjacent foothills of eastern and southern New Guinea). According to Hitz and Hauschild (2000), specimens fitting this subspecies are also known from the vicinity of Merauke town and islands on the eastern and north-eastern coast of New Guinea, while the populations from the north coast of Papua New Guinea have been assigned to *T. g. gigas*. Populations from Kai Islands and Aru Islands in the eastern margin of the Maluku archipelago are reported as a distinct subspecies *T. g. keyensis* Oudemans, 1894 (Shea 2000; Hitz and Hauschild 2000). The Tanimbar and Babar Islands on the southern margin of the Maluku archipelago are inhabited by *T. scincoides.* Shea (2000) described this form as *T. s. chimaerea* Shea, 2000. The remaining subspecies *T. s. scincoides* (White, 1790) and *T. s. intermedia* Mitchell, 1955 are distributed in southeastern/eastern and northern Australia, respectively (Unverzagt 2004; Cogger 2000).

In this paper, we examined genetic relationships among blue-tongue skinks of the genus *Tiliqua* from the Maluku Islands, Biak Island, and Irian Jaya "mainland". To date, molecular markers have in these populations have been unexplored, although they may improve our understanding of the systematics and population history. Thus, we sequenced two mitochondrial and one nuclear gene to (1) explore phylogenetic relationships among examined populations, (2) calculate and draw haplotype networks, and (3) discuss colonization scenarios.

Material and methods

Species selection and specimens used

We examined DNA samples from 128 individuals belonging to the *Tiliqua scinciodes-gigas* complex from this project (29 individuals), European zoos, and private collections. All these individuals or their maternal ancestors had been legally imported from Indonesia and with recorded geographic origin as the Indonesian co-author (DG) has personally verified all the available locality information in the most cases. The specimens came from the following regions: Aru, Biak, Halmahera, Kai Islands, Seram, Tanimbar Islands, and three regions of Irian Jaya "mainland": Manokwari, Jayapura, and Merauke (Fig. 2). We sequenced their mitochondrial ND4, 12rRNA, and nuclear cmos genes. In addition, two captive specimens of *T. scincoides intermedia* with presumably Australian ancestors as well as two skinks of *Egernia* group - *Cyclodomorphus gerrardii* (Gray, 1845) and *Bellatorias frerei* (Günther, 1897) were sequenced as well. These species, together with *Corucia zebrata* Gray, 1855 and *Egernia whitii* (Lacépéde, 1804) (sequences from GenBank: *C. zebrata* – JQ898450, AB028793, HQ655201; *E. whitii* – AY169640, AF280119, AY818774) were included as outgroups in the phylogenetic analyses.

DNA extraction, PCR amplification, and nucleotide sequencing

The buccal swabs were taken from each sampled live animal, the fingertips were taken from the dead ones and both stored prior to DNA extraction in Eppendorf tubes with 96% ethanol. Total genomic DNA was isolated with DNAeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines.

Using standard conditions and primers (see Online resource 1), we amplified the mitochondrial protein-encoding ND4 gene and parts of $tRNA^{His}$, $tRNA^{Ser}$ for all 128 sampled individuals. In each ND4 haplotype, we sequenced ribosomal gene 12S rRNA and nuclear protein-encoding gene cmos for one individual (see Table 1 for details).

Sequence alignment and phylogenetic analyses

Sequences of ND4, 12S rRNA, and c-mos were initially aligned with ClustalX (Thompson et al. 1997) and adjusted manually where necessary using Chromas Lite 2.01 (Technelysium Pty Ltd), BioEdit (Hall 1999) and Clustal X 1.81 (Thompson et al. 1997). Additional steps of alignments' preparation were done using the online fasta sequence toolbox FaBox 1.41 (Villesen 2007). Three alignments were analysed in this study. The first alignment containing all sampled individuals was prepared using the mitochondrial ND4 gene only.

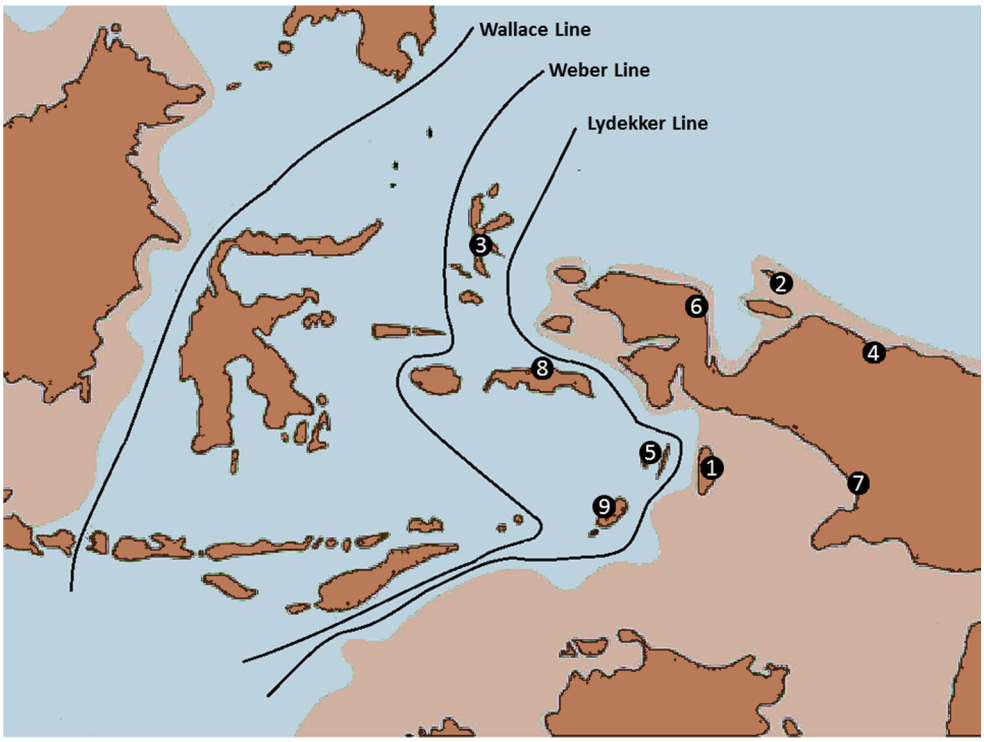

Fig. 2 Map of Sunda and Sahul, containing the present-day mainland and mainland extensions by the lower sea levels during glacial maxima. All sampled localities are highlighted 1: Aru, 2: Biak, 3: Halmahera, 4: Jayapura, 5: Kai Islands, 6: Manokwari, 7: Merauke, 8: Seram, 9: Tanimbar

This alignment performed by FaBox 1.41 was used for haplotype analyses. The second and third alignments containing one individual from each haplotype were prepared using a combination of the following genes: mitochondrial ND4 + 12S rRNA and all genes ND4 + 12S rRNA + c-mos. We used one individual of *E. whitii* and *C. zebrata* as outgroups, sequences were downloaded from GenBank (*E. whitii*: AY169640, AY649109, AY818774; *C. zebrata*: JQ898412, AY308334, HQ655201). We also sequenced *C. gerrardii* and *E. frerei* as a part of this study.

Alignment data were reviewed in MEGA6 (Tamura et al. 2013) and PAUP* version 4.0b10 (Swoford 2002). The pairwise distances were calculated and the information about aligned length, included sites, Pi sites, and variable sites of all alignments was obtained.

Bayesian trees (BA) were calculated using MrBayes 3.2.4 (Huelsenbeck et al. 2001; Ronquist 2003) by two independent runs with a random starting tree for 10×10^6 generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 25% of trees (2,500,000 generations), which was generated before the chain reached equilibrium in the trees' distribution.

A haplotype network was calculated from ND4 sequences of *T. gigas* samples. For this purpose, we adopted Median-Joining (MJ) algorithm (Bandelt et al. 1999) as implemented in Network 4.6.1.4 (http://www.fluxus-engineering.com/). MJ algorithm identifies groups of closely related types and introduces hypothesised ancestral types in order to unite the types into a parsimonious network.

Polymorphisms within each sampled population were worked out by the statistic software DnaSP v5 5.10.01 (Librado and Rozas 2009) which estimated the following: haplotype diversity (*h*), segregating sites (S), nucleotide diversity (π), 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 π indicate a constant large population size. However, a low value of π and high value of *h* signifies a recent expansion.

Results

We detected 54 haplotypes of the mitochondrial ND4 gene in 128 sampled individuals belonging to the genus *Tiliqua* (for haplotypes, localities, and further details see Table 1). Representatives of each haplotype were included in further phylogenetic analyses.

We calculated BA trees for each of the following three alignments: ND4; ND4 + 12S rRNA and ND4 + 12S rRNA + c-mos sequences. Each of these alignments contained 58 sequences, i.e. 54 corresponding to haplotypes and four representing the outgroups (Table 2).

The topologies of all three resulting trees were almost identical. Slight differences not concerning major branches are visible just in the tree resulting from the shortest alignment (*ND4,* see Online resource 2). Thus, below we focus on the topology of the BA tree based on all three sequenced genes (Fig. 3). The examined

Table 1 Information about haplotypes contribution, localities of examined speciemens and GenBank numbers of sequenced DNA fragments. Samples from Irian Jaya don't have known more specific locality of origin

Sequences belonging to the haplotype	GenBank numbers (ND4, cmos, 12S rRNA)	Locality
101, 105, 107, 127, 137, 138	MK462034, MK461980, MK448013	Irian Jaya
102, 114, 136, 230, 233, 427	MK462035, MK461981, MK448014	Merauke
103	MK462036, MK461982, MK448015	Irian Jaya
104, 429	MK462037, MK461983, MK448016	Merauke
106, 111, 113, 116, 120, 124, 125, 126, 129, 131, 132, 133, 134, 139, 142, 144, 145, 146, 148, 150, 224, 226	MK462038, MK461984, MK448017	Merauke
108, 135, 140	MK462039, MK461985, MK448018	Irian Jaya
109, 110, 112, 115, 117, 123, 141, 149, 227	MK462040, MK461986, MK448019	Merauke
118, 143	MK462041, MK461987, MK448020	Irian Jaya
119	MK462042, MK461988, MK448021	Irian Jaya
122, 210, 225, 430	MK462043, MK461989, MK448022	Merauke
128	MK462044, MK461990, MK448023	Irian Jaya
147	MK462045, MK461991, MK448024	Irian Jaya
201, 205	MK462046, MK461992, MK448025	Kai Island
202, 204, 207, 208, 219, 220	MK462047, MK461993, MK448027	Kai Island
203, 213, 215	MK462048, MK461994, MK448026	Seram
206, 418	MK462049, MK461995, MK448028	Halmahera
209, 216, 237, 412	MK462050, MK461996, MK448029	Halmahera
211	MK462051, MK461997, MK448030	Irian Jaya
212	MK462052, MK461998, MK448031	Seram
214, 221, 416	MK462053, MK461999, MK448032	Halmahera
217, 407	MK462054, MK462000, MK448033	Jayapura
218	MK462055, MK462001, MK448034	Tanimbar
222, 223, 229, 232	MK462056, MK462002, MK448035	Merauke
228	MK462057, MK462003, MK448036	Irian Jaya
231	MK462058, MK462004, MK448037	Irian Jaya
234	MK462059, MK462005, MK448038	Irian Jaya
235	MK462060, MK462006, MK448039	Irian Jaya
236	MK462061, MK462007, MK448040	Irian Jaya
401	MK462062, MK462008, MK448041	Aru Island
402	MK462063, MK462009, MK448042	Tanimbar
403	MK462064, MK462010, MK448043	Jayapura
404, 406	MK462065, MK462011, MK448044	Jayapura
405	MK462066, MK462012, MK448045	Jayapura
408	MK462067, MK462013, MK448046	Jayapura
409, 411	MK462068, MK462014, MK448047	Biak Island
410	MK462069, MK462015, MK448048	Biak Island
413, 414	MK462070, MK462016, MK448049	Halmahera
415	MK462071, MK462017, MK448050	Halmahera
417	MK462072, MK462018, MK448051	Halmahera
419	MK462073, MK462019, MK448052	Halmahera
420	MK462074, MK462020, MK448053	Halmahera
421, 422, 423, 424	MK462075, MK462021, MK448054	Manokwari
425	MK462076, MK462022, MK448055	Merauke
426, 428	MK462077, MK462023, MK448056	Merauke
501, 502	MK462078, MK462024, MK448057	*T. s. chimereae*
503	MK462079, MK462025, MK448058	*T. s. intermedia*
504	MK462080, MK462026, MK448059	unknown

Table 1 (continued)

Sequences belonging to the haplotype	GenBank numbers (ND4, cmos, 12S rRNA)	Locality
507	MK462081, MK462027, MK448060	Seram
508	MK462082, MK462028, MK448061	Seram
510	MK462083, MK462029, MK448062	unknown
511	MK462084, MK462030, MK448063	unknown
512	MK462085, MK462031, MK448064	unknown
516	MK462086, MK462032, MK448065	*T. s. intermedia*
517, 518, 519	MK462087, MK462033, MK448066	*T. g. evanescens*

sequences of the genus *Tiliqua* formed a monophyletic group (posterior probability = 1.00) which split into major clades corresponding to *T. scincoides* (1.00) and *T. gigas* (1.00).

Within the *T. scincoides* clade, *T. s. chimaerea* from Tanimbar Islands (0.98) formed a sister branch to the Aru sample, while two *T. s. intermedia* samples represented basal offshoots.

Within the *T. gigas* clade, there was a well-supported clade (1.00) including *T. g. keyensis* as well as samples from the Seram Island, Biak Island and Jayapura. We further refer to this as the "northern" clade. The latter two localities formed exclusive (1.00 and 1.00, respectively) but closely related branches (1.00 for Biak-Jayapura clade). *T. g. keyensis* samples also appeared clearly distinct (1.00).

The remaining *T. gigas* samples from Merauke, Manokwari and the Halmahera Island (including samples of uncertain origin, presumably from S and W parts of Irian Jaya) formed another branch which also received a statistical support (1.00). We further refer to this as the "southern" clade. Samples from the Halmahera Island represented a strongly supported clade (1.00) within this group. This Halmahera clade was placed close to Manokwari, the locality on the Irian Jaya mainland which is geographically close to the Halmahera Islands.

Uncorrected p-distances in ND4 gene between *T. s. intermedia* of a presumably Australian origin and *T. scincoides* samples coming from Indonesia were small: the mean for the Tanimbar Islands (*T. s. chimaerea*) was 1.97% (range: 1.72–2.24%) and 1.52–1.93% for the Aru Island. The distances between the Aru sample and those of *T. s. chimaerea* ranged from 0.60 to 0.90%. In contrast, the distances between non-Australian *T. scincoides* and *T. gigas* ranged from 6.99 to 9.20% (mean = 8.03%). The divergences among *T. gigas* belonging to different populations ranged from 0.25 to 6.51%; especially *T. gigas keyensis* is well differentiated from the others (see Table 3 for details).

We computed a haplotype network of the mitochondrial ND4 gene for all 122 *T. gigas* samples (Fig. 4). The network pattern was clear with distinct clusters. The haplotypes coming from the same geographic region tended to cluster together.

The neutrality tests of the „northern" population and all samples population resulted in positive values. The neutrality tests resulted in negative, but non-significant values of the „southern" population only. Also, the „southern" population shows a low value of π and high value of h (see Table 4).

Discussion

The phylogenetic trees estimated from our sequence data reveal a clear branching pattern (see Results). The deepest node of our trees corresponds to the split of *T. gigas* and *T. scincoides*. As the samples coming from the same island

Table 2 Information about alignments used in this study for calculating the phylogenetic analyses

Gene region	Aligned length	Included sites	Pi sites	Variable sites
ND4	824	818	212	330
12S rRNA	423	423	107	139
c-mos	397	393	5	22
Total	1644	1634	324	491

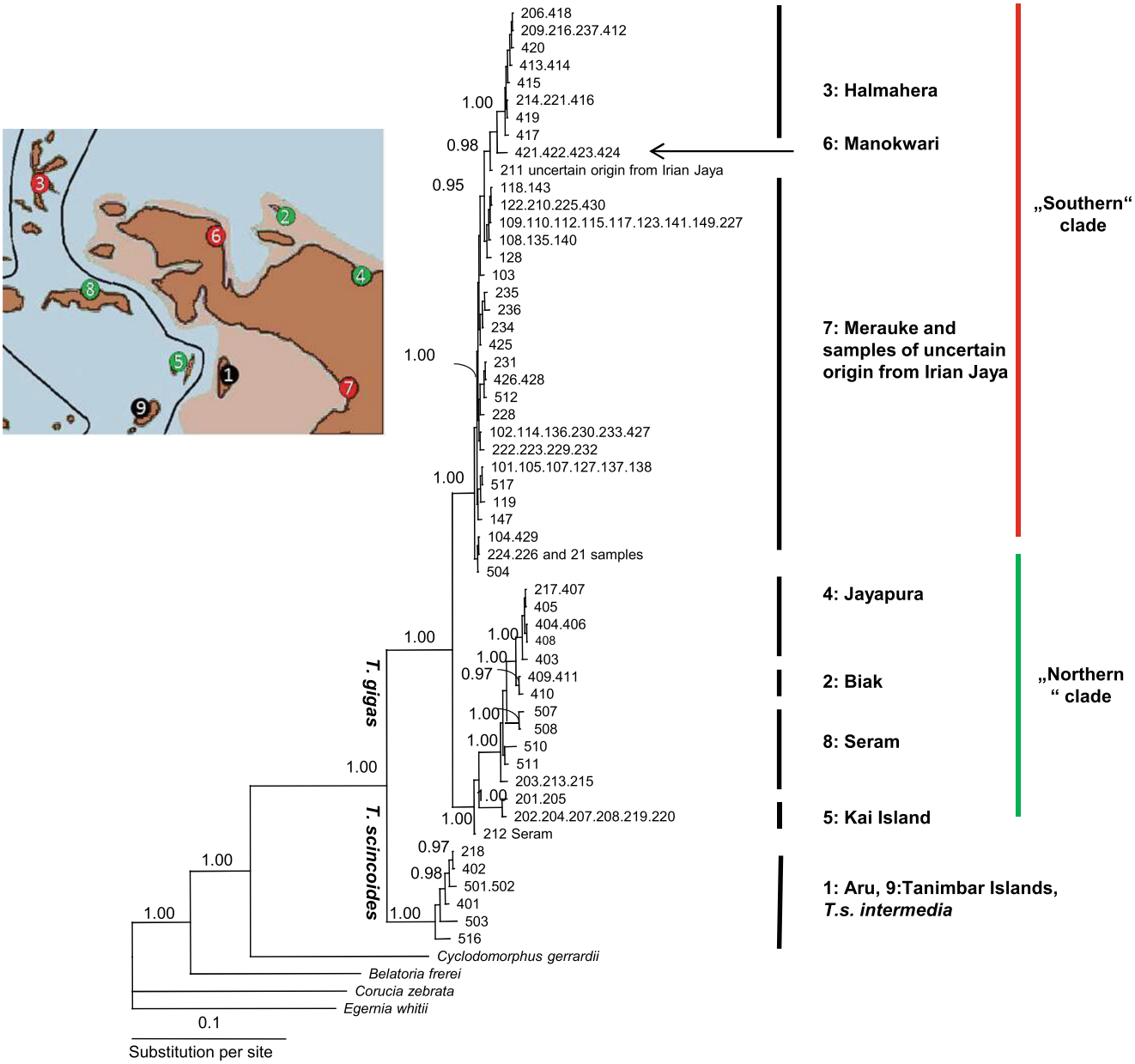

Fig. 3 BA tree constructed using a combined alignment of all three sequenced genes - ND4, 12S rRNA, cmos, total length - 1644 bp, populations generated - 10,000,000. Posterior probability values 0.95 and higher are displayed only. The tree contains labels of locality, numeric code of locality and split to the "northern" and "southern" clades

and/or region tend to form monophyletic (e.g., the Halmahera Island, Kai Islands, Biak Island, and Jayapura) or paraphyletic groups (e.g., "Merauke" group with respect to the Halmahera Island and Manokwari on the Bird's Head), these trees are highly congruent with the geographic origin of the sampled individuals. This also provides indirect verification for proper localization of the sampled animals (Table 1).

Tiliqua scincoides The sampled specimens from the Tanimbar Islands, assigned to *T. s. chimaerea,* are closely related to a specimen from the Aru Island and two captive individuals, assigned to *T. s. intermedia,* which presumably have ancestors from continental Australia. Thus, the examined specimen from Aru unequivocally belongs to *T. scincoides* and represents the first record of this species on the Aru Island. Placement of the Aru Island on the Sahul shelf makes likely a scenario suggesting colonization of this island from the Australian continent during the periods of glacial transgression. Previous authors (Shea 1992, 2000) assigned all bluetongues from this island to *T. gigas,* specifically to *T. g. keyensis.* Thus, the populations of bluetongues inhabiting the Aru Island require further verification.

Table 3 Estimates of evolutionary divergence over sequence pairs between haplotypes of *T. gigas* population

	Halmahera	Manokwari	Merauke	Jayapura	Biak	Seram	Kai Islands
Halmahera	0.13–0.50% (0.24%)						
Manokwari	1.01–1.27% (1.16%)	–					
Merauke	0.63–2.18% (1.61%)	0.89–1.66% (1.25%)	0.13–1.27% (0.47%)				
Jayapura	5.68–6.23% (5.96%)	4.98–5.11% (5.03%)	4.70–5.81% (5.15%)	0.13–0.38% (0.22%)			
Biak	5.40–6.10% (5.76%)	4.70–4.98% (4.84%)	4.70–5.68% (5.17%)	0.25–0.76% (0.48%)	0.25%		
Seram	4.16–6.10% (5.53%)	3.48–4.98% (4.61%)	3.22–5.40% (4.63%)	0.88–2.82% (1.37%)	0.63–2.56% (1.14%)	0.13–2.17% (0.89%)	
Kai Islands	6.09–6.51% (6.31%)	5.66%	5.38–6.08% (5.58%)	4.29–4.56% (4.40%)	4.02–4.29% (4.16%)	2.04–3.89% (3.42%)	–

The mean number, minimum, and maximum of base substitutions per site overall sequence pairs within each group is shown. Analyses were conducted using the maximum composite likelihood model implemented in MEGA6 (Tamura et al. 2013)

Tiliqua gigas splits into two well-differentiated clades. The „northern" clade come from areas of the Kai Islands and Seram, as well as localities along the northern coast of Irian Jaya (Jayapura, Biak Island). Shea (2000) designated the specimen collected on Ambon as a neotype of *T. gigas*. Thus, the "northern" clade involves a nominotypic subspecies *T. g.*

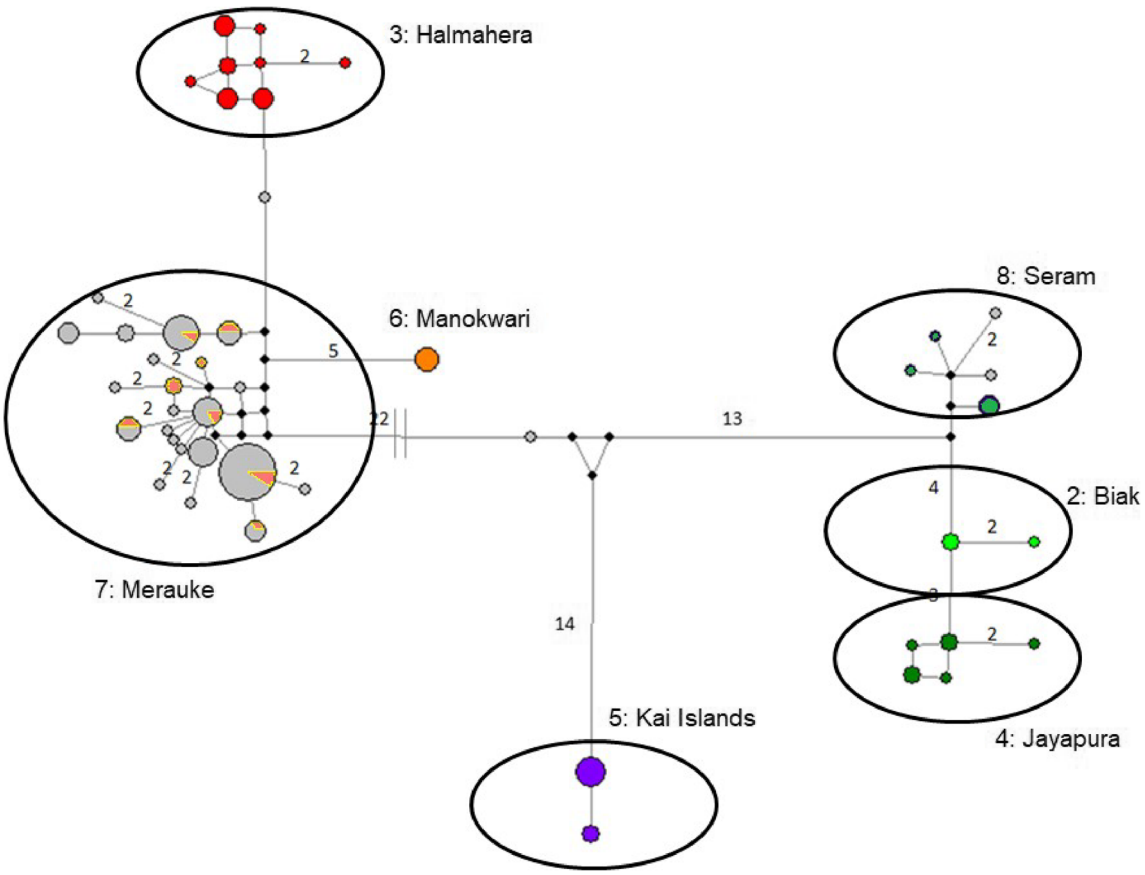

Fig. 4 Haplotype network calculated from 825-bp ND4 gene sequences of *T. gigas* samples. Median Joining algorithm was used for calculation (samples from each locality are marked with different colour, grey colour marks samples with the unknown locality of origin). "northern" clade is highlighted in green, "southern" clade in red

Table 4 Demographic characteristics for the *T. gigas* based on the 825-bp mitochondrial *ND4* gene alignment

Clades	Ns	S	H	h	π	Fu & Li's *F**	F u & Li's *D**	Fu's *Fs*	Tajima's *D*	Exp
All samples	120	82	32	0.92	0.022 4	0.730 46	0.781 5	2.075	0.380 75	4.57
„northern"	26	46	2	0.88	0.021 56	1.022 65	0.529 1	3.847	1.599 57	2.630 6
„southern"	94	34	23	0.88	0.007 5	−1.013 14	−1.103 55	−2.617	−0.447 47	5.640 3

Sequences: number of individuals sequenced (Ns), number of segregating sites (S), number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), Fu & Li's F*, Fu & Li's D*, Fu's FS, Tajima's D and expansion coefficient (exp)

gigas. Ambon is an island close to Seram, thus we can reasonably expect that the Seram samples we examined represent *T. g. gigas*. Nevertheless, we detected two distinct branches within the "northern" clade, (1) a cluster of haplotypes from the northern part of Irian Jaya (Jayapura) and the Biak Island and (2) a deeply divergent one from the Kai Islands (cf. Shea 2000; Hitz and Hauschild 2000), which have never been connected to Sahul by a land bridge (Voris 2000). The latter cluster corresponds to the *T. g. keyensis* subspecies.

The other main clade within *T. gigas* is the "southern" one, which inhabits S and extreme NW of Irian Jaya as well as the Halmahera Islands. A distinct cluster of mutually related haplotypes from the Halmahera Islands may be explained by a recent colonization event and/or subsequent lineage sorting. According to our phylogenetic trees, the likely source of this Halmahera colonization is the Bird's Head region of Irian Jaya. The sequence divergence between the "northern" and" southern" clades was surprisingly large, which might signify that these clades represent distinct species. However, with no samples from the southwestern part of Papua New Guinea, a possible relationship of the latter clade to *T. g. evanescens*, described by Shea (2000) from that area, remains uncertain.

The studied area is full of natural barriers (sea, mountains) preventing the gene flow between the populations. This explains the emergence of clear distinctness of regional reptile populations. As a result, multiple species and/or subspecies of varanids (Koch et al. 2009; Ziegler et al. 1999, 2007) and pythons (Harvey et al. 2000; Natusch et al. 2020) were described from this relatively small area. In these groups, as well as in the case of bluetongues, such taxonomic decisions based initially on morphological characters have received an increasing support by molecular data. Nevertheless, a surprising ability of highly divergent reptilian lineages to hybridize once the barriers disappear (for a review see Jančúchová-Lásková et al. 2015a, b) should also be considered.

Genetic differentiation among populations inhabiting various parts of New Guinea and adjacent islands have been studied in multiple taxa of animals. These studies typically reported a sharp contrast between the north and south of the island (Donellan and Aplin 1989; Harvey et al. 2000; Rawlings and Donnellan 2003; Austin et al. 2010). We also found a strong genetic difference between *T. gigas* populations inhabiting the northern coast and those from the Bird's Head and the southern coast of Irian Jaya. Nevertheless, the pattern of affinities of Irian Jaya populations to those inhabiting the islands of Wallacea is less intuitive. Specifically, populations of *T. gigas* from the Kai and Seram Island (situated in the south and east of Irian Jaya) clustered unequivocally with those from the northern part of the Irian Jaya "mainland" (Jayapura and Biak populations). As these islands remained separated from the Sahul mainland even during glacial maxims, this unexpected pattern can be explained by stochasticity of colonization events. Passing through the sea barrier may depend not only on the geographic proximity, but also on the direction of winds and sea currents. Nevertheless, to resolve phylogeographic scenarios, additional information is still needed, especially precise and congruent timing of both phylogenetic and geological events.

Author's contributions Designed the research (FD and GD), provided the material (GD), designed and performed the laboratory work (SB), computed the trees and statistics (SB) and wrote the paper (FD and SB).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval In this article all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. No animals were killed or harmed. In this study there were used the biological samples only.

Consent for publication We consent with publication of this article and all supplementary materials.

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8.2.

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Barbora Somerová is the first author.

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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

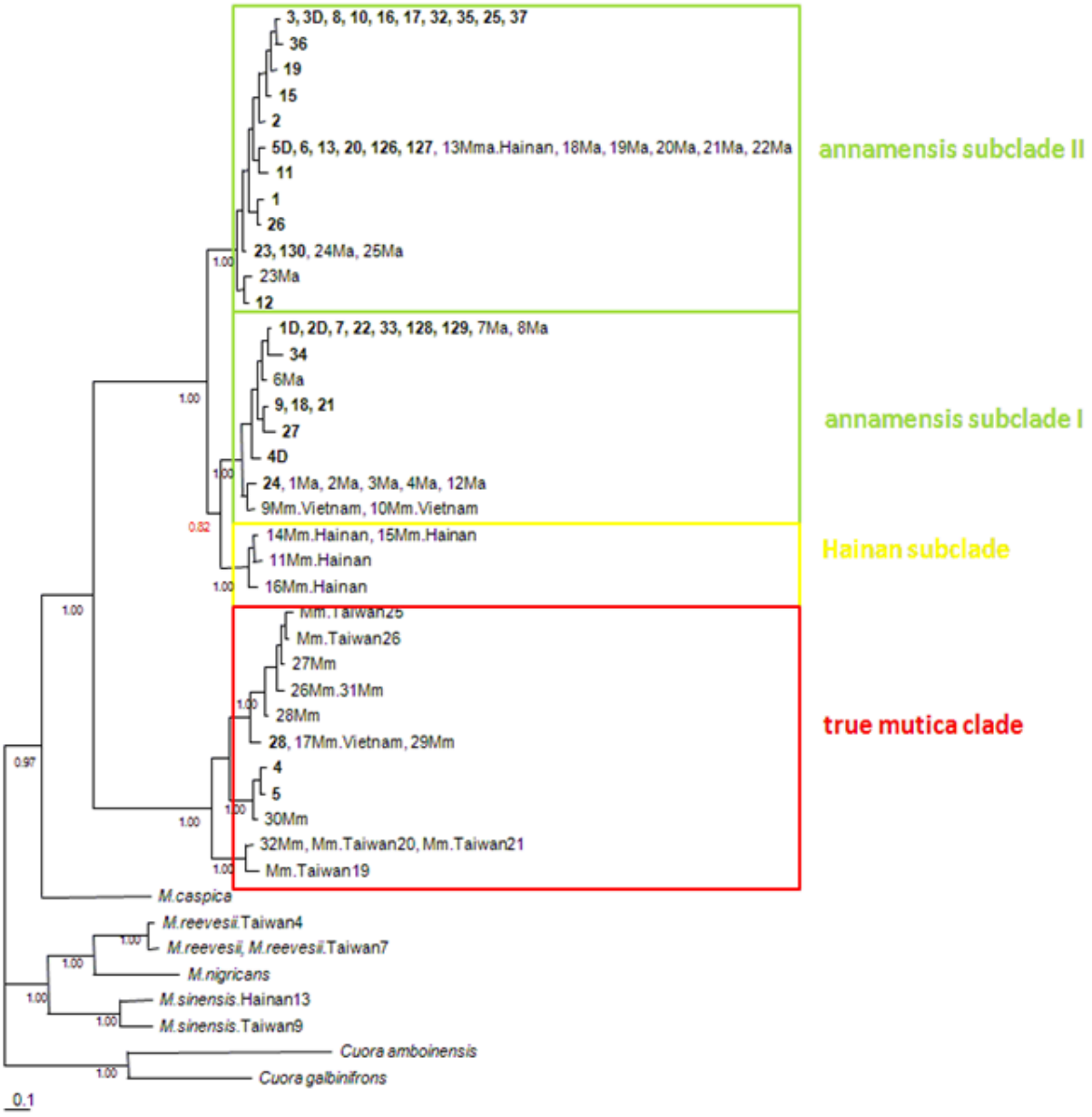

Fig. 1. Bayesian tree of mitochondrial DNA (ND4) of the genus *Mauremys*. Numbers at branches are support values, only values > 0.95 are shown. Samples sequenced in this study, which correspond to Table 1, in bold, remaining samples were sequenced by previous authors (Fong et al., 2007; Fong and Chen,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

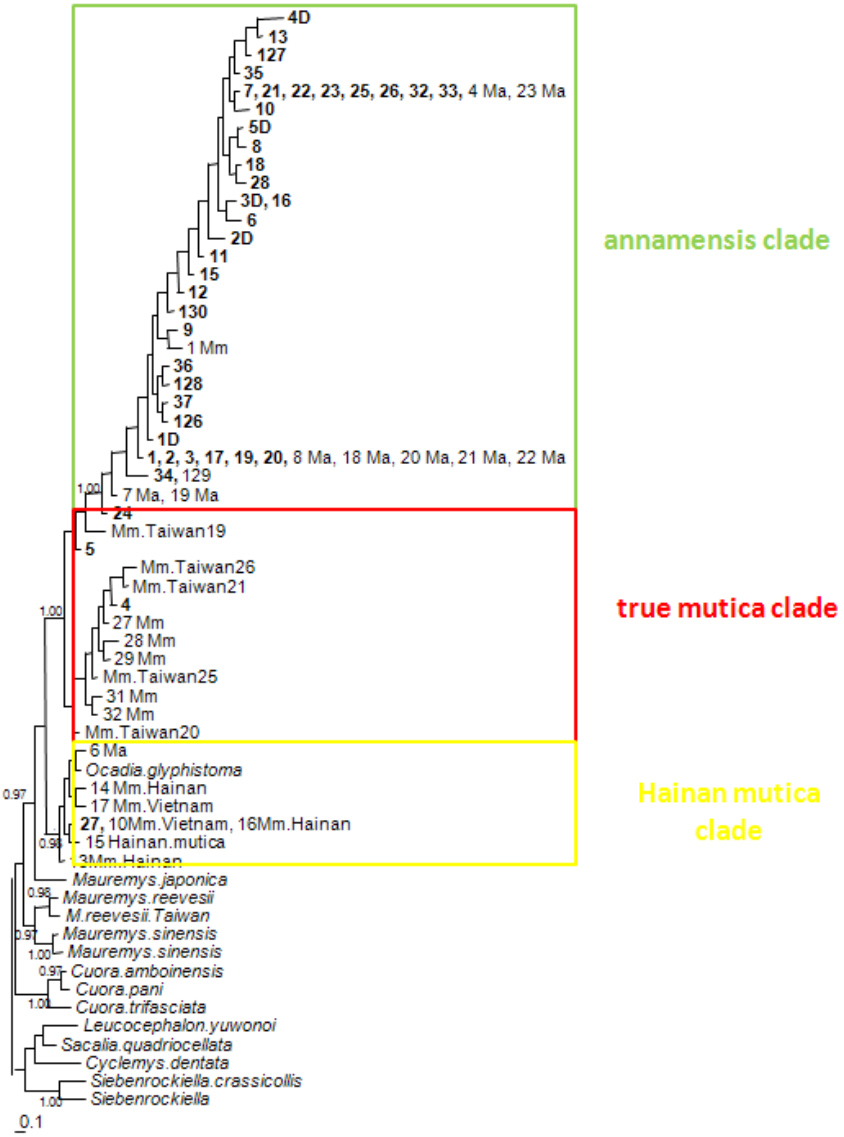

Fig. 2. Bayesian tree of nuclear DNA (R35) of the genus *Mauremys*. For further explanation, see Fig. 1

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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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

8.3.

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*(1), 56-66.

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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. borneensis*. 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 specimen	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 (π) 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 π indicate a constant large size of population. However, a low value of π 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).

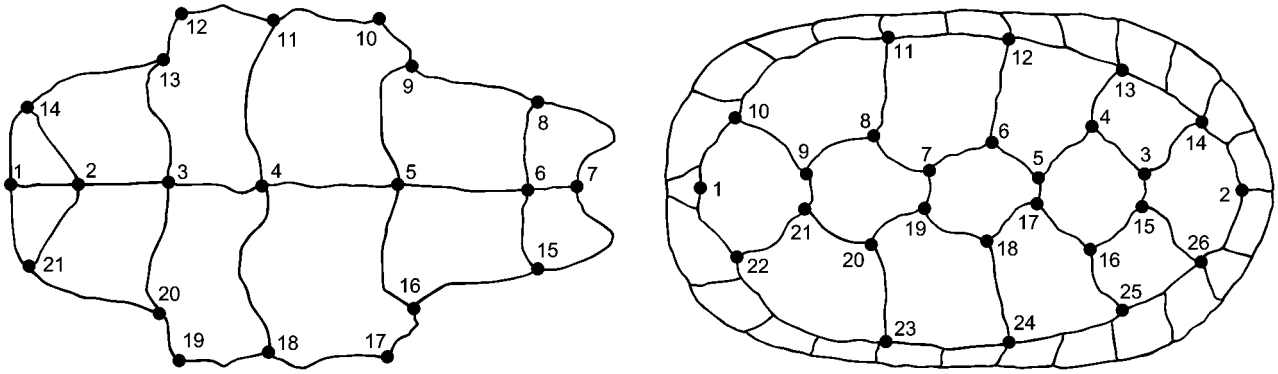

FIGURE 1. Ventral and dorsal view of a shell of *Orlitia borneensis* showing the landmarks used in this study.

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 (π), Fu & Li's F*, Fu & Li's D*, Fu's F_S, Tajima's D and expansion coefficient (exp).

N_s	S	h	π	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.

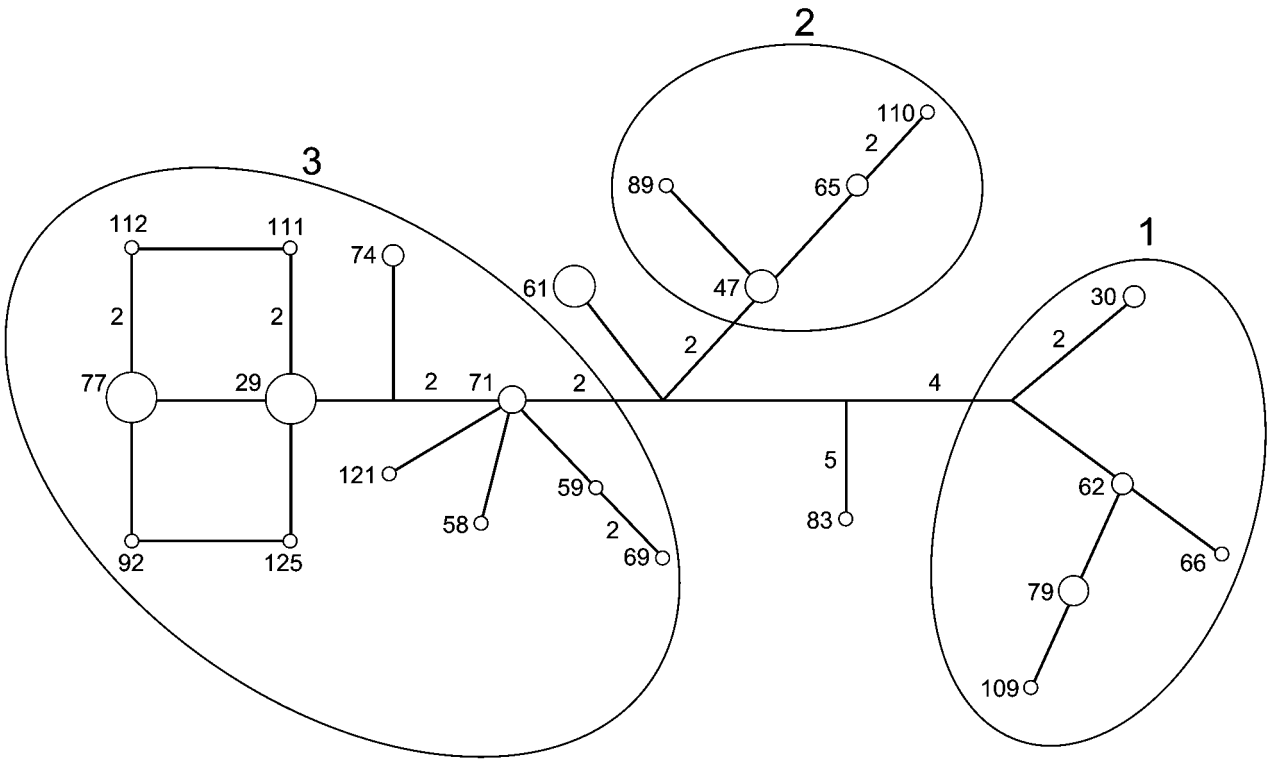

FIGURE 2. Median-joining network indicating relationships among haplotypes of *Orlitia borneensis* based on cytochrome *b* sequences. Haplotypes are denoted as circles, their size is proportional to number of individuals carrying respective haplotype. Numbers at branches represent numbers of mutational steps (displayed for n > 1). Three main haplogroups are marked by ovals.

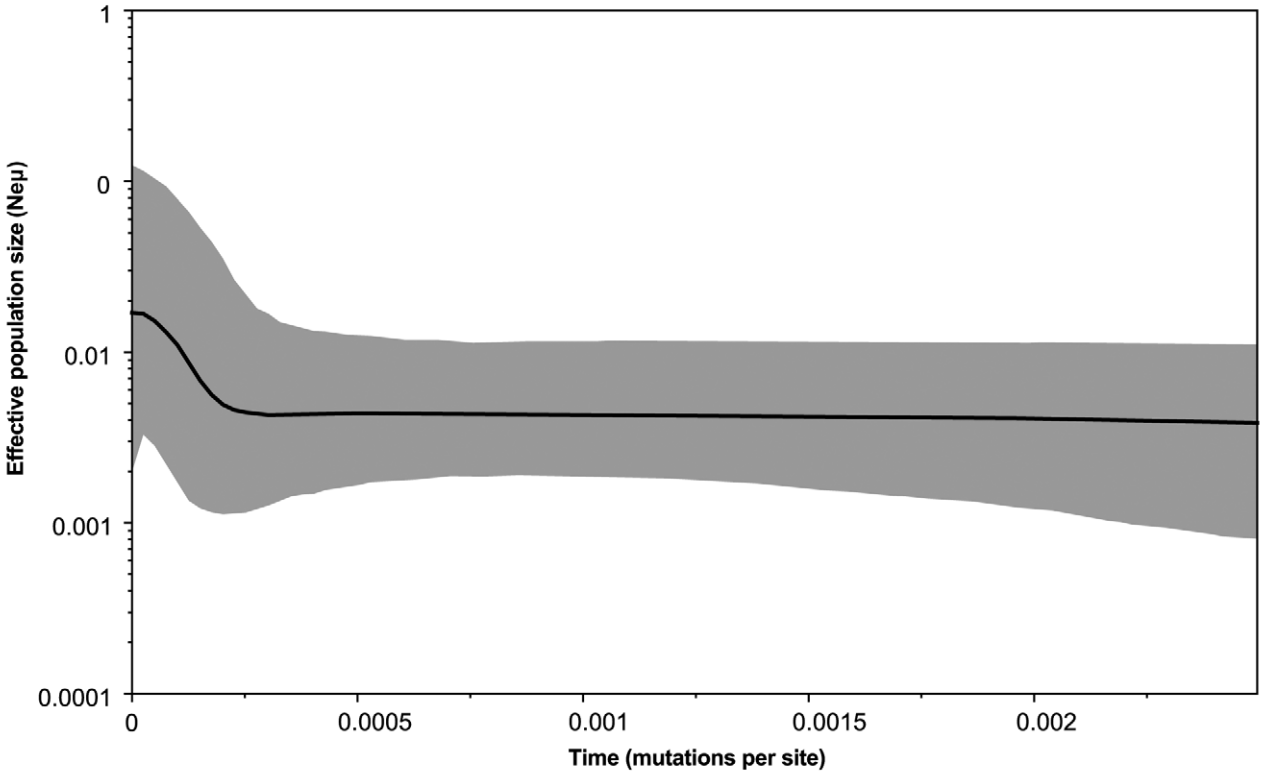

FIGURE 3. Bayesian skyline plot demonstrating changes in effective population size in *Orlitia borneensis* based on mitochondrial data. Thick solid line represents median of the estimate, borders of grey area delineate the highest 95% posterior density interval.

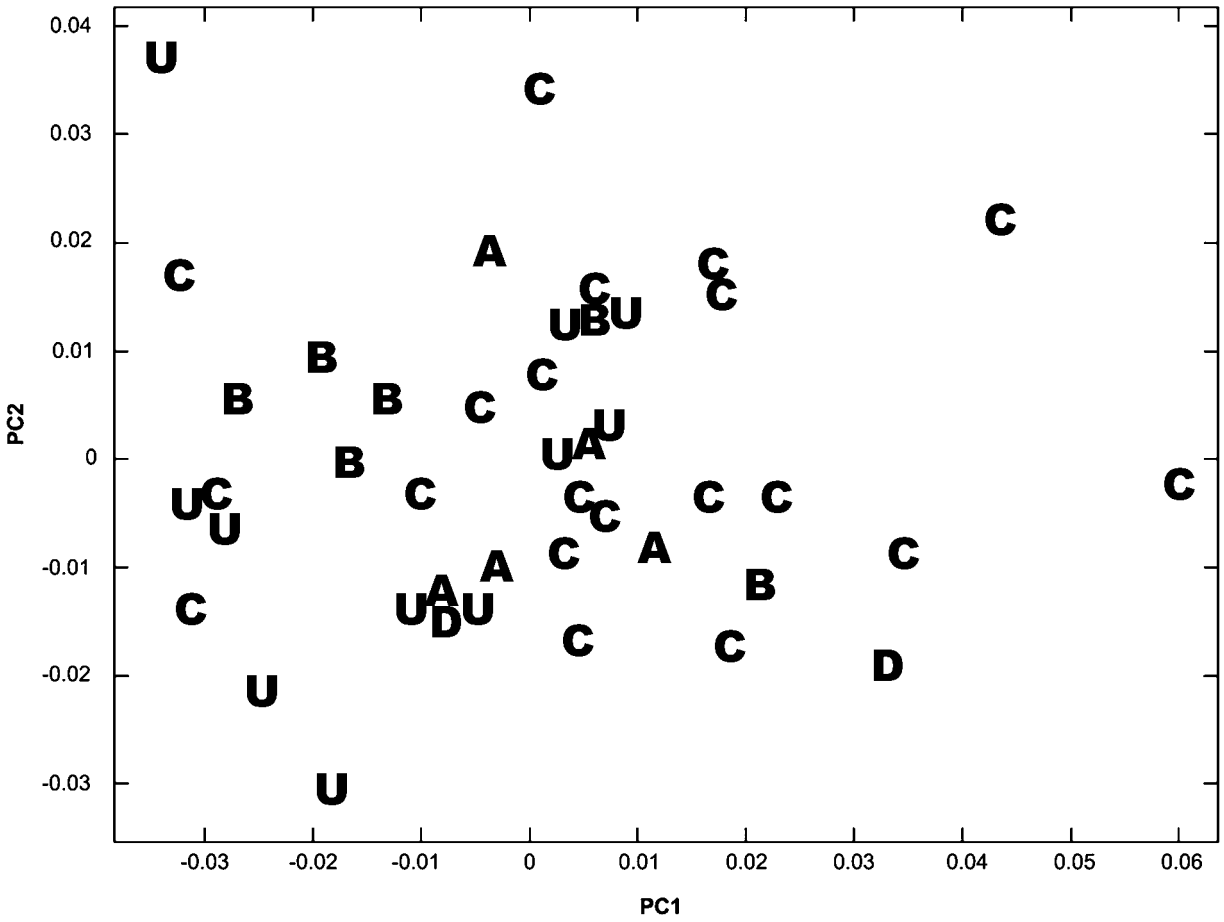

FIGURE 4. PCA for plastron shape. Specimens are marked according to haplotype group. A—haplotype group 1, B—group 2, C—group 3, D—haplotype 61, U—unknown. The first axis PC1 explains 29.79% and second axis PC2 explains 14.14% of the total variance in the shapes.

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.

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8.4.

Rehák, I., Šťáhlavská, I., **Opelka Somerová B.**, Šimková O. & Frynta D. (2021). A deep divergence and high diversity of mitochondrial haplotypes in an island snake: the case of Cuban boa, *Chilabothrus angulifer* (Serpentes: Boidae). *Acta Societatis Zoologicae Bohemicae*, in print.

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Barbora Somerová is the third author.

A deep divergence and high diversity of mitochondrial haplotypes in an island snake: the case of Cuban boa, *Chilabothrus angulifer* (Serpentes: Boidae)

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Abstract. For the needs of proper management, we analysed the mitochondrial haplotype structure of the European *ex situ* population of Cuban boas. The results showed its extraordinary diversity, we sequenced 96 specimens and detected 25 distinct haplotypes. Besides this haplotype diversity, the results revealed a deep divergence among three principal haplogroups. Bayesian estimates of the divergence time (3.57 and 2.26 Mye) suggest that within the currently only recognized species *Chilabothrus angulifer* (Bibron, 1843) there are evolutionary lines whose distance corresponds to or is greater than among some other - taxonomically recognized - species of the genus. This indicates that the Cuban boas represent in fact at least two cryptic subspecies or species. Nevertheless, after considering the current state of the *ex situ* population, given the current knowledge of the phylogeography of Cuban boas and the fact that they inhabit a single large island (and its nearby coastal islands and islets) at present, we recommend to manage the current *ex situ* population as a whole.

Key words. Conservation genetics, population management, *ex situ* population, evolution, phylogeography, adaptive radiation, speciation, boid snakes, Caribbean herpetofauna, Great Antilles.

INTRODUCTION

Terrestrial fauna of the Great Antilles is characterised by an extraordinary high endemism and species diversity. It is due to unique biogeographical history of the Caribbean (cf Hedges et al. 2019). A local Cretaceous biota was originally exterminated by Chicxulub impact at the Cretaceous-Tertiary boundary (Lyons et al. 2020). In spite of a strong permanent isolation by the Caribbean Sea, this region has been repeatedly colonized from mainland. It was proposed that the immigrant lineages dispersed through "Gaarlandia", the putative incomplete temporary land bridge connecting NE of the South American Continent with precursors of the Great Antilles during Oligocene and Early Miocene periods. Currently, geological data rejected "Gaarlandia" hypothesis and molecular clock suggest that immigration events were not confined to this period (Ali & Hedges 2021 and references herein, but see Philippon et al. 2020). Thus colonization of the Great Antilles should be attributed to events of overwater dispersal (Ali 2012). The earliest vertebrate fossil record is a frog of the genus *Eleutherodactylus* coming from Puerto Rico Oligocene, 29 Mye (Blackburn et al. 2020). Nevertheless, the colonization events were extremely rare and thus current species richness of the Great Antillean region is mainly a result of subsequent speciation within a few immigrant lineages. While the diversity of native mammalian fauna of the Great Antilles has been substantially reduced by recent extinctions, squamate reptiles representing another clade of terrestrial non-volatile vertebrates have remained less affected and may serve as a model for phylogenetic and population studies of Caribbean endemism. Studies performed in principal endemic clades of squamates as anoles (*Anolis* sensu lato; Losos 2009 and references herein, Cádiz et al. 2018) and iguanas of the genus *Cyclura* (Malone et al. 2000) clearly demonstrated that geographic isolation among large Antillean Islands typically corresponds to the deepest divergences. However, both adaptive radiation and geographic speciation contributed to further speciation. The geographic ranges of individual species of squamates are typically restricted to a certain part of the island as it is well documented in the largest island of Great Antilles - Cuba (Glor et al. 2004, Rodríguez Schettino, González & Rodríguez 2010, Rodríguez Schettino, Mancina & González 2013). Traditionally, four main Zoogeographical regions were recognized: Western, Central, Camagüey-Maniabón and Eastern (Estrada & Ruibal 1999). Species richness tend to be associated with higher elevations. Cladistic analysis of endemism revealed a complex pattern with nested areas of endemism concentrated in the Western and Eastern parts of the island (Murray & Crother 2019).

Great Antillean boas of the genus *Chilabothrus* Duméril et Bibron, 1844 belong to the earliest reptilian colonists of the Great Antillean region. Its relatives inhabit the South American continent which was unequivocally revealed as the source area (Tolson 1987, Reynolds et al. 2014a). According to recent molecular phylogenies, a common ancestor of anacondas of the genus *Eunectes* Wagler, 1830 and rainbow boas of the genus *Epicrates* Wagler, 1830 represents a sister clade of *Chilabothrus* (Reynolds et al. 2013, 2014a). Divergence between *Chilabothrus* and *Eunectes* + *Epicrates* was

estimated to 30.2 [CI 24.5, 35.8] Mye (Reynolds et al. 2013). A fossil *Chilabothrus stanolseni* (Vanzolini, 1952) from Florida 18.5 Mye supports the view that ancestors of current *Chilabothrus* reached Great Antilles as early as in lower Miocene period (Onary & Hsiou 2018). Nevertheless, at that time boids successfully dispersed from South America also to continental Central America as documented by a fossil record of a snake of the genus *Boa* Linnaeus, 1758 from Panama 19.3 Mye, i.e., prior the Great American Faunal Interchange (Head et al. 2012).

Currently, 14 extant species of the genus *Chilabothrus* are recognized (Pyrron et al. 2014, Reynolds & Henderson 2018, Landestoy et al. 2021). Phylogenetic relationships within the genus were studied repeatedly (Sheplan & Schwartz 1974, Tolson 1987, Kluge 1989, Burbrink 2004, Reynolds et al. 2013, Reynolds et al. 2014a). Molecular studies (Reynolds et al. 2013, Rodríguez-Robles et al. 2015, Landestoy et al. 2021) confirmed five principal clades that diverged in Lower Miocene: (1) Cuban - *Chilabothrus angulifer* (Bibron, 1843), (2) Puerto Rican – *C. inornatus* (Reinhardt, 1843), *C. monensis* (Zenneck, 1898) and *C. granti* (Stull, 1933), (3) Jamaican - *C. subflavus* (Stejneger, 1901), (4) Hispaniolan - *C. ampelophis* Landestoy, Reynolds et Henderson, 2021, *C. fordii* (Günther, 1861), *C. gracilis* Fischer, 1888, and (5) Hispaniola-Bahamian one - *C. argentum* Reynolds, Puente-Rolón, Geneva, Aviles-Rodriguez & Herrmann, 2016, *C. exsul* (Netting & Goin, 1944), *C. chrysogaster* (Cope, 1871)*, C. schwartzi* (Buden, 1975), *C. striatus* (Fischer, 1856) and *C. strigillatus* (Cope, 1862).

Molecular phylogenies placed *C. angulifer* as a sister taxon of either the other *Chilabothrus* species with divergence time estimated to 21.7 [16.9, 26.0] Mye (Reynolds et al. 2013) or the Puerto Rican clade with divergence time estimated to 15.3 Mye (Pyron et al. 2013, Reynolds et al. *2*015, 2016a, Landestoy, Reynolds & Henderson 2021). In any case, *C. angulifer* represents a phylogenetically deep and distinct lineage of boids. Besides, its evolutionary history, there are multiple phenotypic characters distinguishing this species from its relatives (see also Reynolds et al. 2016a). *Chilabothrus angulifer* is (1) the largest form of the genus *Chilabothrus* (Tolson 1987, Rodríguez-Robles & Greene 1996), (2) with small litter size, (3) extremely large newborns and (4) heavy maternal investment per offspring (e.g., Tolson 1987, Frynta et al. 2016). A deep divergence of *C. angulifer* from other *Chilabothrus* species and other boids as well as presence of numerous unique morphological, physiological and behavioural characters, makes this island species a good candidate for conservation concern.

Conservation genetics of both wild and *ex situ* populations was thoroughly studied in multiple species of the genus *Chilabothrus*, especially in *C. subflavus* (Tzika et al. 2008, 2009, Newman et al. 2020), *C. inornatus* (Aungst et al. 2020, Puente-Rolón et al. 2013, Reynolds et al. 2014b), *C. monensis* (Rodríguez-Robles et al. 2015, Reynolds et al. 2015), *C. argentum* (Reynolds 2016b) and *C. chrysogaster* (Reynolds et al. 2011). The within-species genetic variation reported by these studies was rather small, well-corresponding to limited population numbers and fragmented distribution range of these endangered species (Harvey & Platenberg 2009, Newman et al. 2016, Tucker et al. 2020). In

contrast to a considerable efforts devoted to genetics of these *Chilabothrus* species, *C. angulifer* has remained nearly neglected in this respect. There are just papers concerning chromosomal evolution (Augstenová et al. 2019) and parthenogenesis (Seixas et al. 2020).

The Cuban Boa remains the only one of the three largest Cuban reptiles for which a more detailed population and habitat viability assessment (PHVA) has not yet been performed, while the PHVA is available for the Cuban crocodile, *Crocodylus rhombifer* (Soberon et al. 2000) and for the Cuban iguana, *Cyclura nubila* (Rodríguez et al. 2003). The species is listed as Near Threatened in the Cuban national Red List assessment (González et al. 2012), as Least Concern according to the IUCN Red List (Fong et al. 2021). However, the real conservation status of *Chilabothrus angulifer* is not well known (although a number of recent studies have significantly expanded our knowledge of this species - e.g. Dinets 2017, Rodríguez-Cabrera et al. 2015, 2016, 2020). The species is also granted international protection under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Appendix II). An important element of species protection is - in accordance with the modern concept of the conservation strategy (the One Plan Approach to Conservation) - adequate perspective management of the *ex situ* population in human care. The conservation breeding is listed among the recommended elements for the protection of Cuban boas in the Cuban National Red List assessment (Rodríguez Schettino 2012).

The effort to keep Cuban boas in human care is old (Rehák, studbook data). The Philadelphia Zoo, USA, imported twenty Cuban boas already in the 19th century (from 1876 to 1893) and another ten between 1900 and 1901, and by 1956 another fifteen specimens. At that time, their survival was usually very poor and they did not reproduce (or the reproduction was unsuccessful as in the Berlin Zoo, Germany, where the Cuban boa was also kept at the beginning of the last century). At that time, the *ex situ* population consisted exclusively of specimens imported from the wild. The birth of live young in captivity was achieved in the late 1950s (1958 in the Smithsonian National Zoological Park, USA).

In 60s, 70s and 80s, owing to close political and economic relationships with Cuba, Cuban boas were repeatedly imported to Zoos and private holders in the former Czechoslovakia (e.g. Prague Zoo imported the first Cuban boas directly from Cuba in 1963 and 1965 - Rehák, studbook data), German Democratic Republic, Soviet Union, Hungary and Poland. An independent source of imports represented the U.S. Naval Base, Guantanamo Bay, Cuba. From the end of the 1970s, in addition to imports from the wild, the successful captive breeding began to make a significant contribution to the formation of the *ex situ* Cuban boa population in human care (e.g. Huff 1976, Nowinski 1977, Murphy et al. 1978, Vergner 1978, Tolson 1983, Bloxam & Tonge 1981, Vergner 1989, Rehák 1992). Captive propagation of *C. angulifer* (although originally regarded as a difficult species to breed) began to be very successfull despite its small litter size and extremely slow life-history. Cuban boas have become common in Zoos, public and also some private collections (Marešová & Frynta 2008).

With the political changes at the turn of the eighties and nineties of the last century, imports from nature came to an almost complete end. The future of the *ex situ* population in human care began to depend almost exclusively on captive breeding. It became clear that Zoos must focus on the long-term perspective management of the ex situ population. At the initiative of the Prague Zoo and the Amphibian and Reptile Taxon Advisory Group of the European Association of Zoos and Aquaria (chaired by I. Rehák), the Studbook for Cuban boa has started (the first issue - Rehák 1994, the European Studbook for Cuban boa is maintained and continuously updated in an electronic form - I. Rehák, studbook keeper) and the European Endangered Species Programme (newly the EAZA Ex situ Programme) for Cuban boa was proposed. Consequently, the Cuban boa EEP - coordinated by Ivan Rehák/Prague Zoo - was approved by the EAZA and launched in 1993.

The contemporary European Zoo population may represent descendants of many founders originated in multiple localities across Cuba and its neighbouring small islands. Consequently, mitochondrial lineages from European Zoos may be viewed as more or less representative population sampling of Cuban population. Now, more than thirty years after the end of imports from the wild (after 1989, only one import from the wild is registered in the European studbook - Rehák, studbook data) to Europe and several generations of captive breeding, however, founding individuals already died (at present, only the last two specimens collected from the wild are still alive - Rehak, studbook data) and records about their geographic origin are available only in handful exceptional cases.

The aim of this paper was to provide a first insight to conservation genetics of *C. angulifer.* For this purpose, we described and analysed mitochondrial genetic variation within a population of *C. angulifer* kept in European Zoos and associated private holders. The sampling was performed two decades ago during the first years of this millennium. We addressed two main questions. (1) What can the variability estimated within *ex situ* population tell us about variability and historical demography of wild populations? (2) Is there molecular evidence that founders of this *ex situ* Zoo population were variable enough to provide a good prospect for further maintenance of this population?

MATERIAL AND METHODS

Sampling

We analysed 96 new samples of *C. angulifer* (Figs 1-3) and one new sample of *C. inornatus* (see Table 1). The geographic origin of the sampled specimens or their maternal ancestors was certain in just few cases representing localities across the Cuba island (Fig. 4).

The sampling, sequencing and preliminary analyses were carried out during the years 2002-2009. We decided to rely on non-invasive sampling causing no harm and minimizing stress in sampled specimens. Thus, we selected buccal swabs as a source of DNA. We sampled specimens kept by European Zoological gardens and collaborating private breeders. We aimed to include putative

178 founders or their maternal descendants (daughter, granddaughter etc.). In order to avoid multiple
179 sampling of the same maternal lineage, we inspected pedigree data if available.
180
181 **DNA Extractions and Sequencing**
182 We sequenced two mitochondrial genes, combined the new sequences with previously published data
183 (Campbell 1997, Reynolds et al. 2013, Rivera et al. 2011), and supplemented them with additional
184 sequences from GenBank (Phylogenetic analyses).
185 The two genes included 1133 base pairs (bp) of cytochrome b (CYTB) and 814 bp of NADH
186 dehydrogenase subunit 4 (ND4) gene, partial cds; tRNA-His and tRNA-Ser genes. These genes were
187 chosen according to their phylogenetic information content in previous studies with the same
188 taxonomic scope and availability of sequences. Genomic DNA was extracted from 96% ethanol-
189 preserved buccal swab with NucleoSpin Tissue kit (Macherey-Nagel) according to manufacturer's
190 protocol for buccal swab isolation. Extracted DNA was stored at – 18 °C until used as a template for
191 Polymerase Chain Reaction (PCR, Sambrook, Fritsch & Maniatis 1989). Individual markers were
192 amplifed by PCR using the same combination of primers like in previous studies. The entire CYTB
193 gene 1113 bp long was amplified using primers L14910 (5'-GAC CTG TGA TMT GAA AAC CAY
194 CGT TGT-3') and H16064 (5'-CTT TGG TTT ACA AGA ACA ATG CTT TA-3') and conditions
195 from Burbrink, Lawson & Slowinski (2000). For ND4 gene we used a modified primer pair (L) ND4
196 (5'-TGA CTA CCA AAA GCT CAT GTA GAA GC-3') and (H) Leu (5'-TRC TTT TAC TTG GAT
197 TTG CAC CA-3') (Keogh et al. 2008) and conditions from Arevalo, Davis & Sites (1994).
198 **Phylogenetic and demographic analyses**
199 We prepared four data sets for phylogenetic analyses performation – CYTB alignment 1059 bp long,
200 ND4 alignment 814 bp long and two alignments combining CYTB and ND4 sequences of total lenght
201 1874 bp containing sequences downloaded from GenBank used as outgroup (*C. fordii* – KP746467,
202 KP746549; *C. striatus* – KP746472, KP746555; *C. monensis* – KP746425, KP746430, KP746507,
203 KP746512; *C. inornatus* – KC819501, KC819502, KP746552, 746553; *C. exsul* – KC329926,
204 KC329959; *C. chrysogaster* – KC329925, KC329958; *C. subflavus* – KC329948, KC329973;
205 *Epicrates cenchria* – KC329950, KC329974; *Epicrates maurus* – KC329951, KC329976; *Eunectes*
206 *murinus* – KC329952, KC329977; *Eunectes nataeus* – KC329953, JN967256).
207 The estimates of evolutionary divergence over sequence pairs between haplotypes of *C.*
208 *angulifer* population were conducted using the maximum composite likelihood model implemented in
209 MEGA6 (Kumar et al. 2018; Tamura et al. 2013). The relationship within population of *C. angulifer*
210 was represented by using the Median-Joining network approach (Bandeltet al. 1999) in the program
211 Population Analysis with Reticulate Trees (PopART; Leigh and Bryant 2015). Two haplotype
212 networks were worked out – for alignment containing all 96 CYTB sequences of *C. angulifer* and for
213 alignment ND4 containing one sequence of each CYTB haplotype.

Phylogenetic reconstructions were conducted using Bayesian analysis (BA), maximum likelihood (ML) and Bayesian interference (BI) for alignments combining the CYTB and ND4 sequences. Bayesian analyses (BA) of both multilocus alignments (CYTB, ND4) were performed using MrBayes v3.2.6 (Huelsenbeck et al. 2001; Ronquist et al 2012). The best-fit models of sequence evolution were selected under MrModeltest 2.3 (Nylander 2004). The six partitions were set following way: three for CYTB and three for ND4 gene (CYTB: GTR+I+G pos1, GTR+I+G pos2, GTR+I+G pos3; ND4: GTR +I+G pos1, GTR+I+G pos2, GTR+I+G pos3). Two independent runs of BA were conducted with a random starting tree and run for 20,000,000 generations, with trees sampled every 1000 generations and with 25% burn-in. The ML analysis of multilocus alignment (CYTB, ND4) was performed in IQ-TREE (Nguyen et al. 2015) using the online web interface W-IQ-TREE (Trifinopoulos et al. 2016). The best substitution model for both genes was selected automatically by ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE. The best model of nucleotide substitution for the partition by gene scheme was identified as follows (CYTB: TIM2e+G4 pos1, TN+F+I pos2, TIM2+F+G4 pos3; TIM2+F+G4 pos1, HKY+F+I pos2, HKY+F+G4 pos3).

BEAST v.2.6.6. (Bouckaert et al. 2014) was used for the BI analysis, the two partitions were set. The GTR model was selected as the best model of nucleotide substitution by jModelTest v.2.1.6. (Darriba et al. 2012) for both partitions, the CYTB and ND4. Site and clock models were unlinked across partitions. Strict clock model was used for all partitions. According to Reynolds et al. (2013), we set the prior of *Chilabothrus* root node to 21.7 Mye (SD = 1.8).

Polymorphisms within *C. angulifer* population were worked out by the statistic software DnaSP v6 6.12.03 (Rozas et al. 2017) which estimated the following: haplotype diversity (h), segregating sites (S), nucleotide diversity (π), and Tajima's D, Fu & Li's F*, Fu & Li's D*, and Fu's FS tests. According to Russell, Medellín & McCracken (2005), high values of h and π indicate a constant large population size. However, a low value of π and high value of h signifies a recent expansion. To estimate population dynamics through time we have constructed a model in BEAUti, 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 0.02 per 3 million years in BEAST (Rodríguez-Robles et al. 2015). We have analysed the outputs in TRACER v1.7.1 and displayed them as Bayesian skyline plot drawing the effective population sizes over time.

RESULTS

We sequenced cyt b in 96 specimens of *C. angulifer.* We identified 25 distinct haplotypes (Table 1). While 13 of them were detected exclusively in a single specimen, the remaining ones were found in multiple individuals (2, 2, 3, 4, 4, 5, 5, 5, 7, 8, 9 and even 28 times).

Median-Joining Network (MJN) revealed a presence of three clearly distinct main groups, further referred to as Haplogroup I, Haplogroup II and Haplogroup III (Fig. 5). Maximum uncorrected

p-distances among cyt b haplotypes belonging to different groups were 0.0220 (mean = 0.0183), 0.0340 (0.0228) and 0.0293 (0.0277), for I vs II, I vs III and II vs III, respectively. Corresponding values for within group comparisons were 0.0121 (0.0076), 0.0083 (0.0033) and 0.0293 (0.0101), for groups I, II and III, respectively (Table 2).

Bayesian spline-plot of this dataset revealed a long-term stability of effective population size during the last three millions of years followed by a recent decline (Fig. 6). Population parameters estimated separately for each haplogroup, as well as for pooled ones, are provided (Table 3). These parameters are congruent with relative stability of the population numbers in the past. The only exception represents Haplogroup II exhibiting negative values of parameters indicating recent population expansion (significant at $P < 0.05$).

In addition, we sequenced ND4 gene in representatives of each cyt b haplotype. This proved presence of 19 ND4 haplotypes. The resulting network pattern of ND4 sequences (Fig. 7) clearly resembled that of cyt b one (Fig. 5).

In order to recover phylogenetic relationships among *C. angulifer* haplotypes, we run Bayesian analysis. The analysed alignment contained both examined mitochondrial genes for all 25 *C. angulifer* haplotypes and outgroups. The results corroborated that the groups II and III form well-supported monophyletic clades (posterior probability = 1). In contrast to this, the group I splits into two lineages. One of them has a sister relationship to a clade including both remaining groups (II+III), while the other represents a basal most offshoot of *C. angulifer* tree (Fig. 8).

Next, we examined an alignment (cyt b and ND4) including two representatives of each *C. angulifer* haplogroup, other *Chilabothrus* species and outgroups (*Eunectes* and *Epicrates*). We employed Bayesian and Maximum Likelihood approaches. The results of both computation methods were virtually the same and confirmed the above described topology (Fig. 9).

Finally, we run BEAST and constructed time-calibrated tree to estimate timing of divergence among *C. angulifer* haplogroups (Fig. 10). In contrast to the previous analyses, it placed group III as a sister of groups I + II. The last common ancestor of all *C. angulifer* haplogroups was estimated to 3.57 (95% CI: 2.37-4.82) Mye, while the split between the group I and II to 2.26 (95% CI: 1.43-3.09) Mye.

DISCUSSION

Haplotype diversity

We sampled 96 individuals and detected presence of 25 mitochondrial haplotypes in examined captive population of *C. angulifer*. Although, we tried to avoid sampling of close maternal relatives, we repeatedly found multiple occurrence of the same haplotype coming from the same institution (cf. Table 1). It is likely, that we sampled multiple maternal descendants of the same founder in some cases. Thus, we even underestimated haplotype diversity among the founders of the examined captive

288 population. This clearly supports the view that haplotype diversity in the source natural populations of
289 this endangered snake was extremely high.
290 **Divergence among haplogroups**
291 The deepest divergence among principal haplogroups of *C. angulifer,* we report here (3.57 Mye, 2.37-
292 4.82), suggest that they split already in the Pliocene period. This estimate is of comparable magnitude
293 as those previously reported between clearly distinct species of the genus *Chilabothrus* belonging to
294 the same major clade of this genus (for these clades see under Introduction). Specifically, divergences
295 within *ampelophis-fordii*, *exsul-schwarzi-argentum-striatus-strigillatus* and *monensis-granti* clades
296 are possibly even more recent (cf. Landestoy et al. 2021). Of course, it can be assumed that in widely
297 distributed Cuban boas inhabiting a relatively very large island (and its nearby coastal archipelagos)
298 with a complicated geological history, the genetic structure and phylogeography will be much more
299 complicated, making the interpretation of detected divergences much more difficult and complex than
300 in the case of isolated congeneric species from small islands or islets. Current advanced genomic
301 studies performed in continental rattlesnakes of the genus *Crotalus* showed complex evolutionary
302 history of these snakes. Episodes of temporal allopatry accompanied by genetic drift and divergent
303 selection were repeatedly followed by secondary contacts with pervasive gene flow even between
304 anciently diverged lineages (Schield et al. 2015, 2017, 2018, 2019).
305 Sequence divergence of mitochondrial genes among mainland species of boas tend to be
306 higher than among the haplogroups of *C. angulifer*, e.g., *Epicrates* (Passos & Fernandes 2008, Rivera
307 et al. 2011), *Boa* (Hynková et al. 2009), *Corallus* (Colston et al. 2013), *Eryx* (Eskandarzadeh et al.
308 2020a,b), *Acrantophis* (Vences & Glaw 2003) and *Candoia* (Austin 2000). There are, however,
309 multiple exceptions, e.g., sequence divergence between the South American *Epicrates cenchria* and
310 its sister species *E. maurus* with predominantly Central American range is very close to that between
311 *Chilobothris angulifer* haplogroups III and I+II (Rivera et al. 2011). Similarly, geographically
312 localized haplogroups within *Boa imperator* exhibiting distinct parapatric geographic ranges may be
313 viewed as cryptic species or at least subspecies (Suárez-Atilano et al. 2014, Card et al. 2016). Island
314 populations of *B. imperator* are challenging. The discordance between very small sequence
315 divergence and parallel change in morphological and developmental traits reminds us that magnitude
316 of the adaptive evolution is not necessarily proportional to expired time (Boback 2006, Boback &
317 Siefferman 2010, Green 2010, Bushar et al. 2015, Card et al. 2019). In pythons, the snakes bearing
318 adaptive strategies comparable to that of boas (Esquerré & Keogh 2016), we can found multiple
319 examples demonstrating above discussed phenomena (Rawlings et al. 2008, Esquerré et al. 2020).
320 **Hybridization between snakes belonging to different mitochondrial clades**
321 Sequence divergence in mitochondrial genes can be used as a predictor of ability to hybridize
322 (Jančúchová Lásková et al. 2015a). Although, the 3% genetic divergence among haplogroups of *C.*
323 *angulifer* is comparable with that among distinct species of boids and pythons (see above), it is still
324 much smaller than that between the most distant species of squamates that are still able to interbreed

and produce fertile hybrids (Jančúchová Lásková et al. 2015b, for a review see Jančúchová Lásková et al. 2015a). In captive *C. angulifer*, we already proved fertility of hybrids between individuals belonging to different haplogroups (unpublished data).

Why are the haplogroups so divergent within a single island

We report here a surprisingly deep divergence among mitochondrial haplogroups of *C. angulifer*. This requires persistence of maternal lineages within this species for a long period of last few millions of years. According to the phylogeographic and coalescent theory (cf. Avise 2000), this may be explained either by (1) an extremely large and stable population size, or by (2) spatial subdivision of the species into multiple isolated populations, each locally maintaining a certain haplogroup.

The first hypothesis, requiring an extremely large population size, is compatible with the view that prior colonization of the island by humans, *C. angulifer* belonged to principal top terrestrial predators of Cuba. Compared to "warm blooded" predators, the ratio between biomass of predators and their prey is much higher in the case of "cold-blooded" predators like snakes. Thus, we cannot exclude that *C. angulifer* was present in high densities and distributed throughout the territory of this large island. Although recent records of *C. angulifer* are absent in some zones of the island (Rodríguez Schettino et al. 2013), the historical distribution range could be fairly continuous. Although, *C. angulifer* was repeatedly reported from several Pleistocene localities (cf. Syromyatnikova et al. 2021 and reference herein), we may only speculate about real population size of *C. angulifer* in the Pleistocene or Pre-Colombian periods. Our Bayesian-spline estimating demography for a quasi-unstructured population revealed that except very recent decline the population was stable during the last three millions of years.

Although, slow life history of *C. angulifer* may also contribute to extension of coalescence time (but see Rodríguez-Cabrera et al. 2016 reporting early sexual maturation in natural population), the first hypothesis is not in accord with a strong phylogeographic structure and/or endemism reported in other Cuban reptiles (see below for rock-iguanas).

The second hypothesis might be supported by the fact that Cuba consists from multiple continental fragments, originally representing separate islands. During the Lower Middle Miocene (14-16 Mye), there were at least four main islands, i.e., Western, West Central, East Central and Eastern islands that have been joined only recently. The Western island was the most distant from the others. Formation of Cuba in its current form has been completed in Pliocene, roughly 4 Mye ago (see Iturralde-Vinent 2006). Therefore, estimated divergence time among *C. angulifer* haplogroups (3.57 Mye) is not old enough to be compatible with the scenario suggesting a secondary contact of haplogroups initially evolved in isolation on precursor islands. Nevertheless, as the confidence interval of our estimate is too wide (2.37-4.82 Mye), this scenario cannot be ruled out entirely.

Putative subdivision of *C. angulifer* populations after final formation of the island during Pliocene is more consistent with our estimates of divergence time among three principal haplogroups. We have no evidence about permanent geographic barrier dividing the island during the Pliocene-

Quaternary period, however, lowland parts of the island were periodically inundated during the interglacial maxima. This process temporarily separated the area into three or even more pieces. On the contrary, the Isla de la Juventud would merge with mainland Cuba when the sea level would drop by about 18 m, while during the last glacial maximum (cca 20,000 years ago) the sea level was about 125 meters lower than it is today (Fairbanks 1989, Tolson & Henderson 1993, Poore et al. 2000, Steadman & Franklin 2017).

A strong male-biased dispersal was reported in many snake species (e.g., Rivera, Gardenal & Chiaraviglio 2006, Keogh et al.2007, Dubey et al. 2008, Pernetta et al. 2011, Folt et al. 2019). This mechanism may contribute to a limited flow of maternally inherited mitochondrial genes and consequent geographically limited distribution of haplotypes. Therefore, an isolation by distance should be also considered, besides a true geographic barrier. A study performed in continental coralsnakes recently demonstrated that spatial sorting of their mitochondrial haplotypes can be attributed to genetic surfing caused by stochastic and demographic processes rather than to allopatric divergence (Streicher et al. 2016).

In general, all scenarios involving the second hypothesis predict presence of a clear geographic distribution pattern of the haplogroups. Below we discuss evidence supporting this prediction.

Geographic distribution of the haplogroups

We sampled captive population, decades after last imports from wild. Thus, we rely on only anecdotic records about the precise geographic origin of examined haplotypes.

The sample belonging to the maternal descendant of the founder coming from Viñales in the Western part of Cuba is the only clearly localized sample of the Haplogroup I comprising 7 haplotypes (23 individuals). The most common origin of imported specimens of *C. angulifer* were probably provinces surrounding the capital Havana. Multiple founders possessing haplotypes belonging to a haplogroup II, which is the most represented in our sampling (8 haplotypes, 48 individuals), probably come from this part of the island, nevertheless we can be sure about it in just one case (locality Matanzas). Among 10 haplotypes (24 individuals) belonging to a haplogroup III, only four are of known geographic origin. All of them come from Central (Trinidad) and Eastern Cuba (Nicaro and Sierra Maestra). Another record of Haplogroup III from Eastern part of Cuba represents cyt b sequence (accession No. KC329922) belonging to our haplotype AA1 which was reported from Guantánamo by Reynolds et al. (2013)**.** This may suggest, that Haplogroup III is distributed predominantly in the Central-Eastern parts of the Island, while Haplogroups I and II in the Western areas (see below for a comparison with Cuban rock iguanas).

Although, it is likely that *C. angulifer* haplogroups follow the above suggested geographic pattern, we have no sufficient data to finally prove this hypothesis and further research in the wild is needed to solve this problem.

A comparison with phylogeography of other species

Cuban rock-iguana (*Cyclura nubila*) belongs to the most charismatic species of squamates inhabiting Cuba. Because its large body size and similar history on the Great Antilles, this species provides a reliable comparison with *Chilabothrus angulifer*. Similarly, as in *C. angulifer*, there is a deep divergence among mitochondrial haplotypes of this iguana (Frynta et al. 2010). Recently, Shaney et al. (2020) sampled multiple populations of *Cyclura nubila* and demonstrated that each geographically defined population of *C. nubila* is characterized by an exclusive group of closely related haplotypes. Moreover, the haplotypes coming from the western and eastern parts of Cuba form mutually clearly distinct clades exhibiting parapatric distribution. Each of these clades further splits into several local haplogroups. The split between these principal clades is deep, they estimated their divergence to 6.8 Mye (Shaney et al. 2020). Nevertheless, confidence interval of their estimate (4.2–9.7 Mye) partly overlaps that we computed for a split between major mitochondrial clades (I+II versus III) of *Chilobothris. angulifer* (2.37-4.82 Mye). Thus, the divergence patterns recovered in these two charismatic reptilian species may reflect the same underlying process.

A deep split between Western and Eastern clades of cyt b (5.2%) was also reported in endemic rodents of the genus *Capromys*. Nevertheless, as a result of more rapid substitution rate in rodents, a divergence time between these clades was estimated just to 1.1 Mye (Upham & Borroto-Páez 2017). Interestingly enough, geographic distribution of these *Capromys* clades follows almost precisely the pattern reported in *Cyclura nubila* (Shaney et al. 2020).

Phylogenetic relationships among the haplogroups I, II and III

The topologies recovered by MrBayes and maximum likelihood proved that haplogroups II and III are monophyletic and mutually exclusive. These methods, however, failed to support monophyly of Haplogroup I and placed lineages belonging to this group as sister clades of the remaining haplotypes belonging to groups II and III (Figs 8 and 9). In contrast to this topology, a time-calibrated tree produced by BEAST reveal basal split between Haplogroup III and the clade including mutually sister clades formed by haplogroups I and II. We prefer the topology of the time-calibrated tree (Fig. 10) because of following reasons: (1) The divergence between *C. angulifer* and its closest relatives of the the Puerto Rican clade (15.3 Mye) is much longer compared to the deepest split between *C. angulifer* haplogroups (3.57 Mye). Thus, finding a proper root of the haplotype tree is really difficult and uncertain. (2) The split between groups III and I+II is in accord with the haplotype networks, (3) In the analyses placing haplogroup III with II, the branch leading from their common ancestor to the Haplogroup III is long.

Conservation genetics of European ex situ population

On one hand, we reported the deep splits among principal mitochondrial haplogroups and thus we have to expect that natural populations of *Chilabothrus angulifer,* similarly like those of the Cuban rock-iguanas (*Cyclura. nubila*), are divided into multiple conservation units according to coalescence

of their genes (cf. Shaney et al. 2020). After several generations of captive breeding (the generation time for *C. angulifer* is about 11.0 years, the oldest captive born Cuban boa, who contributed to the current European population with offsprings, is a female born in 1973 - Rehák, studbook data) and interbreeding among *C. angulifer* originated from multiple populations, pure-bred lineages are rare and thus not sufficient for establishing viable population. Such an attempt, reflecting our finding, that Cuban boas represent at least two different subspecies or species (depending on the criteria and applied species concept). would require derivation of new *ex situ* populations from the wild (as pointed out already by Rehák 2006, 2008).

On the other hand, Zoo populations of the reptiles are typically extremely small (Frynta et al. 2010, Marešová & Frynta 2008) and thus suffer from inbreeding rather than outbreeding depression. The number of maternally unrelated founders of the European Zoo population reflected by haplotype diversity reported in this study is large. It clearly confirms the view that the number of founders and their genetic variability at the beginning of this millennium were large enough to create a viable *ex situ* population of this endangered snake species. Nevertheless, an initial expansion, outbreeding, maintenance at a sufficient population size and regular breeding are required to prevent loss of genetic variation and viability of the population.

Recent population of Cuban boas in zoos (as well as in private collections) consists mostly of captive born animals originated of founders imported from wild – especially in 1970´ and 1980´ (Rehák, studbook data). The species is currently mainly found in European institutions, with just a few others in Asia and North America, so the management at the European level (European *ex situ* population) is a convenient option. The current population is descended from at least of fifteen founders, but any more accurate number is not possible to calculate because about 80% of specimens have unknown pedigree. For the same reason, a more detailed population assessment cannot be performed, population projections cannot be accurately created and thus important sources for the well based establishing of the Long-term Management Plan for Cuban boas in human care are missing. Currently, 233 living Cuban boas (68 males, 80 females and 85 of undetermined sex - held in 63 cooperating institutions) are registered in the European studbook for Cuban boas (Rehák, ed.), which is a sufficient and adequate number for management (the current population size may be able to minimize random demographic and catastrophic events for the long-term) and at the same time a number requiring adequate management. We do not have any data to suggest reduced fitness, survival or reproductive problems in connection with crossbreeding.

At the same time, we consider that although the divergence among some populations of Cuban boas is greater than that of some other, taxonomically recognized, species (see above), it is important to note that most of these species are isolated island species "doomed" to evolve independently, whereas in the case of Cuban boas, the individual evolutionary lines previously formed during Cuba's complex geological history, currently inhabit a single island (with the surrounding

nearby coastal archipelagos), and it can be assumed that their future evolution is likely to be associated with the unavoidable hybridization.

In conclusion, we therefore recommend the management of the existing *ex situ* population of Cuban boas in human care as a whole, as a single unit. At least until a more accurate picture of the phylogeography of Cuban boas, the geographical distribution of their individual evolutionary lines, their natural hybridization and the possible existence of hybrid zones is available.

Limitations of the study.

Our study is based on genetic samples from captive animals. Thus, we relied solely on maternally inherited mitochondrial genes. As the examined individuals were mostly descendants of individuals coming from different regions of the Island, the utility of biparentally inherited nuclear genes was greatly limited. Moreover, sex chromosomes of *C. angulifer* are not clearly differentiated (Augstenová et al. 2019), which prevented us to employ Y-chomosome. Therefore, further genetic examination of wild populations of *C. angulifer* including genomic approach is urgently needed to complete the picture.

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Table captions:

Table 1. List of samples collapsed to 25 haplotypes according 1059 bp long allignment of cyt b with description of three haplogroups (red highlighted haplotypes were not identified by analyse of 553 bp long alignment of cyt b, these sequenced were included in haplotype 23). The samples names were created by using unique code helping to identify the Zoological garden or breeder, where the samples were collected (AA – Aalborg Zoo, Denmark; B –Budapest Zoo, Hungary; BAR – Barcelona Zoo, Spain; BOJ – Bojnice Zoo, Slovakia; BRF –Burford Zoo, UK; BRS – Bristol Zoo, UK; C – Colchester Zoo, UK; CH – Kharkov Zoo, Ukraine; CHS – Chester Zoo, UK; KAL – Kaliningrad Zoo, Russia; L – Lisbon Zoo, Portugal; PL –Plock Zoo, Poland; R – Rotterdam Zoo, Netherlands; RG – Riga Zoo, Latvia; RO – Rostock Zoo, Germany; TOR –Toruň Zoo, Poland; rest of samples came from Prague Zoo, CZE and collaborating private holders). If available, the geographic origin of the sampled specimen or its matriline is indicated in parentheses (in bold)

Table 2**.** Estimates of evolutionary divergence over sequence pairs between haplotypes of *C. angulifer* population. The mean number, minimum, and maximum of base substitutions per site overall sequence pairs within each group is shown. Analyses were conducted using the maximum composite likelihood model implemented in MEGA6 (Tamura et al. 2013). alignment 1059 bp of CYTB

Table 3**.** Demographic characteristics for the *C. angulifer* based on the 1059 bp mitochondrial CYTB alignment. Sequences: number of individuals sequenced (Ns), number of segregating sites (S), number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), Fu & Li's F*, Fu & Li's D*, Fu's FS, Tajima's D

Figure captions:

Fig. 1. Adult female of *Chilabothrus angulifer* bearing haplotype UD4 (Haplogroup I).

Fig. 2. Subadult female of *Chilabothrus angulifer* bearing haplotype F8 (Haplogroup II).

Fig. 3. Adult male of *Chilabothrus angulifer* bearing haplotype 93 (Haplogroup III).

Fig. 4. Map of Cuba island with locality specification (1: Sierra Maestra, 2: Nicaro, 3: Trinidad, 4: Matanzas, 5: Viñales).

Fig. 5. Median-joining haplotype network computed using CYTB 1059 bp alignment containing all 96 samples of *C. angulifer* (Bibron), the size of the circle is proportional to the frequency of haplotypes and the mutational steps are indicated on the branches.

Fig. 6. The Bayesian skyline plot (BSP) of sequence variability in CYTB 1059 bp long for the *C. angulifer* population, visualizing the effective population sizes over time.

Fig. 7. Median-joining haplotype network computed using ND4 814 bp alignment containing all 96 samples of *C. angulifer* (Bibron), the size of the circle is proportional to the frequency of haplotypes and the mutational steps are indicated on the branches.

Fig. 8. Bayesian tree computed from multilocus alignment (CYTB and ND4 genes). Statistical support of the nodes is expressed as a posterior probability (posterior probabilities > 0.95 are shown)

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Haplogroup number	Name	Nr of sequences	Samples (Locality)	Cyt b	ND4
I	UD4	2	K1, UD4	OL158846	OM648935
I	UHO	4	F9, S101, UD3, UHO	OL158848	OM648937
I	ML1	1	ML1	OL158847	OM648936
I	R703	1	R703	OL158849	OM648938
I	17	9	17, 18, 77, BN1, E42, EX1, F3 (**Viñales**), U1, U4	OL158850	OM648939
I	P1	5	P1, P5, PR1, PR2, PR6	OL158851	OM648940
I	E43	1	E43	OL158852	OM648941
II	14	7	14, 15, EA, KHN0, M14, M18, ST1	OL158855	OM648944
II	U3	29	16, AA3, B333, BAR2, BLL, BOJ3, BR4, BR5, BR7, BR6, BRF156, BRS1, BRS2, BRS3, C1, C2, F16, F17, DR115, DR117, DR118, KAL1, KAL0, L6504, M19, MD114, RG4, U2, U3	OL158854	OM648943
II	KP1	8	P2, P3, P4, PR3, PR4, PR5, KP1, KT0	OL158858	OM648947
II	F8	1	F8	OL158856	OM648945

II	78	1	78	OL158860	OM648949
II	MAT1	1	MAT1 (**Matanzas**)	OL158853	OM648942
II	BRF157	1	BRF157	OL158857	OM648946
II	BRF158	1	BRF158	OL158859	OM648948
III	M2	1	M2	OL158867	OM648956
III	TRI	1	TRI (**Trinidad**)	OL158865	OM648954
III	KSC	3	CH1, KSC (**Sierra Maestra**), RG3	OL158866	OM648955
III	93	4	93 (**Nicaro**), TO0, TO1, UH1	OL158862	OM648951
III	PLB	5	PLB, PLA, PLC, TOR0L, TOR1L	OL158863	OM648952
III	RO1	1	RO1	OL158861	OM648950
III	KN1	1	KN1 (**Nicaro**)	OL158864	OM648953
III	H	2	DR116, H	OL158868	OM648957
III	AA1	5	AA1, AA2, BAR3, BAR1, CHS1	OL158869	OM648958
III	BRF155	1	BRF155	OL158870	OM648959

1037

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	Haplogroup I			Haplogroup II			Haplogroup III		
	Min	Max	Average	Min	Max	Average	Min	Max	Average
Haplogroup I	0.000628	0.012102	0.007595	-------	-------	-------	-------	-------	-------
Haplogroup II	0.016076	0.022047	0.018310	0.000627	0.008270	0.003298	-------	-------	-------
Haplogroup III	0.024066	0.034391	0.028801	0.023397	0.329260	0.027666	0.000627	0.029300	0.010094

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Clades	Ns	S	H	h	Π	Fu & Li's *F**	F u & Li's *D**	Fu's *Fs*	Tajima's *D*
All samples	96	62	24	0.876	0.02698	1.05000	1.09863	2.075	0.58061
Haplogroup I	23	25	7	0.791	0.00825	1. 98528	0. 67390	5.368	1.34480
Haplogroup II	49	19	8	0.613	0.0075	-3.24849	-3.31041	-0.373	- 1.65269
Haplogroup III	24	57	10	0.891	0.01261	-0.24742	-0.23195	4.380	-0.16467

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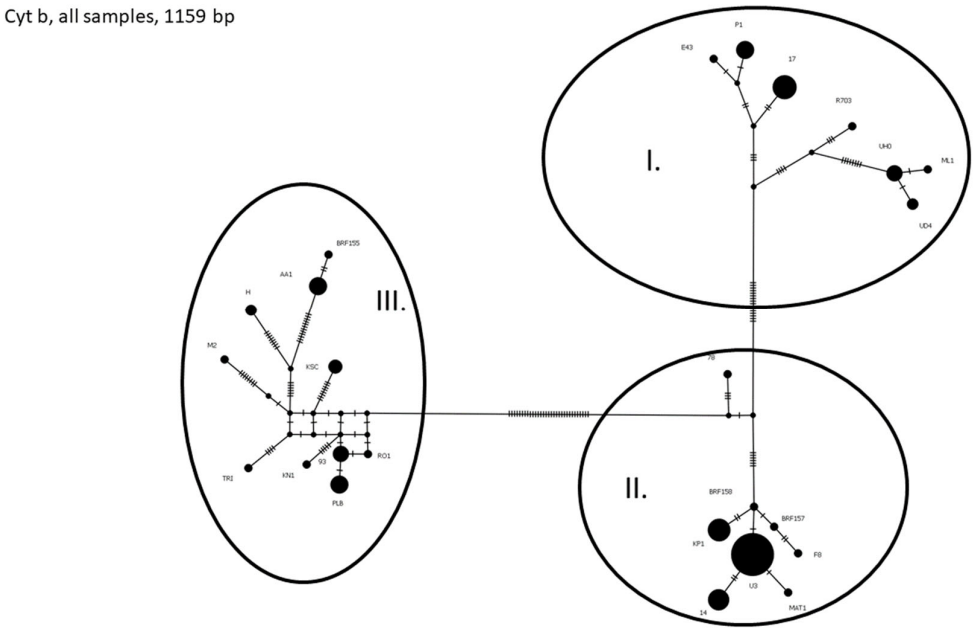

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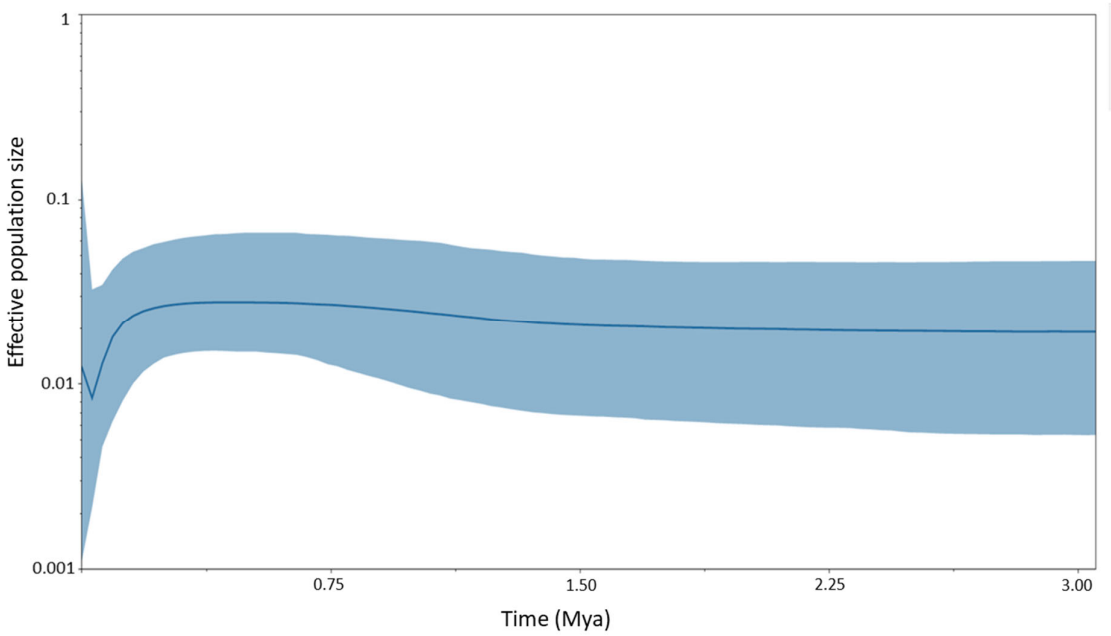

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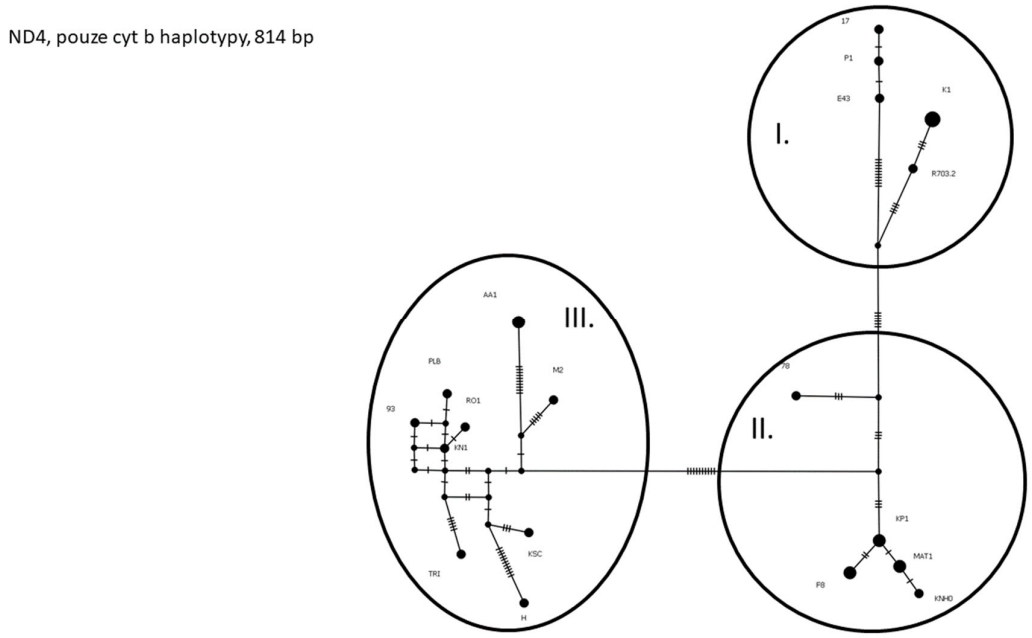

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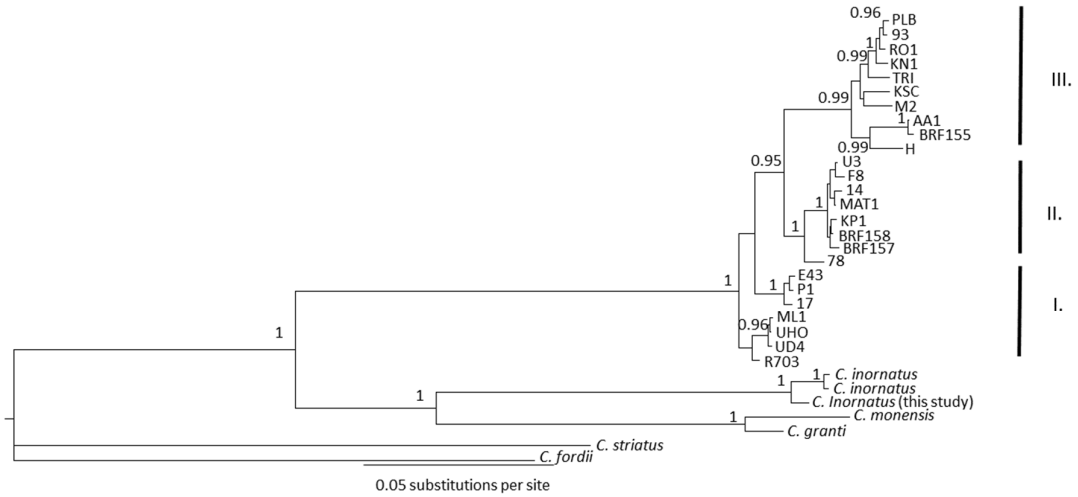

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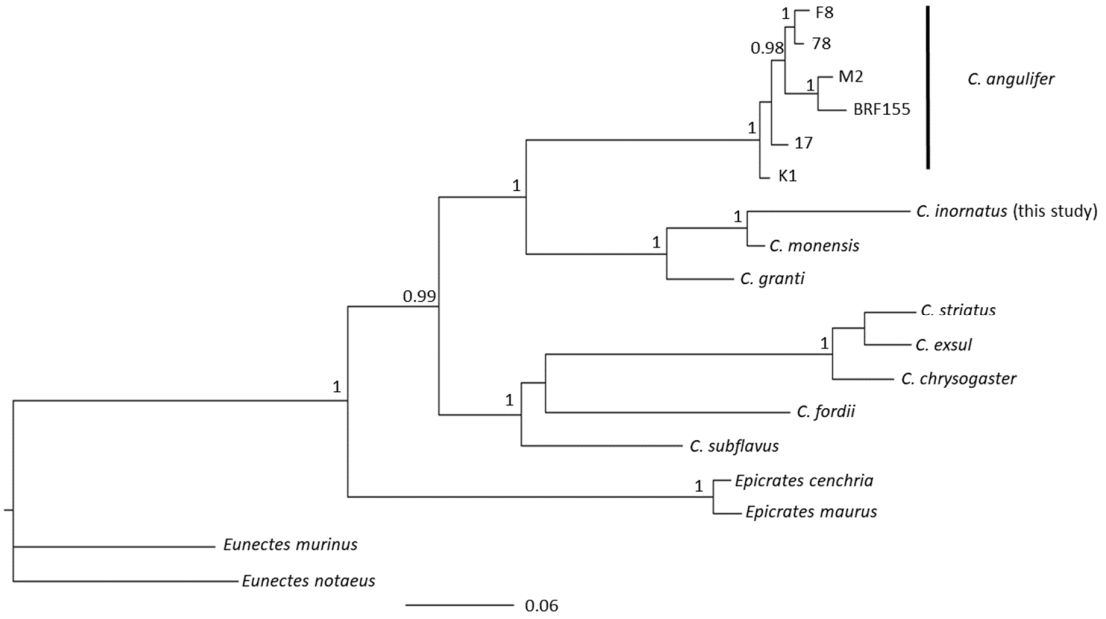

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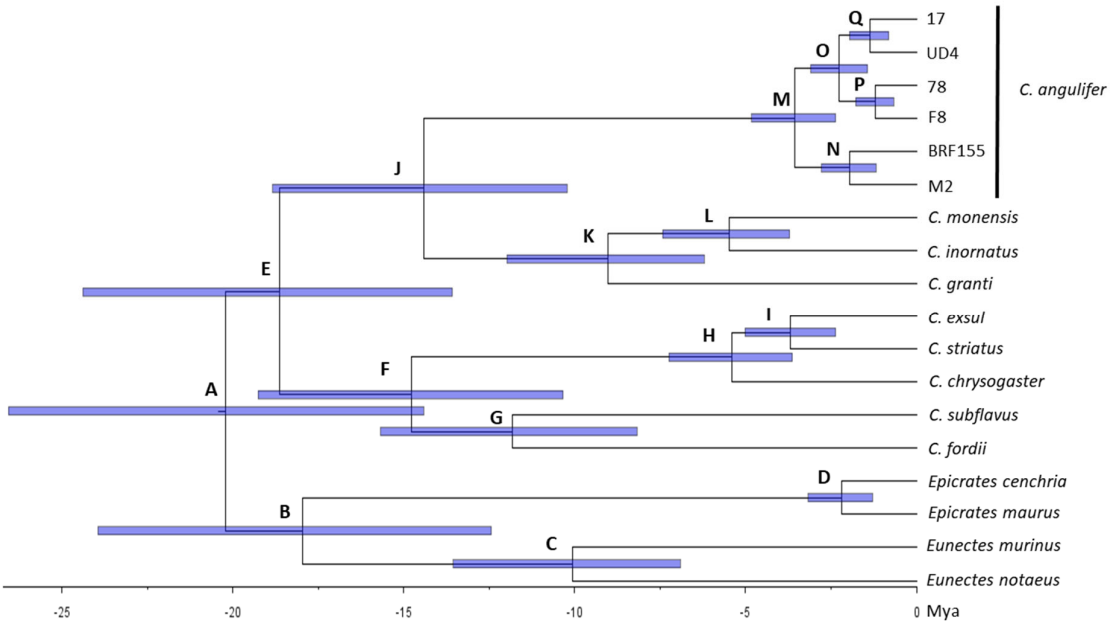

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8.5.

Balasanyan, V., Yavruyan, E., **Somerová, B.**, Abramjan, A., Landova, E., Munclinger, P., & Frynta, D. (2018). High diversity of mtDNA haplotypes confirms syntopic occurrence of two field mouse species Apodemus uralensis and A. witherbyi (Muridae: Apodemus) in Armenia. *Russian Journal of Genetics*, 54(6), 687-697.

Author's contributions: Designed the research (FD and YE), collected the material in the field (FD, LE, AA, BV, YE), **designed and performed the laboratory work** (**SB** and BV), **computed the trees, haplotype network and statistics (SB** and MP) and **wrote the paper** (FD, **BS**, AA, LE, BV).

Barbora Somerová is the third author, nevertheless, she is the first one from our institution. The first two authors are affiliated to Russian-Armenian University in Yerevan.

High Diversity of mtDNA Haplotypes Confirms Syntopic Occurrence of Two Field Mouse Species Apodemus uralensis and A. witherbyi (Muridae: Apodemus) in Armenia

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ANIMAL GENETICS

High Diversity of mtDNA Haplotypes Confirms Syntopic Occurrence of Two Field Mouse Species *Apodemus uralensis* and *A. witherbyi* (Muridae: *Apodemus*) in Armenia[1]

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Abstract—Wood mice of the genus *Apodemus* belong to the most frequent and epidemiologically important rodents of Europe and adjacent regions. Previous studies showed that in the Middle East region species of this genus exhibit extraordinary morphological similarity precluding their proper determination without application of molecular characters. In order to determine the species of the studied populations and to obtain an insight into their phylogeographic history, we analyzed their genetic variation. We sequenced 1139 bp fragment of the mitochondrial DNA control region and flanking tRNA genes in samples collected from six localities. Phylogenetic analyses revealed presence of distinct clades corresponding to species *A. uralensis* and *A. witherbyi*. In most localities we confirmed presence of both species which suggests their large sympatric and syntopic occurrence. We recognized an extensive genetic variability, 38 specimens of *A. uralensis* belong to 32 distinct haplotypes, while 19 specimens of *A. witherbyi* to 14 haplotypes. We confirmed presence of several distinct haplotypes that may originate from multiple wood mouse colonization waves from distinct geographic regions.

Keywords: Apodemus, Transcaucasus, phylogeography, mitochondrial DNA, D-loop

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INTRODUCTION

The Republic of Armenia is a biogeographically distinctive territory with significantly diverse habitats despite of its small area. It lies between Anatolian and Iranian plateaus and nearly half of its territory is consisted by the Lesser Caucasus. Within a relatively limited region, we can find arid subtropical semi-deserts at the very south, alpine landscapes at altitudes of 4000 meters above the sea level, or humid mixed forests at the north. The local biodiversity has been substantially influenced by geological processes, ice ages and related climatic changes in the past, therefore various species from Europe, Middle East and Central Asia meet in this region, as well as many others are endemic to local mountains. Numerous mountain ridges and valleys may have played role in dispersal and speciation of fauna by functioning as barriers, migration routes or refugia [1–8]. However, relatively little research has been done on this topic. Due to complexity of the local biodiversity, not only genetic affinities and variation of the Armenian populations of particular species, but also species identity of some populations, has remained obscure up to these days and often much of accessible information on the local fauna is decades old and very obsolete.

This applies also to the complex of murid species which we aimed on in our study. Wood mice of the genus *Apodemus* Kaup, 1929 sensu lato belong to the most widespread rodents of Eurasia [9, 10]. Their ability to inhabit wide spectrum of habitats from semi-deserts and steppes to woodlands allows these animals to be present in relatively high densities across the landscapes. This predetermines these mice to become valuable component of mammalian communities and food webs and consequently also agricultural and forestry pests and transmitters of diseases (zoonoses). In the Western Palearctics, the wood-mouse species of the genus *Apodemus* (except for *A. agrarius, A. epimelas* and *A. mystacinus*) form a monophyletic group of species which are closely related and morphologically similar to each other [9, 11–17]. Over the past few decades there has been significant number of research on the evaluation of genetic and morphological criteria enabling identification of traditionally recognized European species, i.e., *A. sylvaticus* (Linnaeus, 1758), *A. flavicollis* (Melchior, 1834), and *A. microps* (Krato-

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chvíl and Rosický, 1952) which was currently synonymized with *A. uralensis* (Pallas, 1811) [18–30]. Within the main European clade of the genus *Apodemus* there is a low level of interspecific morphological variation, which can be a result from a bush-like pattern of radiation [31] leading to nearly simultaneous emergence of many species. However, because of this pattern it is also difficult to infer mutual relations of the species, although they are well defined monophylla themselves [12, 30, 32]. Using molecular data along with morphological assessment is especially important in the case of research on wood mice in the Middle East, where there is no accurate taxonomy within the genus *Apodemus*. The latter is a consequence of the difficulty in identifying the species because of the high morphological and ecological similarities of representatives of various species. Studies concerning *Apodemus* phylogeny published over 10–15 years until today used such molecular methods like allozyme analysis (which was one of the first biomolecular methods used by many authors for species determination) [11, 33], restriction fragment length polymorphism (RFLP) [32], random amplified polymorphic DNA (RAPD) [34], nuclear and mitochondrial DNA sequencing [12, 15, 16, 31, 32, 35–39]. Different studies often gave contradictory results about the relationships among *Apodemus* species, but all agree on the distinctness of examined species. Phylogeographic analyses were performed in *A. sylvaticus* and *A. flavicollis* [12, 40–42].

Information on the taxonomy and distribution of *Apodemus* species in the territory of Middle East and Transcaucasus is still incomplete, because published data are based mainly on morphology patterns and molecular methods were used at a limited number of localities across the country [12, 25, 43].

In the neighbourhood of Armenia, five valid species of the genus *Apodemus* have been reported until now [cf. 11, 14, 16, 38, 44–47]: *A. uralensis* (Pallas, 1811), *A. witherbyi* (Thomas, 1902), *A. flavicollis* [42], *A. hyrcanicus* [44] and *A. mystacinus* [43, 48]. Based on a large scale phylogenetic comparison of mitochondrial cyt b gene sequences of *A. uralensis* across the Europe and Central Asia, the eastern populations have been recently recognized as a separate species *A. tokmak* (Severtsov, 1873). The range of this new species is, however, restricted to the Central Asia and separated from the Transcaucasus region by the Caspian Sea.

In this paper, we present new genetic data for wood mice of the genus *Apodemus* from Armenia. We sequenced a fragment of mitochondrial DNA including highly variable control region, performed phylogenetic analysis and visualized haplotype networks. The aim of this study was to (1) verify putative species identity of the collected mice using molecular characters; (2) analyze sequence variation on population level and (3) discuss phylogeographic and demographic interpretations of the results.

MATERIALS AND METHODS

A total of 58 mouse specimens belonging to *Apodemus* species were examined, including pygmy wood mice *A. uralensis* and steppe field mice *A. witherbyi* [49] (Table 1). The geographic origin of samples is presented in Fig. 1. Aghavnadzor (40°35′03″ N 44°41′29″ E; number of specimens $n = 30$), Hankavan (40°37′09″ N, 44°34′29″ E; $n = 11$) Kotayk province, central Armenia, 1900 m a.s.l. *Biotope*: the localities Hankavan and Aghavnadzor include a valley of the river Marmarik and the several altitudinal zonation such as foothill, mountain, subalpine and alpine zones of the Pambak and Tsaghkunyats ridges. River valley is surrounded by mountain meadows and mixed forests at higher elevations, mainly on the northern slopes. Aygut (40°41′03″ N, 45°10′23″ E; $n = 1$), Gegharkunik province, NE Armenia, 1400 m a.s.l. *Biotope*: Lesser Caucasus, river valley surrounded by semi-arid mountain steppe with dispersed mixed forests at higher altitudes. Dilijan (40°44′27″ N, 44°51′47″ E; $n = 6$), Tavush province, NE Armenia, 1300 m a.s.l. *Biotope*: Lesser Caucasus, humid mixed forests. Animals were captured in the Dilijan town and the territory along the Agstev River. Khosrov State Reserve (39°56′42.1″ N, 44°51′35.5″ E; $n = 9$), Ararat province, central-south Armenia, 1300 m a.s.l. *Biotope*: slopes descending to the Ararat valley, arid foothills, slopes covered with dispersed woodlands. It is a noted area in the Caucasus region for unique European and Asian flora and fauna. Yerevan (40°11′ N, 44°31′ E; $n = 1$), Capital district, south-central Armenia, 1000 m a.s.l. *Biotope*: Ararat valley, predominantly arid with dry steppe vegetation. One individual was caught in the south-western suburbs of the capital Yerevan. In addition, one specimen of *Mus macedonicus* from Yerevan was also included.

Total genomic DNA was isolated from finger or tail tip preserved in absolute ethanol at –20°C. The DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol.

The DNA amplification was performed with polymerase chain reaction (PCR) using primers suggested by Bellinvia (2004) for the mitochondrial DNA control region (D-loop) and flanking tRNA genes (about 1000 bp). Selected part of mtDNA was amplified in two overlapping segments. PCR reactions were carried out in 25 μL volume including 1 μL of each 10 μM primer, 12.5 μL of PPP Master Mix (TopBio), 9.5 μL of PCR H_2O and 1 μL of template DNA following manufacturer's protocol. The PCR amplification protocol consisted of 31 cycles of denaturation at 95°C for 30 s, annealing at 50°C (using primers 1 + 2bis) or 55°C (using primers 3 + 4) for 1 min, and extension at 72°C for 1 min; a further 15 min elongation step at 72°C followed the last cycle. For some samples the temperature of annealing had to be decreased to 46°C in case of using combination of primers 1 + 2bis to

Table 1. List of localities of genetically determined specimens of *Apodemus uralensis* and *A. witherbyi*

	A. uralensis		*A. witherbyi*	
	number of individuals (N)	number of haplotypes (N_h)	number of individuals (N)	number of haplotypes (N_h)
Hankavan	11	11	–	–
Dilidjan	3	3	3	3
Aygut	1	1	1	1
Aghavnadzor	23	18	5	5
Khosrov Reserve	–	–	9	6
Yerevan, SW	–	–	1	1

obtain usable PCR products. All the PCR products were purified using the ethanol precipitation.

The entire D-loop and flanking region sequences were aligned and manually checked using Chromas Pro 1.7.5 (Technelysium Pty Ltd), BioEdit [50] and Clustal X 2.0.11 [51]. An online toolbox FaBox 1.41 (http://users-birc.au.dk/biopv/php/fabox/index.php) was also used for work with the sequences. We prepared three alignments, the first one in length of 1139 bp for phylogenetic analyses where we included sequences of all 59 samples—and also sequences of *Apodemus* species from GenBank (numbers AY588254, AY588250, AY588251, AY623063, AY623064, AY623065, AY623066, AY623067, AY588263, AY588260, AY588252, AY588255, AY588256, AY588257, AY588259, AY588264, AY588253, AY588262, AY588258, AY588261). We added two sequences of *Mus macedonicus macedonicus* as outgroup to this alignment (collected specimen and a GenBank sequence EU106248.1).

The second and third alignment was prepared for construction of haplotype networks. The second alignment included only 19 of our samples marked as *A. witherbyi*, its total length was 1041 bp. The third alignment included 38 of our samples marked as *A. uralensis* and its total length was 1024 bp.

Neighbour-joining (NJ) and maximum parsimony (MP) analyses were performed under PAUP* version 4.0b10 [52], and Bayesian analysis (BA) was con-

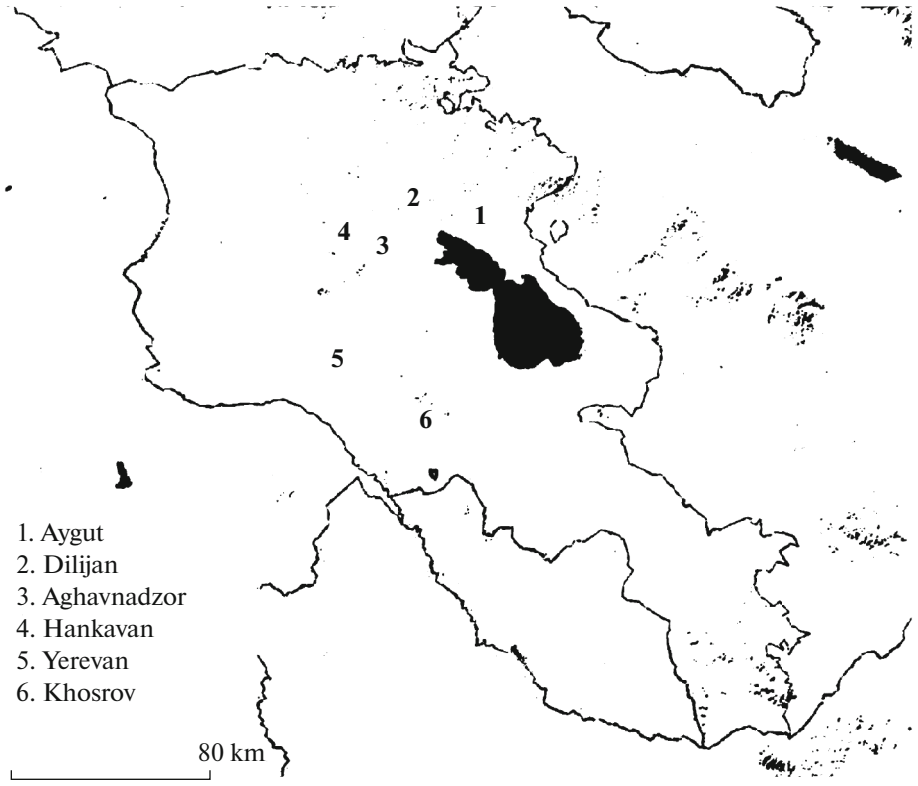

Fig. 1. Map of collection localities. 1—Aygut, 2—Dilijan, 3—Aghavnadzor, 4—Hankavan, 5—Yerevan, 6—Khosrov Reserve.

ducted with MrBayes 3.1 [53, 54]. Tree search with NJ algorithm was done with Kimura two-parameter distance and support within the final topology was assessed through 10000 bootstrap pseudoreplicates. For MP, we conducted heuristic search analyses using tree-bisection and reconnection (TBR) branch swapping and 1000 random replicates of taxa additions. The branch support was evaluated using 10000 bootstrap pseudoreplicates [55]. All characters were equally weighted and unordered. Bayesian analysis was conducted with a random starting tree and run for 15×10^6 generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 15000 trees (1500000 generations). As the best-fit model, TVM+I+G by hLRT was selected in Modeltest 3.7 [56]. In addition, we applied the median-joining method available in NETWORK, version 4.6.1.2 [57] to construct haplotype networks for each examined *Apodemus* species.

Polymorphism for each of populations *A. witherbyi* and *A. uralensis* was detected by the statistic software DnaSP v5 5.10.01 [58] which quantified the following: haplotype diversity (*h*), segregating sites (S), nucleotide diversity (π) and performed neutrality tests [59]: Tajima's *D*, Fu and Li's *F**, Fu and Li's *D**, and Fu's *Fs* tests [59–61]. According to Russell et al. (2005) [62], high values of *h* and π indicate a constant large size of population. However, a low value of π and high value of *h* signify recent expansion. To detect signals of population expansion, we also used simple expansion coefficients [60], defined as S/Π, where Π is the average number of pairwise nucleotide differences (also quantified in DnaSP v5 5.10.01).

RESULTS

In this study we sequenced a fragment of the mitochondrial DNA containing the entire D-loop region and flanking tRNA genes: part of the $tRNA^{Thr}$ and the entire $tRNA^{Pro}$ at 5', as well as part of the $tRNA^{Phe}$ at 3'. For 59 specimens this part of DNA varied from 1019 to 1076 bp.

The phylogenetic analyses were based on the alignment of 1139 bp including our samples and 22 sequences from GenBank (including 12 of the genus *Apodemus*). We performed Bayesisan, maximum parsimony and neighbour joining analyses; these methods mutually agreed in main topology of the resulting trees (see Fig. 2 for Bayesian tree and node supports). Monophyly of two distinct branches including 38 and 19 *Apodemus* samples from the territory of Armenia was strongly supported (Bayesian posterior probabilities >0.98, MP and NJ bootstraps >99 and 91, respectively). Inclusion of the GenBank sequences of known species identity into the phylogenetic analyses allowed us to unequivocally assign 57 of 58 sequenced *Apodemus* specimens (except the sample no. 1421, see below) into *A. uralensis* (38 specimens) or *A. witherbyi* (19 specimens). A sequence of the additional sample belonging to the genus *Mus* (no. 1386; locality Yerevan) fits *M. m. macedonicus*; this conclusion was further confirmed by a separate phylogenetic analysis of this sequence in the context of *Mus macedonicus* samples previously studied by Macholán et al. (2007) [63], in which no. 1386 fell within the clade of *M. m. macedonicus* samples closely to the sequences from Iran (data not shown).

The detailed phylogenetic placement of the sequence of the specimen no. 1421 has remained unstable, although it obviously belongs to the *Apodemus* clade. The sequence has no clear affinities to those of any previously sequenced *Apodemus* species. Uncorrected p-distances of this sequence to those of other *Apodemus* species range from 4.04 to 21.00% (for *A. uralensis* and *A. agrarius*, respectively). The supports of the detailed branching patterns within *A. uralensis* and *A. witherbyi* clades were rather low, although the Armenian samples tend to cluster together and/or with samples from geographically close areas of the Middle East (Fig. 2).

Next we separately analyzed the sequences from Armenian populations of *A. uralensis* and *A. witherbyi*. For *A. uralensis* we constructed a nucleotide alignment consisting of 1025 bp, of which 106 were variable and 64 were parsimony-informative. The uncorrected *p*-distance among haplotypes within this dataset ranged from 0.10 to 4.91%. Similarly, we obtained an alignment for *A. witherbyi* consisting of 1041 bp, of which 50 were variable and 20 were parsimony-informative. Within this dataset the uncorrected *p*-distance among haplotypes ranged from 0.20 to 2.37%.

These alignments were further used for construction of haplotype networks (Fig. 3 and Fig. 4) and computation of indices characterizing haplotype diversity. In both clades, the results revealed presence of some very distinct haplotypes, as well as the possibility of recent expansion. The latter suggestion was supported by high haplotype diversities, low nucleotide diversities and high values of simple expansion coefficients, also the neutrality tests resulted in negative, although non-significant values (Table 2).

External characters of speciemens possessing haplotypes belonging to *A. uralensis* and *A. witherbyi* clades overlapped considerably. The mean body length was 90.7 (range 82–96) and 94.6 mm (90–101); the ear length 15.0 (14.0–16.1) and 14.9 mm (14.2–16.1); and hind-foot lengths 20.61 (19.9–21.9) and 21.17 mm (20.3–22.5), for *A. uralensis* and *A. witherbyi*, respectively. The only examined measurement with significant between-species difference was the tail length (*t*-test: $t = -6.00$, $P < 0.0001$, *A. uralensis*: mean = 85.7 mm, range 77–95, *A. witherbyi*: mean = 99.9 mm, range 93–110). The individuals identified as *A. witherbyi*, were characterized by a bit paler and more yellowish dorsal surface than in *A. uralensis*. They tend to possess a bit larger and smoother yellow pectoral

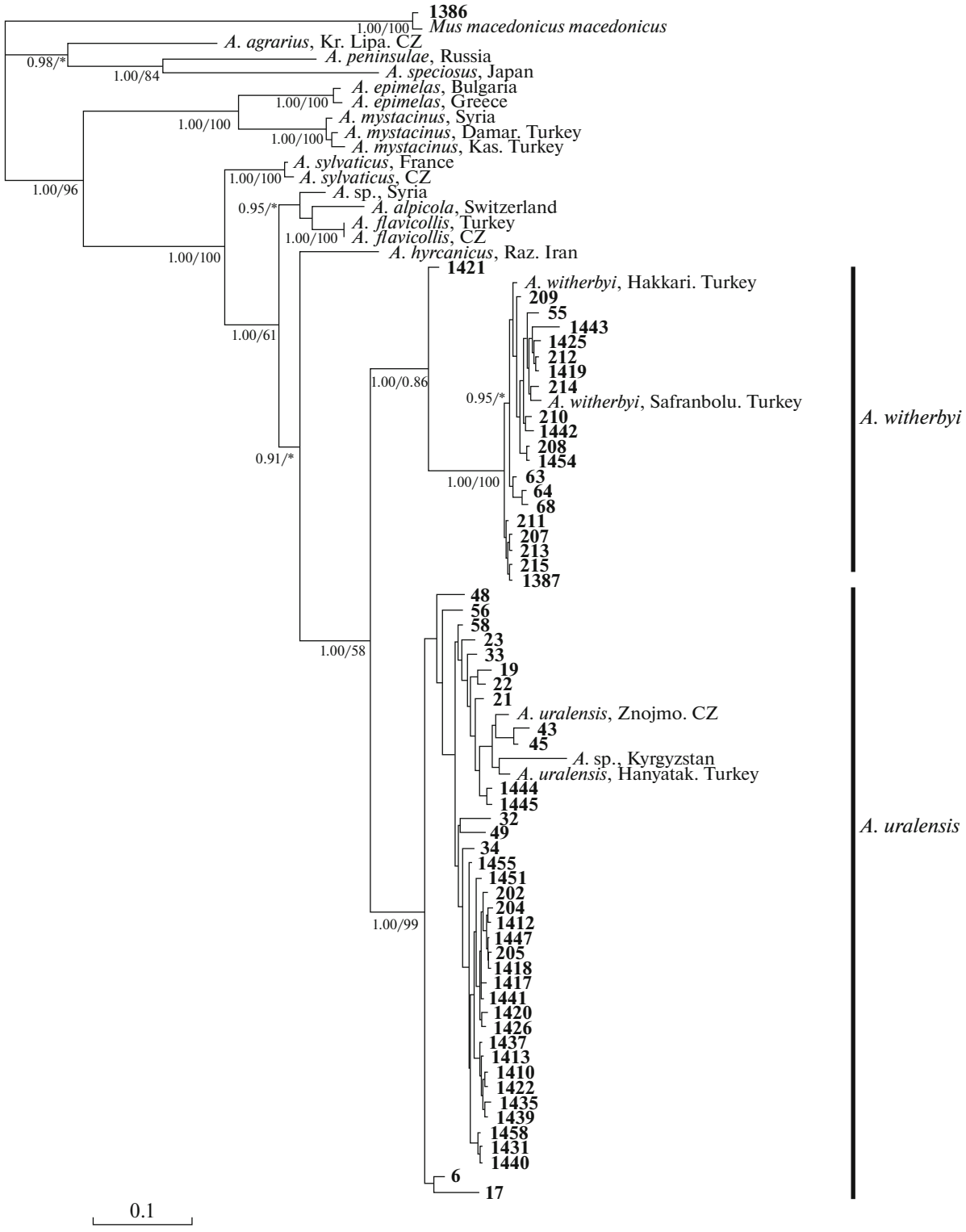

Fig. 2. Phylogenetic tree constructed using Bayesian analysis with Maximum parsimony posterior probability (posterior probability >0.90/bootstrap values >50 shown only, * posterior probability <50).

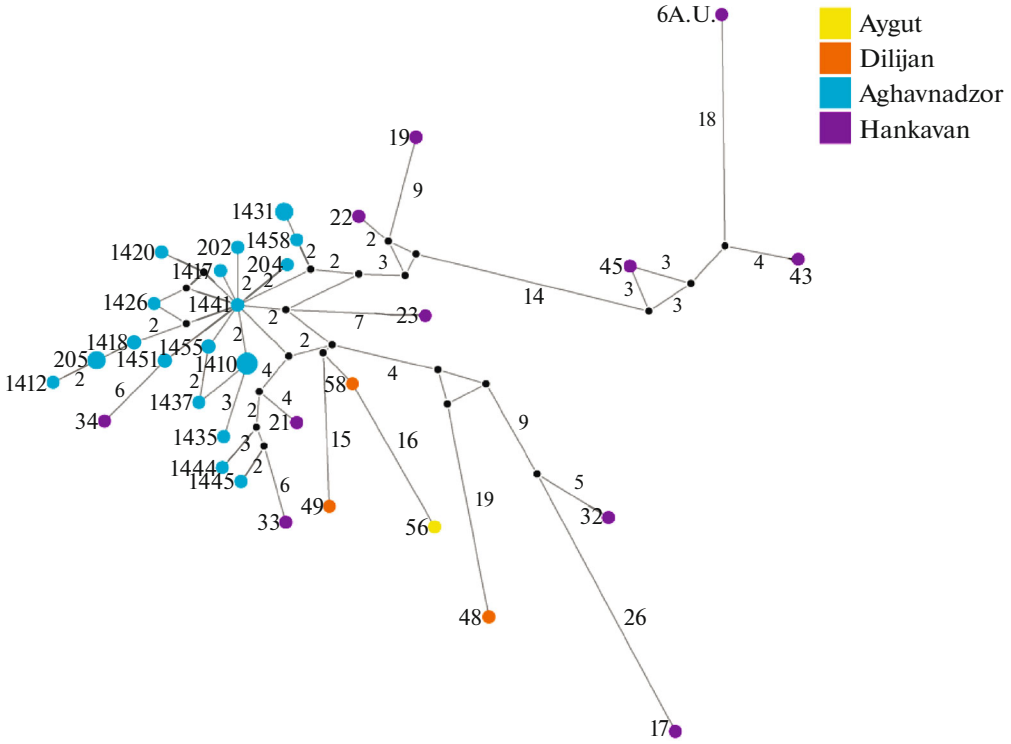

Fig. 3. Median-joining network obtained for the D-loop sequences of *A. uralensis*. Circle sizes are proportional to the number of the same haplotypes observed in the data set. Values at branches represent numbers of mutational steps (displayed for $n > 1$).

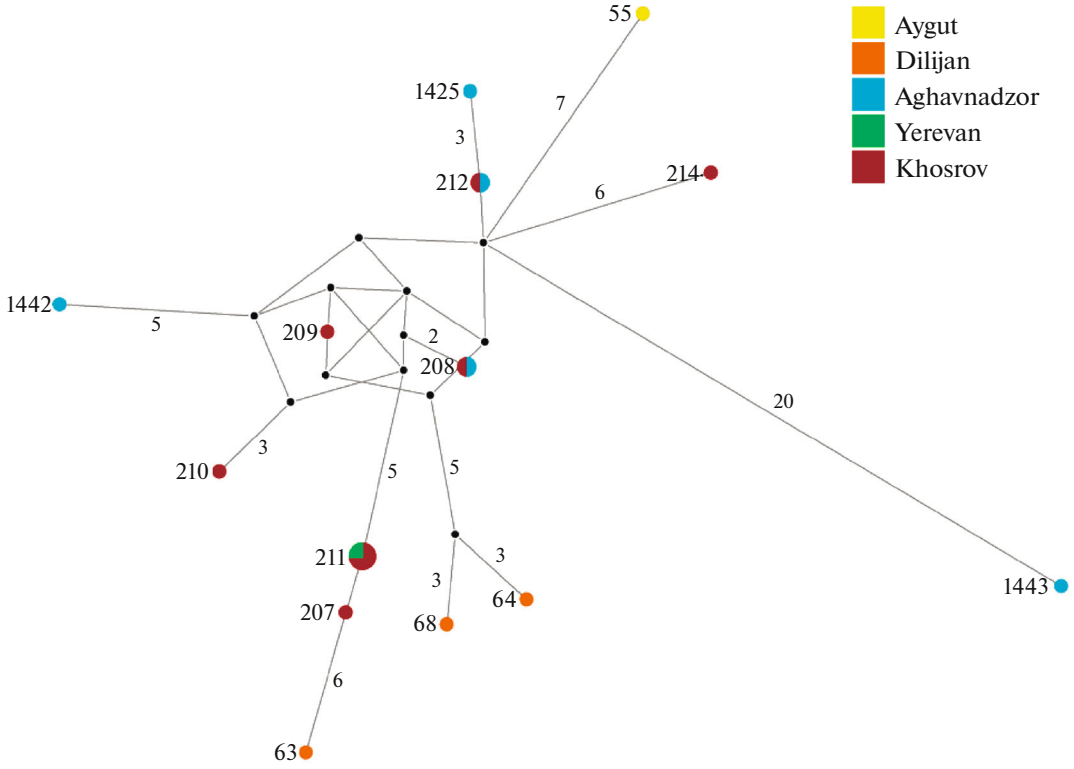

Fig. 4. Median-joining network obtained for the D-loop sequences of *A. witherbyi*. Circle sizes are proportional to the number of the same haplotypes observed in the data set. Values at branches represent numbers of mutational steps (displayed for $n > 1$).

Table 2. Sequence polymorphism and demographic characteristics for *A. uralensis* and *A. witherbyi* based on mitochondrial D-loop

	N_s	S	N_h	H	π	Fu and Li's *F**	Fu and Li's *D**	Fu's *Fs*	Tajima's *D*	Exp
A. witherbyi	19	45	14	0.95	0.01	−1.7612	−1.6579	−2.352	−1.1711	4.70354
A. uralensis	38	96	29	0.98	0.015	−1.6442	−1.2774	−8.009	−1.5749	6.69876

Samples are defined as species in phylogenetic tree. Sequences: number of individuals sequenced (N_s), number of segregating sites (S), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (π), Fu and Li's *F**, Fu and Li's *D**, Fu's *Fs*, Tajima's *D* and expansion coefficient (exp).

spots. Nevertheless, these differences in colouration were not distinct enough to reliably predict species identity as revealed by sequence data.

DISCUSSION

Five *Apodemus* species are currently recognized to inhabit the regions neighbouring Armenia. (1) *A. uralensis* (Pallas, 1811) a widespread species ranging from the Central Europe [19, 64] usually referred to as *A. microps* to the Central Asia [65]. The geographic variation of this taxon was examined by Orlov (1996). (2) *A. witherbyi* (Thomas, 1902). This species was described from Israel [66, 67] as *A. hermonensis* [66], but it was later found to be widespread throughout the non-desert areas of the Middle East (including Rhodos Island [68]). Later on Filippucci et al. (1996) [69] suggested that *A. hermonensis* probably can be conspecific with the species reported as *A. falzfeini* (Mezhzherin & Zagorodnyuk, 1989) or *A. fulvipectus* (Ognev, 1924) from Turkmenistan, the Transcaucasus, the Caucasus, and neighboring steppes up to Crimea [70]. The presence of *A. hermonensis* in Armenia was reported by Suzuki et al. (2008) [26]. Currently, *A. hermonensis* is treated as a junior synonym of *A. witherbyi* [43, 45, 48, 71]. (3) *A. flavicollis*. The phylogeographic analyses of the entire distribution range of this species suggest that Middle East populations represent a distinct clade [42]. Wood mice populations from the Transcaucasus referred to as *A. ponticus* (Sviridenko, 1936) may probably represent a sister lineage to *A. flavicollis* and therefore are often treated as conspecific [43, 72]. (4) *A. hyrcanicus* (Vorontsov et al., 1992), a recently described species, probably restricted to the area of Hyrcanian forests on the Southern shore of the Caspian Sea [14, 43, 44]. Its occurrence in Armenia is thus improbable. (5) *A. mystacinus* (Danford and Alston, 1877) inhabiting rocky habitats in urban or woodland areas throughout Anatolia, Levant, Georgia and Crete, sometimes attributed together with its sister species *A. epimelas* to the subgenus *Karstomys* [43, 48, 49].

The phylogenetic analyses performed by Bayesian and maximum parsimony methods mutually agreed and both revealed presence of two distinct *Apodemus* clades corresponding to species *A. uralensis* and *A. witherbyi*. Thus, the species determination using the sequenced fragment of mitochondrial DNA including the control region is unequivocal. The sequence obtained from the specimen 1421, however, does not fit to any of the previously sequenced species. This may be caused by a possible presence of nuclear pseudogenes [73], though we cannot rule out it may represent a new mitochondrial lineage. On the other hand, topology of both species within the phylogeny of *Apodemus* species remains unclear, when compared with the data in literature. In our results, *A. uralensis* and *A. witherbyi* grouped together into a clade, while *A. hyrcanicus, A. flavicollis* and *A. sylvaticus* formed ancestral groups. According to other authors, whose studies were based on cytochrome b, 12S rRNA or nuclear genes, *A. uralensis* usually groups together with *A. sylvaticus*, *A. flavicollis* and *A. hyrcanicus*, though their exact positions may vary, leaving *A. witherbyi* (or *A. hermonensis*, respectively) as a sister species to the whole group [12, 38, 43].

In both examined species, the patterns revealed by haplotype network analyses may suggest recent demographic expansion of the populations. The presence of some very distinct haplotypes in both examined species requires further examination. Geography and climatic history of Armenia (glacial history of the Caucasus [4]) allows us to speculate that this pattern may be a result of successive colonization waves.

In most localities we confirmed the presence of both *A. uralensis* and *A. witherbyi* which suggests their considerable sympatric and syntopic occurrence. This further supports previous results from Eastern Turkey [11, 13] where these two species, and sometimes also *A. flavicollis* were frequently found on the same localities. We found no obvious microhabitat separation in sympatric populations. Nevertheless, the predominance of *A. witherbyi* in Khosrov Reserve and *A. uralensis* in Hankavan, conforms to a general tendency of the former species to prefer steppe while the latter species rather forest and/or mountainous habitats.

The genetic distinctness of sympatric *Apodemus* species sometimes sharply contrasts with their morphological uniformity, the phenomenon that was previously demonstrated, e.g., in the Balkans (cf. [74]). Also in Armenia and adjacent regions, high degree of genetic differentiation of sympatric *Apodemus* species [11, this paper] contrasts with their apparent mutual

similarity in appearance (colouration, body dimension) and craniodental morphometry (cf. [13, 14]). The external dimensions and colouration of our genetically determined specimens of *A. uralensis* and *A. witherbyi* fit the variation reported by Kryštufek and Vohralík (2009) [71]. The only little overlapping trait was the tail length, however, its reliability for field determination requires further verification in larger sample of genetically determined specimens.

There may be three possible explanations for this kind of variation: (1) gene introgression among sympatric species in the Middle East [75–77] and/or (2) convergent morphological evolution of multiple clades in the Middle East [78, 79] and/or (3) divergent morphological evolution in European taxa combined with persistence of ancestral phenotypes in the Middle East [14]. These let us to discuss some possible ecological causes of small interspecific variation in the Middle East when compared to that in Europe. Habitats suitable for the survival of *Apodemus* species can be more differentiated in European landscapes (like field and forest) than in the Middle East.

Our results based on maternally inherited mitochondrial genes are unable to detect genetic introgression. Although, Macholán et al. (2001) [11] failed to detect interbreeding among sympatric *Apodemus* species in the Middle East, nuclear genes have to be examined to prove their findings. In conclusion, our study confirmed that the two syntopic species belong to the species *A. uralensis* and *A. witherbyi*, each characterized by a distinct cluster of haplotypes.

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9. Přílohy

Následující práce výslovně nejsou součástí souboru publikací zařazených to této disertační práce. Byly součástí jiných disertačních prací a podíl Barbory Opelka Somerové na nich není dominantní. Jsou přiloženy výhradně pro dokreslení publikačního profilu uchazečky.

9.1. Příloha 1

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Why is the tongue of blue-tongued skinks blue? Reflectance of lingual surface and its consequences for visual perception by conspecifics and predators

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ORIGINAL PAPER

Why is the tongue of blue-tongued skinks blue? Reflectance of lingual surface and its consequences for visual perception by conspecifics and predators

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Abstract Blue-tongued skinks of the genus *Tiliqua* (Scincidae) are characterized by their large blue melanin-pigmented tongues, often displayed during open-mouth threats, when the animal feels endangered. It is not clear whether this unusual coloration is a direct anti-predation adaptation or it may rather serve intraspecific communication, as ultraviolet-blue color is a frequent visual signal in a number of lizard species. We used spectrophotometry and visual modeling to compare blue tongues of *Tiliqua gigas* with tongues and skin coloration of other lizard species, and to examine their appearance through the eyes of both the conspecifics and avian predators. Our results show that (1) the tongue coloration is probably not substantially influenced by the amount of melanin in the skin, (2) lingual and oral tissues are UV-reflective in general, with blue colored tongues having chromatic qualities similar to UV-blue skin patches of other lizard species, (3) UV-blue tongues are more conspicuous than pink tongues, especially in the visual model of conspecifics. We hypothesize that blue tongues may possibly serve as a semantic (honest) signal analogous to UV-blue skin patches of other lizard species due to greater UV-bias in the vision of diurnal lizards. Regarding the social behavior and high aggressiveness in *Tiliqua* and their relatives, such signal might serve, e.g., in intraspecific long-distance communication between conspecifics in order to avoid aggression, and its anti-predation effect may only be a secondary function (exaptation).

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Introduction

Color signals play an important role in both intra- and interspecific communication in a number of animal species. Apart from serving as cues for individual or species recognition, they cover a variety of semantic meanings (Williams and Rand 1977; Losos 1985; Couldridge and Alexander 2002; Creel and Creel 2002). The size or intensity of particular coloration can correlate with the hierarchic status of its bearer and/or indicate his health or aggressiveness. This is common in males of many fish, bird, or reptile species which indicate in this way their fighting ability to their competitors. This influences their mating success and therefore, the corresponding traits are often subject to sexual selection (Fernald and Hirata 1977; Andersson et al. 1998; Sinervo et al. 2000; Senar 2006; Hamilton et al. 2013). Conspicuous colors and patterns can also serve to deter potential predators. Aposematic patterns are usually present on the animal's body surface, being exposed either permanently (e.g., in coral snakes), or only at the moment of threat, which is typical for otherwise cryptic species (e.g., in fire-bellied toads). Some species rely on a strategy of startling the predator for a moment, flashing suddenly a hidden warning sign and thus gaining some time to escape (e.g., sunbitterns, frilled lizards or some species of butterflies; Ruxton et al. 2004; Hill and McGraw 2006; Hamilton et al. 2013).

Within the wide variety of color signals, blue coloration deserves special attention, as it is relatively uncommon among vertebrates, but mostly has a semantic function (Bagnara et al. 2007; Perez i de Lanuza and Font 2010; Umbers 2013). In this case, vision and coloration are related in a sense that many

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animals having blue patches on their surfaces are also capable of seeing ultraviolet light. Patches that we perceive as blue are in fact often UV-blue, as their peak reflectance is mostly in the UV range. Such signs are therefore conspicuous to these species, allowing them to communicate through this channel without being detected by UV insensitive predators (Endler 1992; Håstad et al. 2005; Lind et al. 2013; Marshall and Stevens 2014). UV-blue crowns in blue tits or throats, lateral eyespots, and outer ventral scales in many lacertids (e.g., *Timon*, *Gallotia*, *Lacerta*, *Podarcis* etc.) represent not only intraspecific recognition traits (they can be sexually dichromatic in the UV spectrum in many species), but also serve as honest signals of male quality (Andersson et al. 1998; Font et al. 2009; Bajer et al. 2011; Molina-Borja et al. 2006). In African flat lizards of the genus *Platysaurus* (Cordylidae) as well as in the lacertids, the intensity of UV-blue coloration positively correlates with the aggressiveness and the ability of males to mate successfully (Whiting et al. 2006; Font et al. 2009; Fleishman et al. 2011; Perez i de Lanuza et al. Page: 5 2014a). On the other hand, a blue color is very rarely involved in warning signals and its potential aposematic function remains questionable (Bagnara et al. 2007; Umbers 2013).

Unlike most lizards, blue-tongued skinks of the genus *Tiliqua* (Gray 1825) are special by their non-dermal *warning* blue sign. Current taxonomy recognizes seven species of these mostly robust diurnal omnivorous skinks (*Tiliqua scincoides*, *Tiliqua gigas*, *Tiliqua multifasciata*, *Tiliqua nigrolutea*, *Tiliqua occipitalis*, *Tiliqua rugosa*, *Tiliqua adelaidensis*) distributed throughout Australia, eastern Indonesia, and Papua New Guinea (Shea 2006; Gardner et al. 2008; Cogger 2014). As their name suggests, they are characterized by their large blue fleshy tongues, which are often exposed when the animal feels endangered or irritated. The skink widely opens its mouth and startles a rival or predator by exposing its tongue either inside the mouth by raising its base while keeping the tip down or by sticking the entire tongue out. This can be accompanied by body inflating, hissing, or lunging against the enemy (Carpenter and Murphy 1978; Murray and Bull 2004; Brown 2012; Cogger 2014). *Tiliqua* skinks are frequently hunted by the birds of prey (Aumann 2001; McDonald et al. 2003; Olsen et al. 2010); other avian predators may include kookaburras or corvids, while reptilian predators are mostly elapid snakes and monitor lizards (Hauschild et al. 2000; Koenig et al. 2002; Fitzsimons 2011).

Regardless of lingual coloration, an open-mouth threat is a common reaction among animals (Vitt and Lacher 1981; Sherbrooke 1991; Langkilde and Shine 2005; Godfrey et al. 2012). Herrel (2000) notes that although the tongue's defensive use is quite obvious, it does not really explain the function or presence of the blue coloration. Bright blue tongues are found, e.g., in *T. scincoides* or *T. gigas*, while *T. rugosa* has almost a black tongue and juveniles of a closely related skink *Cyclodomorphus gerrardii* have blue tongues, whereas adults have them pink. Thus, the situation is more complicated than it may seem. Herrel (2000) proposes also alternative hypotheses that the blue coloration can be more crucial as a warning sign in young animals, or it can represent an honest signal in competing males during the mating season.

The latter hypothesis may be plausible as aggressiveness is common among individuals of the genus *Tiliqua* and they are capable of wounding each other fatally (Graves and Halpern 1991; Shea et al. 2005; Godfrey et al. 2012). Males of *T. scincoides* occupy core areas which they defend against other males, while females tolerate each other and their home ranges considerably overlap (Price-Rees et al. 2012). Similar social patterns were also observed in *T. rugosa*, where solitary males were more aggressive than males having bonds with females (Kerr and Bull 2006). For these reasons one would naturally expect the need for advanced communication between blue-tongued skinks in order to avoid aggression. Abbate et al. (2009) have confirmed a presence of melanin in the tongue of *T. scincoides*. Since melanin pigmentation is sometimes positively correlated with dominance or aggressiveness in various species of birds or reptiles (McGraw et al. 2003; Jawor and Breitwisch 2003; Senar 2006; Mafli et al. 2011; Plasman et al. 2015), the *honest signal* hypothesis does not seem unlikely in this case, as the tongue tinges also vary among individuals of the same species. *T. scincoides* or *T. gigas* may have pale gray, cobalt blue or dark (nearly black) blue tongues, so their tongues' intensity or darkness may reflect their status or health.

In fact, pigmented tongues can be found in most species closely related to the genus *Tiliqua* with the exception of *T. adelaidensis* and *Corucia zebrata*, the former being a considerably derived species and the latter being basal to the whole group consisting of the remaining genera: (*Egernia* (*Liopholis* (*Cyclodomorphus*, *Tiliqua*))) (Hutchinson et al. 1994; Shea 1995; Shea and Miller 1995; Pyron et al. 2013). Skinks of the genus *Cyclodomorphus* may have not only their tongues pigmented, but also their oral mucosa, reaching blue, blue-gray, or blue-black tinges (Shea 1995; Shea and Miller 1995). *Egernia* skinks have pigmented tongues as well, though not to such an extent or intensity, having for instance dark tips or an overall pale bluish tint. Variously tinged lingual and/or oral mucosa have also been observed in unrelated species of scincid, agamid, iguanid, gekkonid and other lizards (*Gerrhosaurus flavigularis*, *Gonocephalus chamaeleontinus*, *Amphibolurus muricatus*, *Rankinia diemensis*, *Chamaeleolis* sp., *Strophurus* sp. etc.) which had blue, black, yellow, or orange tongues or mouth interiors (Melville et al. 2004; Ayala-Varela and Omar 2010; Holáňová et al. 2012). The reason for such unusual coloration of lizard tongue is yet obscure.

The aim of our study was to explore qualities of the blue lingual coloration from the perspective of both the honest

signal and the specific communication channel. Is the tongue color related to the overall body pigmentation, or is it an independent trait? Is there a *hidden* UV signal like in other lizards with blue coloration? Which recipient (reptile/bird, predator/conspecific) is this visual signal best adjusted to? To shed more light on these questions, we examined (1) possible correlations between tongue coloration and somatic traits (body length, melanin skin pigmentation), (2) the conspicuousness of blue tongues as it is perceived by two groups of animals with different visual systems: avian predators (mostly raptors; violet-sensitive vision) and conspecifics (diurnal lizards; UV-sensitive vision), and (3) color similarities between blue tongues of *Tiliqua* and (un) pigmented tongues and semantic UV-blue skin patches of other lizard species. For this purpose, we carried out spectrophotometric measurements of lingual, oral, and dermal surfaces and analyzed them using visual modeling.

Materials and methods

Spectrophotometric measurements

We measured 14 captive adult individuals of *T. gigas* imported legally into Europe from Indonesia. The stocks originated from surroundings of Merauke town, Irian Jaya province, Indonesia (SW of New Guinea Island). We assumed that in case of an adaptive function, the blue color of the tongue would be more advantageous than a pink one. Therefore, besides the blue tongue, we also measured the pink color of oral mucosa as an approximation of a pink tongue. We decided to take the skinks' back coloration as an example of background, against which the tongue color contrasts were to be calculated. As their dorsal side is considerably cryptic and no reliable and representative sample of substrates from the skinks' natural habitats was available, we chose their skin as a proxy of their natural environment color. It may reflect the palette of colors typical for their habitats, ranging from forest floors to dry semi-deserts (fallen wood, bark and leaves, dry vegetation, sandy soil, etc.) (Price-Rees et al. 2013; Cogger 2014). From the observed variation of the skinks' backs, four notably distinct shades were chosen as substitutes for their natural background colors: light brown (stripes), dark brown (stripes), orange-reddish (spots/stripes), and grayish (side of the head and neck) (Fig. 1).

Color reflectance between 300 and 700 nm was measured with an OceanOptics USB4000 spectrophotometer and an UV-VIS Pulsed Xenon lamp source PX-2. The spectra were taken in a shaded room with a probe held in a constant 5 mm distance under a 45° angle to avoid possible specular effects caused by the glossy scales or moist surface of the tongue. The device was re-calibrated after every third measurement against an Ocean Optics WS-1 white standard and the probe was always sterilized with ethanol before measuring a new individual. Each color patch was measured three times and its mean reflectance was calculated. Skinks mostly opened their

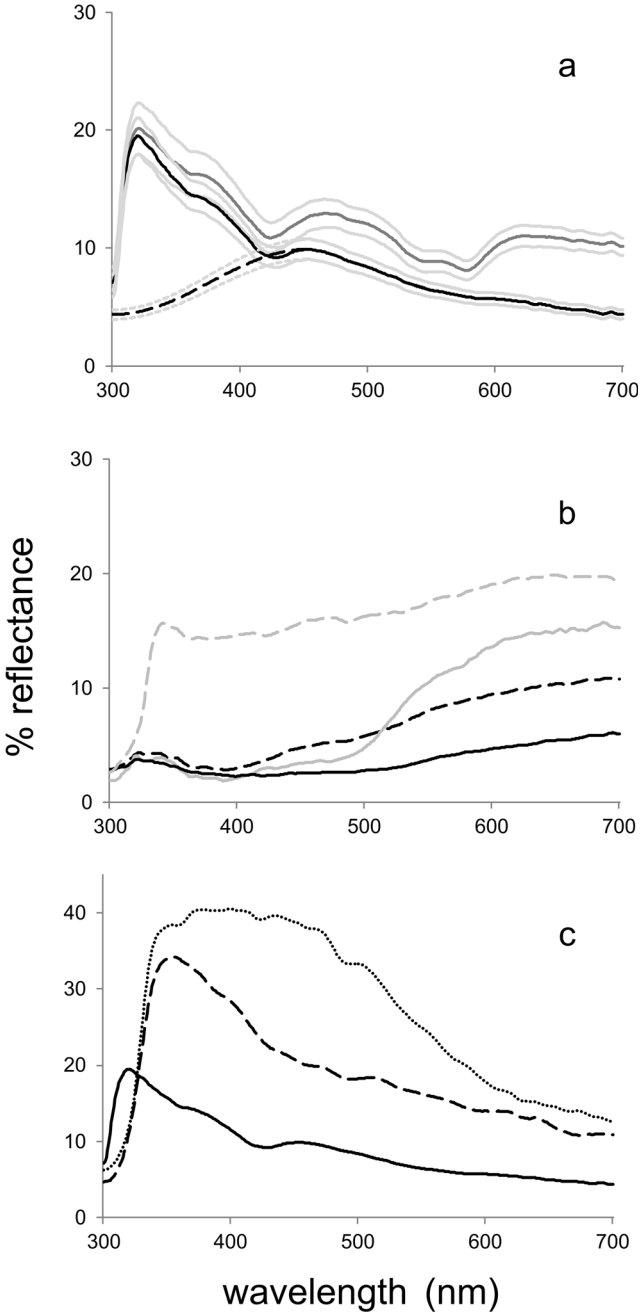

Fig. 1 Reflectance of lingual, oral and skin surfaces. **a** *T. gigas* (n=14). Mean reflectance of blue tongues (*solid black line*), mean reflectance of pink oral mucosa (*solid dark gray line*), manipulated part of spectra in the UV-negative set (*dashed black line*), ± SE (*light gray lines*). **b** Reflectance of background colors taken from skin samples of *T. gigas*: dark brown (*solid black line*), light brown (*dashed black line*), orange-reddish (*solid gray line*), grayish (dashed gray line). **c** Mean reflectance of blue tongues of *T. gigas* (*solid black line*), mean reflectance of blue shoulder spot of a single captive *G. galloti galloti* (*dashed black line*), mean reflectance of blue outer ventral scales (OVS) of a single *D. caucasica* (*dotted line*)

mouths spontaneously in a response to the irritation caused by our manipulation. Most of them kept their mouths open long enough for us to take measurements of the tongue and pink interior. Only when the skinks did not open their mouth themselves, we gently opened it. This seemed to be a sufficient impulse for the individual to keep its mouth open for a while.

As a comparison, we also measured tongues of *Egernia frerei*, *C. gerrardii* and *C. zebrata* (which are closely related to the genus *Tiliqua*; Pyron et al. 2013) and of an unrelated African skink *Trachylepis perrotetii*. With the exception of *C. zebrata*, whose tongue was wholly pink, all of them had their tongues at least partially pigmented: a dark gray tongue in *E. frerei*, a dark gray tip of the tongue in *T. perrotetii*, and an overall faintly bluish tint in *C. gerrardii* (Fig. 2). Each of these species was represented by a single individual.

To compare the blue tongue to UV-blue semantic coloration of other lizard species, we also acquired reflectance spectra from a captive *Gallotia galloti galloti* (shoulder spots) and *Darevskia caucasica* (outer ventral scales—OVS); single specimens in both cases (Fig. 1). Mean reflectance for other species was obtained from literature: *G. galloti galloti* (shoulder spots, n=26, Molina-Borja et al. 2006), *Gallotia galloti eisentrauti* (shoulder spots, n=34, Molina-Borja et al. 2006), *Timon lepidus* (lateral eyespot, n=14, Font et al. 2009), *Podarcis pityusensis* (OVS, n=40, Perez i de Lanuza and Font 2010), *Podarcis lilfordii kuligae* (both OVS and ventrum, n=47, Perez i de Lanuza and Font 2010) and *Platysaurus broadleyi* (throat, n=1, Whiting et al. 2006). The data were extracted with the WebPlotDigitizer (available on arohatgi.info/WebPlotDigitizer/app/ in December 2014). Curves for conspecific males and females were averaged into a single one to approach a *general* signal of the species. Unfortunately, morphology-based sexing of *Tiliqua* is known to be very unreliable (Brown 2012), and it was impossible to determine sex in most cases even with the ultrasound screening. Therefore, we could not look for the hypothetical sexual dichromatism in the coloration of their tongues.

Color processing

To compare whether the overall skink's body pigmentation correlates with its tongue color, we carried out the following procedure. We scanned each individual from its ventral and dorsal sides with a CanoScan 4400 F scanner at 600 dpi resolution, together with BST1 Color and Gray Control Chart as a reference of standardized colors. Both the skink's back and belly (including the top of the head and throat, respectively) were outlined and cut out from the scans in Photoshop CS6 (Fig. 2).

We measured the average brightness of selected areas as an approximation of the amount of melanin-based skin pigmentation presuming that the lighter the skin, the less melanin there would be (Shriver and Parra 2000; McGraw et al. 2005). We did not use spectrophotometric data in this case because the spectrophotometer probe focuses only a very small spot. The color patterns are more complex and we needed to quantify the pigmentation from the overall area. However, we used the spectrophotometer to check the skin

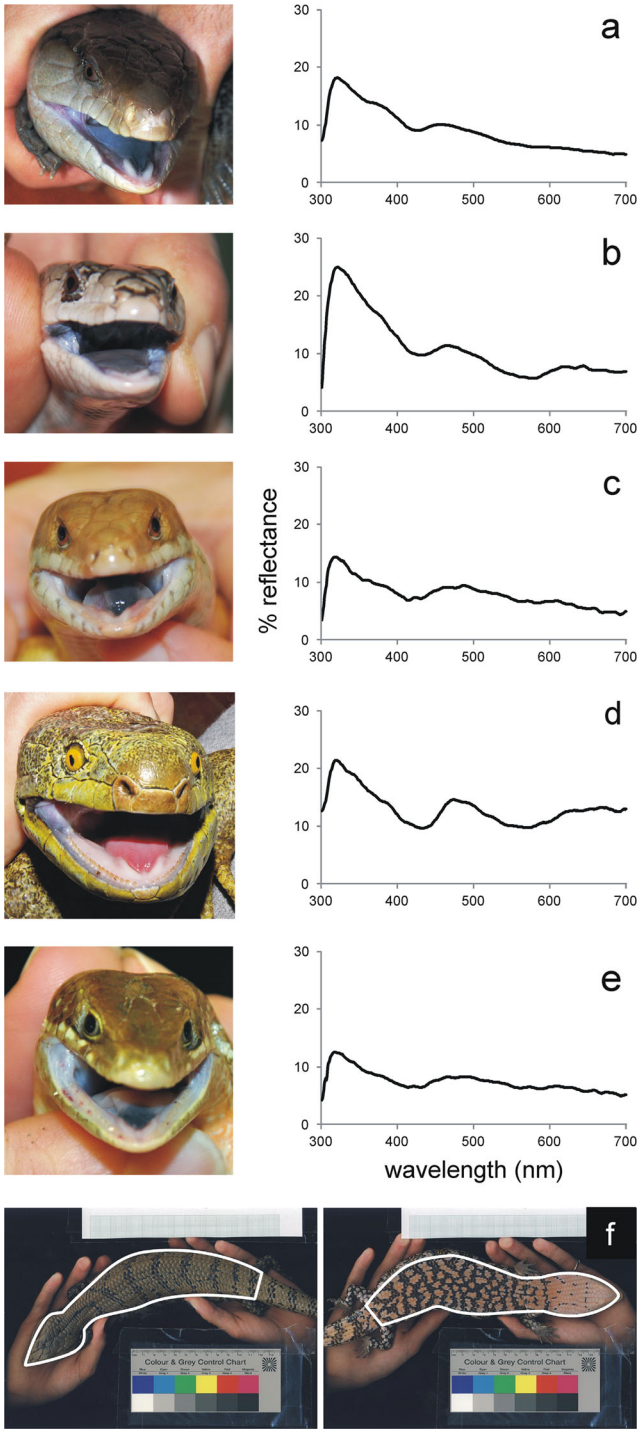

Fig. 2 Tongues and their reflectance spectra of species used in this study: **a** *T. gigas*, **b** *C. gerrardii*, **c** *E. frerei*, **d** *C. zebrata*, **e** *T. perrotetii*, **f** scan examples of dorsal and ventral sides of skinks; *white outline* marks the area used in the lightness analysis

for potential reflectance in UV spectra. No significant reflectance (>5 %) in the UV was detected, therefore we considered the scans to adequately represent the skin lightness as it is perceived.

Selected backs and bellies were converted into grayscale mode, to extract only the brightness information. The area's average brightness was measured with the ImageJ software and displayed as a RGB value. Brightness values were recalculated into percentage by dividing them by 256, which is the total number of steps in the 8-bit RGB brightness scale. Skin melanin pigmentation was represented by two separate values (a) brightness of the back only and (b) averaged brightness of the back and belly, standing for the individual's overall melanin-based body pigmentation.

To evaluate whether the presence of inherent UV reflectance of lingual tissue may be relevant to intra- or interspecific perception, we made an alternative set of spectra with manipulated values. We used sinus function, creating smooth decline in the reflectance curve from the maximum in the human-visible blue range, to the minimal value of the spectrum at 300 nm, therefore depriving the original spectra of the peak in the UV range (Fig. 1).

Visual modeling

We worked with two visual models representing conspecifics and predators. For the representation of conspecifics, we used photoreceptor data for *P. broadleyi*, as those for *Tiliqua* are not known. Diurnal lizards were shown to have a conservative visual system with the retina containing four types of cones, sensitive to UV, short, medium, and long wavelengths (Fleishman et al. 1993; Loew et al. 2002; Whiting et al. 2006; Macedonia et al. 2009), which should apply also to *Tiliqua* (New et al. 2012). *P. broadleyi* belongs to the Scincomorpha clade and hence is the closest relative to *Tiliqua* with available information (Fleishman et al. 2011; Pyron et al. 2013; Perez i de Lanuza et al. 2014b).

Photoreceptor data of *Pavo cristatus* were used for modeling the vision of avian predators (Hart 2002; Ödeen and Håstad 2003). Two types of retina are known in birds, differing in photoreceptors for the shortest wavelengths: an UVS type—with ultraviolet-sensitive cones (peak absorbance under 380 nm), and a VS type, with violet-sensitive cones (peak absorbance above 400 nm) (Endler and Mielke 2005). Birds of prey, but also kookaburras and corvids, have the VS type retina as does the peafowl which is being widely used as a substitute for raptors in visual modeling (Hart 2002; Macedonia et al. 2009; Lind et al. 2013; Ödeen and Håstad 2013; Perez i de Lanuza et al. 2014a).

For calculating chromatic contrasts, we used the TetraColorSpace software (Stoddard and Prum 2008). TetraColorSpace transforms the visual system into a virtual tetrahedral space, following the model of Endler and Mielke (2005), where each vertex represents one of the four photoreceptor types. The perceived color is then projected into the tetrahedron as a point, whose position is calculated from the response of individual photoreceptors to the spectrum and particular illuminant. In our modeling, we have chosen the default illumination set by TetraColorSpace, which represents the standard daylight. The color (chromatic) contrast is then calculated as a Euclidean distance between two points in the color space. Chroma (purity or saturation) of a particular color is expressed as the point's distance from the achromatic origin in the center of the tetrahedron. However, as the maximum distances from the center to the margins are not constant, we counted with the *achieved chroma*, which is the relative distance value. Color contrasts and achieved chroma were calculated for each blue and pink *tongue* (the latter simulated by the oral mucosa) against each of the four background types, for both visual models.

To assess how similar the shades of (1) blue tongues of *T. gigas* and tongues of other skink species and (2) blue tongues of *T. gigas* and UV-blue skin patches of other lizard species look in the eyes of their conspecifics, we calculated color distances, i.e., a chromatic contrast measured in *just noticeable differences* (JND). The calculations for this type of analysis were executed in Avicol v6 (Gomez 2006). The JND measure expresses the discriminability of two colors in a particular visual system, taking into account the receptor noise (Vorobyev and Osorio 1998). A JND value below 1 means that two colors are indistinguishable, a value between 1 and 3 is believed to indicate colors distinguishable only under ideal light conditions, and a value above 3 expresses increasingly distinguishable stimuli (Vorobyev et al. 1998; Siddiqi et al. 2004; Cassey et al. 2009).

First, we calculated color distances for each of the 14 blue-colored tongues to the mean spectrum of *T. gigas*, in order to estimate the individual tongues' intraspecific distances from their overall average. Then we repeated the calculation, this time replacing the average spectrum of blue tongues by a spectrum representing a tongue or a blue skin patch of the selected lizard species. This way we could statistically compare the interspecific distances with the intraspecific ones and thus estimate the proximity of different spectra. We also compared the color distances between UV-positive and UV-negative tongues in both visual models. Input parameters were taken from Marshall and Stevens (2014).

Statistical analyses

The data were tested for normality. The majority of variables had fairly unimodal and symmetric distributions, however, *w* tests revealed multiple cases in which normality was violated at alpha=0.05. Therefore, we selected non-parametric tests instead of parametric ones. We used Spearman's correlation test to detect whether a tinge of the tongue (achieved chroma

and/or mean reflectance) is related to other traits. First, we analyzed the individual body size as an approximation of age, expressed as snout-vent length. For the second trait we chose the amount of skin pigmentation, separately for the back and the whole body.

A Mann-Whitney *U* test was used to compare (a) differences in chromatic contrasts of blue and pink *tongues* for each of the four backgrounds and (b) chromatic contrasts of the same color between the two visual models. To assess whether a transition from pink to blue results in greater boost in both chromatic contrast and achieved chroma in one visual model than in the other, we created the following set of data. For each visual model and background, we calculated chromatic contrast differences (dCC) and achieved chroma differences (dAC). dCCs were achieved by subtracting chromatic contrast values of blue tongues from chromatic contrast values of pink *tongues* in all pair-wise combinations and were expressed in absolute values. Similarly, we calculated dACs as differences between *blue* and *pink* achieved chromas. Then, we compared the dCCs (or dACs) between the two visual models using the Mann-Whitney *U* test.

We used a Kruskal-Wallis test to detect significant differences within the set of intra- and interspecific color distances. When the results proved to be significant, a Mann-Whitney *U* test was applied to compare the individual pairs of data; intraspecific color distances for blue tongues were each time put against color distances between tongues of *T. gigas* and tongues (or UV-blue skin samples, respectively) of other lizard species.

We performed a Wilcoxon matched pair test to compare the color distances between UV-positive and UV-negative tongue spectra within both visual models. All analyses were executed in Statistica 8 (StatSoft, Inc. 2007).

Results

Our spectrophotometric measurements showed that both blue tongues and pink oral mucosa share very similar spectral shape, differing notably in longer wavelengths. Both spectra have primary maximum peak in the UV range at 320 nm and secondary, a slightly lower one, in the blue range, around 460 nm (Fig. 1).

The Spearman's test did not reveal any significant correlations between color qualities of the tongue and selected traits; compared against snout-vent length: for tongue lightness $r_{Spearman}=-0.036$ ($P=0.901$); for achieved chroma of the tongue $r_{Spearman}=-0.493$ ($P=0.073$); compared against pigmentation of the back: for tongue lightness $r_{Spearman}=0.461$ ($P=0.083$); for achieved chroma of the tongue $r_{Spearman}=0.438$ ($P=0.102$); compared against the overall body pigmentation: for tongue lightness $r_{Spearman}=0.354$ ($P=0.196$); for achieved chroma of the tongue $r_{Spearman}=0.232$ ($P=0.405$).

The Mann-Whitney test indicated higher chromatic contrasts of blue tongues compared to the pink *tongues*; for the conspecifics visual model, it was significant against light brown ($Z=-2.80$, $P=0.005$), dark brown ($Z=-2.85$, $P=0.004$), orange-reddish ($Z=-2.80$, $P=0.005$) and grayish backgrounds ($Z=-2.85$, $P=0.004$); for the avian predator visual model, it was significant against light brown ($Z=-3.86$, $P<0.001$), dark brown ($Z=-3.72$, $P<0.001$), orange-reddish ($Z=-3.86$, $P<0.001$) and grayish backgrounds ($Z=-3.86$, $P<0.001$) as well. At the same time, blue tongues were more contrasting against light brown ($Z=2.71$, $P=0.006$), dark brown (2.94, $P=0.003$) and grayish backgrounds ($Z=3.54$, $P<0.001$) in the visual model of conspecifics than in the predator one. Pink *tongues* were significantly more contrasting against light brown ($Z=2.34$, $P=0.019$) and grayish backgrounds ($Z=2.71$, $P=0.007$) in the conspecifics visual model (Fig. 3).

Blue tongues had also higher values of achieved chroma compared to pink *tongues* in both the conspecifics visual model (blue=0.290±SE 0.014, pink=0.192±SE 0.021; $Z=-3.492$, $P<0.001$) and the avian predator visual model (blue=0.271±SE 0.015, pink=0.173±SE 0.010; $Z=-3.583$, $P<0.001$). The difference between pink and blue chromatic contrasts (dCC), and pink and blue achieved chromas (dAC) respectively, was statistically insignificant when the two visual models were compared ($P>0.05$).

The mean intraspecific color distance of *T. gigas*' tongues from their average was 1.57 JND±SE 0.42 (minimum=0.12 JND, maximum=4.96 JND). Overall interspecific differences were significant: for tongues, Kruskal-Wallis test: $H=38.9$, $P<0.001$, and for blue tongues versus UV-blue skin, Kruskal-Wallis test: $H=84.1$, $P<0.001$. The tongue of *C. gerrardii* (2.13 JND±SE 0.34; $Z=1.42$, $P=0.154$) and the shoulder spot of our captive *G. galloti galloti* (1.68 JND±SE 0.34; $Z=0.50$, $P=0.613$) had the closest shades to the tongue of *T. gigas*, with color distances statistically corresponding with its intraspecific span of shades. The second closest hue was measured for the ventrum of *P. lilfordii kuligae*, (2.89 JND±SE 0.34; $Z=-2.39$, $P=0.017$) which fell also into the transitional 1–3 JND range (Fig. 4).

The comparison of UV-positive and UV-negative tongue spectra revealed that they are perceived as substantially distinct colors in the eyes of conspecifics (10.45 JND±SE 0.25), while being hardly distinguishable by avian predators (1.79 JND±SE 0.09; $Z=5.16$, $P<0.001$).

Discussion

We explored the visual qualities of blue lingual surface in the blue-tongued skink *T. gigas* to compare it with similar semantic signals of other lizard species. We have demonstrated that the blue tongue expresses a significant reflectance in the UV

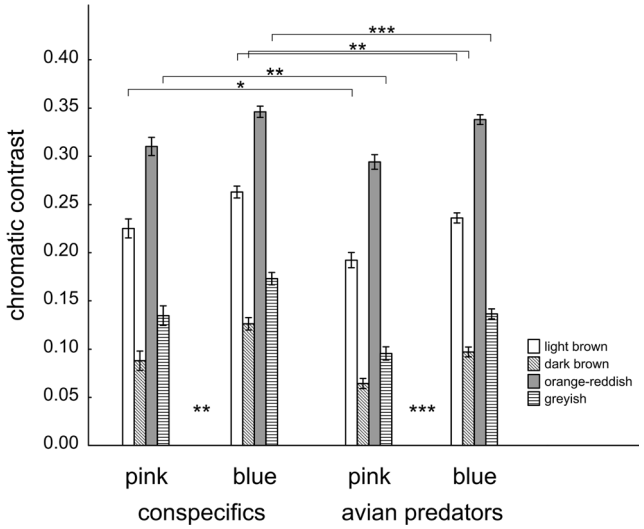

Fig. 3 Mean values of chromatic contrasts of blue tongues and pink *tongues* against four different background colors. *Asterisks* stand for significance of the Mann-Whitney *U* test (those situated between the column sets relate to differences between corresponding values within the visual model): **P*<0.05, ***P*<0.01, ****P*<0.001. *Bars* indicate ±SE

spectrum and a higher color contrast against various backgrounds (especially for the vision of conspecifics). The UV-blue lingual color as well as UV-blue patches of other lizard species fit into the same UV-sensitive visual communication channel of diurnal lizards. This leads us to the assumption that the blue tongue coloration might play an intraspecific signaling role.

The reflectance curve of pink oral mucosa, including the primary peak in the UV range, is obviously a general character of bare tissue, as it corresponds with the spectral reflectance of an open mouth and unfeathered skin of birds' nestlings (Hunt et al. 2003; Jourdie et al. 2004). Pink is not a spectral color and arises from combining two reflectance peaks instead of having just one (Burkhardt 1989; Endler 1990). As reflectance curves of blue tongues match the same shape with the exception of the longer wavelengths part, it is likely that a certain degree of lingual pigmentation suppresses the *red* peak, while retaining the *blue* peak, which leads to the blue coloration.

The shift from unpigmented to pigmented blue tongues notably leads to their higher conspicuousness, especially in their chroma and chromatic contrasts. Although the degree of change between pink and blue is virtually the same, blue was generally more contrasting in the conspecifics model than in the avian predator model. Taking also into account the results of UV-positive and UV-negative spectra comparison, we may assume that blue tongues are more likely to be adjusted to the sight of skinks, rather than to avian predators, which also corresponds to their photoreceptors sensitivity (UVS versus VS cones). This is in accordance with several studies suggesting that UV signs hardly cause any specific response in raptors, which gives their prey the advantage of using the ultraviolet communication channel (Endler 1992; Håstad et al. 2005; Lind et al. 2013; Marshall and Stevens 2014). Neither would be UV likely to work as an aposematic signal to birds with a UVS vision (Lyytinen et al. 2001). Apart from birds, *Tiliqua* are preyed upon by a range of terrestrial predators, but there is even more ambiguity regarding whether some of those can be directly associated with the evolution of lingual coloration.

Elapids and monitor lizards are possible candidates for predators putting *Tiliqua* skinks under evolutionary pressure (Shine and Keogh 1996; Fitzsimons 2011, Fleay 1950 ex Mayes et al. 2005), but little is known about their visual

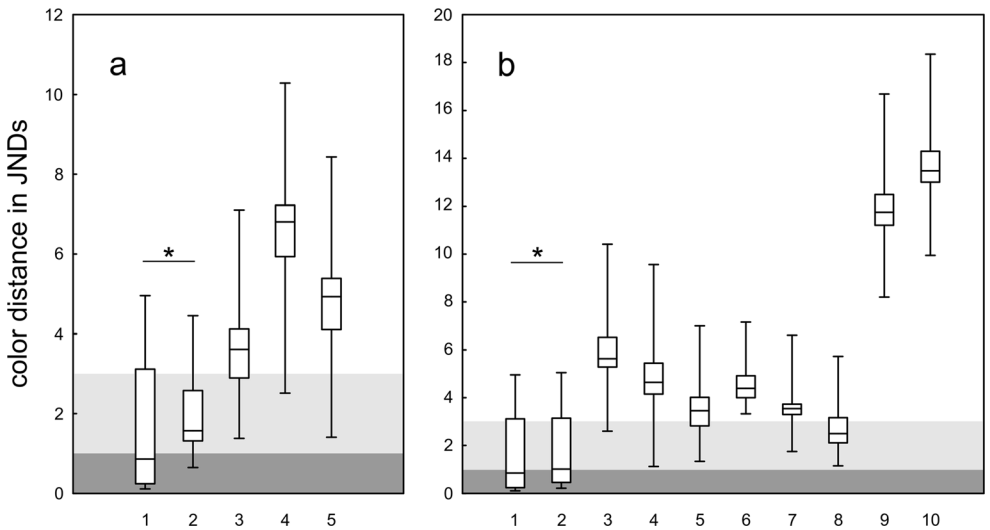

Fig. 4 Color distances of 14 blue tongues of *T. gigas* from mean values of tongues (**a**) and UV-blue skin patches (**b**) of selected species. **a** *1 T. gigas*, *2 C. gerrardii*, *3 E. frerei*, *4 C. zebrata*, *5 T. perrotetii*. **b** *1 T. gigas* (tongue), *2 G. galloti galloti*, captive individual (shoulder spots), *3 G. galloti galloti* (shoulder spots), *4 G. galloti eisentrauti* (shoulder spots), *5 T. lepidus* (lateral eyespots), *6 D. caucasica* (OVS), *7 P. pityusensis* (OVS), *8 P. lilfordi kuligae* (ventrum), *9 P. lilfordi kuligae* (OVS), *10 P. broadleyi* (throat). *Mid-line* median, *box* 25-75 %, *whiskers* minimum-maximum, *dark gray area* less than 1 JND, *light gray area* 1–3 JNDs, **P*>0.05 (Mann–Whitney *U* test)

systems. A study on hydrophiid sea snakes (Hart et al. 2012) shows they are trichromatic and lack UV-sensitive cones, which can be however associated with their transition into the sea, while terrestrial elapids may still have UVS cones preserved as in other snakes (Sillman et al. 1997, 1999, 2001; Macedonia et al. 2009). Monitor lizards have not been examined in this respect so far, but being diurnal, it is likely they are tetrachromatic and UV sensitive like other lizard groups (Perez i de Lanuza et al. 2014b). It is also worth mentioning that marsupials have UV-sensitive cones as well (Deeb 2010), so the UV-reflective tongues could be an appropriate stimulus for their vision too. Recent carnivorous marsupials (belonging to the Dasyuromorphia group) are, however, mostly nocturnal (Miller and Herbert 2010) and therefore these potential predators are unlikely to interact with diurnal *Tiliqua* and to present a serious selective pressure.

If the lingual coloration presents an anti-predation signal, UV-sensitive reptilian predators would be the target recipients more likely than birds. Bustard (1964) gives an account on such defensive gaping reaction of a gecko *Strophurus williamsi* towards a larger gecko of the genus *Oedura* (geckos can be also UV sensitive; Loew 1994; Loew et al. 1996). Like *Tiliqua*, geckos of the genus *Strophurus* (tribe Diplodactylini) have conspicuously colored blue/black or less frequently orange/yellow tongues and mouth interiors, which are exposed during defensive displays (Bustard 1964; Melville et al. 2004). Based on phylogenetic data, Melville et al. (2004) conclude that while pink mouth is ancestral, striking mouth colors may have evolved with a transition of *Strophurus* geckos to diurnal activity. The pattern in *Tiliqua* and its relatives is not so unambiguous. Although both large skinks with pink tongues, *C. gerrardii* and *C. zebrata* are reported to be crepuscular to nocturnal (Mann and Meek 2004; Cogger 2014), there is a number of other crepuscular and/or nocturnal species which do have bluish or dark tongues and mouths; *Liopholis kintorei*, *Cyclodomorphus branchialis*, *Cyclodomorphus casuarina*e, etc. (Shea and Miller 1995; Chapple 2003). Thus, even though blue tongues and/or mouths are a visual signal in both *Tiliqua* and *Strophurus*, diurnal activity is a precondition rather than a trigger for evolution of such trait. Based on the phylogeny by Pyron et al. (2013), the distribution of pigmented tongues rather suggests that they were already present in the common ancestor of the *Egernia-Liopholis-Cyclodomorphus-Tiliqua* group.

The lingual pigmentation itself forms rather a black–blue continuum, which can be noticed on the spectral curves (the lower the shape, the darker the tongue; Fig. 2), but also on their projection into the tetrahedral color space (Fig. 5). Dark pigmented tongues of *E. frerei* and *T. perrotetii* and even a pink tongue of *C. zebrata* lay at the lower end of a color space occupied by the blue tongues (closer to the achromatic center of the tetrahedron), which indicates similar hue, but different lightness and saturation. Their relative proximity is caused mostly by the common dominant reflectance in short wavelengths. Minimum color distance between tongues of *T. gigas* and *E. frerei* (or *T. perrotetii*) was only around 1.4 JND, so there is a certain overlap in the span of chromatic distances—some *T. gigas* have their tongues more similar in color to other species than to some of their conspecifics. The tongue closest in color belonged to *C. gerrardii*, which represents a peculiar case among blue-tongued skinks. Juveniles have blue tongues, but the pigmentation disappears with age and adults have their tongues mostly pink, though—as in our case—some remnants of pigmentation may still remain (Brown 2012).

Similar situation results from the comparison of tongues with UV-blue skin patches. The shade being the worst distinguishable from the blue tongues was the shoulder spot of our single captive individual of *G. galloti galloti*. Data on a much larger sample of wild *G. galloti* from literature resulted however in significantly greater color distances. As our experience with captive and wild animals shows, lizards may partially lose the intensity of their coloration in captivity, but intraspecific variation can be taken into account as well. In addition, the second closest hue was the blue ventrum of *P. lilfordi kuligae*, with the color distance also within the transitional range between indistinguishable and clearly distinguishable colors (1–3 JNDs).

Although we found only two occasions when a tongue or a skin patch had a color statistically interchangeable with the tongue of *T. gigas*, one should keep in mind that the spectra of other species were represented mostly by mean values which would be otherwise surrounded by clouds of raw data points. So at least some partial interspecific overlaps in color spaces can be expected. Since the color space occupied by the blue tongues is surrounded by spectra of UV-blue skin patches, tongues of *T. gigas* may possibly have qualities of a semantic signal, analogous to that of other lizards.

Co-evolution of visual signals with visual systems and behavior has been demonstrated in lizards (Fleishman et al. 2011; Landová et al. 2013; Martin et al. 2015), therefore it is likely that tongue pigmentation may represent such a case and serve in intraspecific communication. Although the skinks of the genus *Tiliqua* are closely related to the *Egernia* group of skinks, characterized by their social behavior and long-term relationships between individuals (Chapple 2003; Main and Bull 1996; Gardner et al. 2008; Fenner et al. 2012), little is known about the social organization of typical blue-tongued skinks of the *scincoides-gigas* group (Price-Rees et al. 2012, 2014; Pyron et al. 2013). Price-Rees et al. (2012) reported that there was no mutual influence of one lizard's location to another, which would suggest low importance of *direct behavioral interactions in these high-density core areas*. However, their study was based on radiotelemetric analyses, which does not allow detection of any visual communication between the lizards. So the putative lack of direct interactions may just as well have been caused by a specific visual signal. Due to

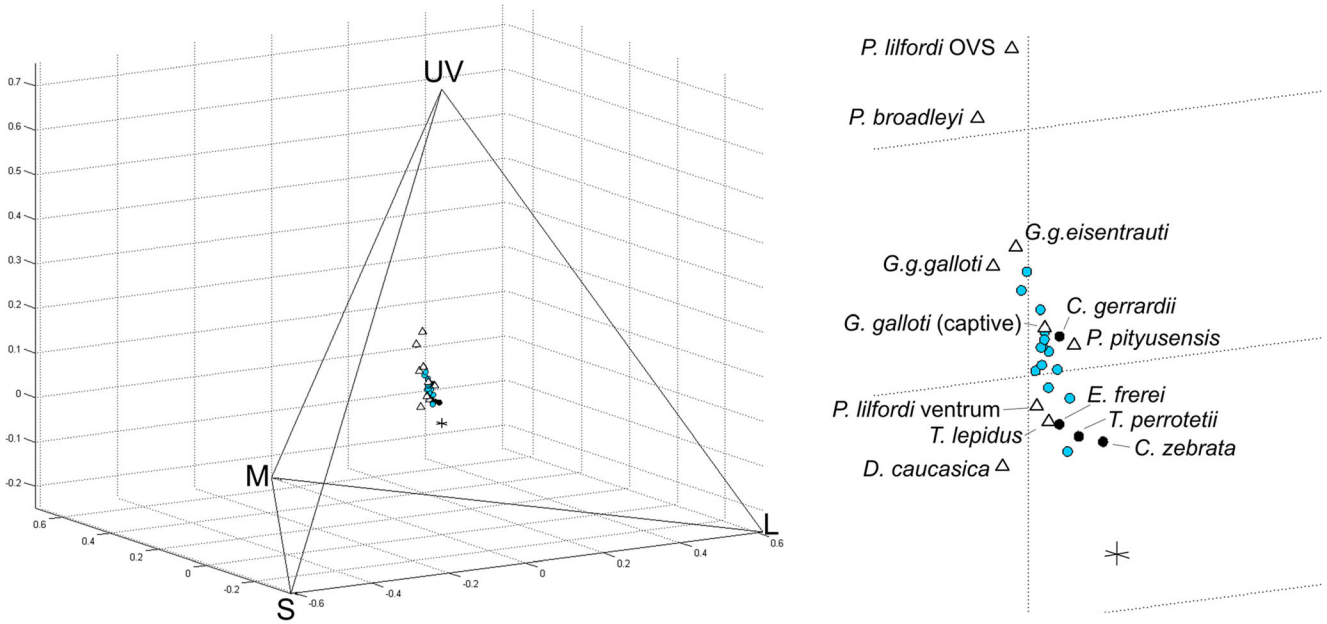

Fig. 5 Visual model of conspecifics with the distribution of analyzed spectra in the tetrahedral color space. Blue tongues of *T. gigas* (*shaded circles*; $n=14$), mean values for tongues (*black circles*), mean values for UV-blue skin patches (*triangles*). *Cross mark* indicates the achromatic center of the color space. *Vertices of the tetrahedron* stand for maximum stimulation values of photoreceptors sensitive to long (*L*), middle (*M*), short (*S*), and ultraviolet (*UV*) wavelengths

considerable aggressiveness of *Tiliqua*, there might be a need to avoid a potential conflict. Murray and Bull (2004), for instance, report higher aggressiveness of *T. rugosa* males towards conspecifics than non-conspecifics and Langkilde and Shine (2005) observed that open-mouth threat often preceded biting in *Egernia*. The tongue coloration therefore may possibly improve the signaling effect.

Our results have not confirmed that the darker the individual, the darker or bluer its tongue. Unless this was caused by a low sample size, we can assume that the intensity of lingual coloration is not much associated with the amount of skin melanin pigmentation, but can still represent an honest signal correlated with other factors like health, endurance, hormone levels, or age. According to our calculations the correlation coefficient suggests possible negative correlation between the body size and tongue saturation, although the result has not proved to be significant. Several other factors, like a growth rate, population, and larger sample size including also juveniles and subadults would give a more accurate picture of this relationship. In any case, even if lingual coloration was independent on the above-mentioned factors, this still would not exclude its possible signaling or recognition function.

Blue-colored tongues may enhance the open-mouth threat during interspecific interactions, and can be also secondarily used as an anti-predatory response which would probably be the most effective towards reptilian antagonists. Yet the tongue coloration need not be involved in close encounters only, but can also boost inter-individual recognition at longer distance interactions. Effective recognition of potential aggressors at distance, where the risk of threat is not immediate, may be of certain importance in avoiding conflicts. We suggest further behavioral tests to be made to clarify interspecific interactions of the blue-tongued skinks, the impact of tongue display on their aggression and predation and to examine possible correlations between tongue coloration and physiological aspects like endurance or hormone levels.

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Ethical standards This study was allowed by Ethical Committee of the Charles University in Prague, Czech Republic and approved by Ethical Committee of Ministry of Education, Youth and Sports, license no. 26582/2012-30.

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9.2. Příloha 2

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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

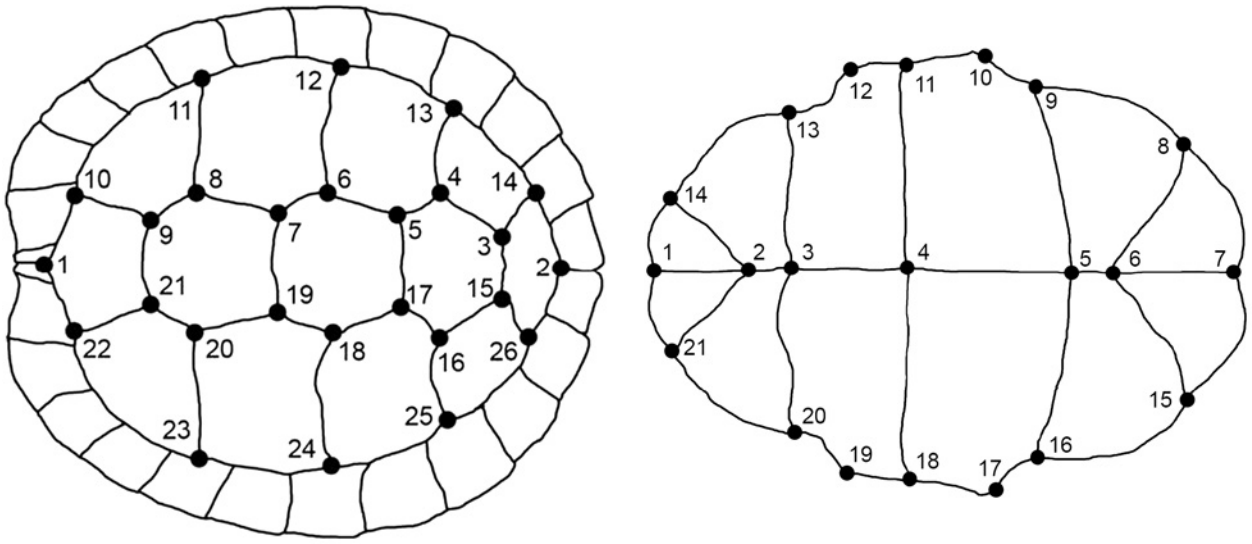

Fig. 1. Carapace and plastron of *Cuora amboinensis* showing the landmarks used in this study.

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 95th 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 ºC for 180 s, followed by 35 cycles composed of denaturation at 94 ºC for 45 s, annealing at 46 ºC for 45 s, and extension at 72 ºC for 1 min and 20 s; and finishing with a further 7 min elongation step at 72 ºC after the last cycle. For some of the samples, the temperature of annealing had to be increased to 50 ºC 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

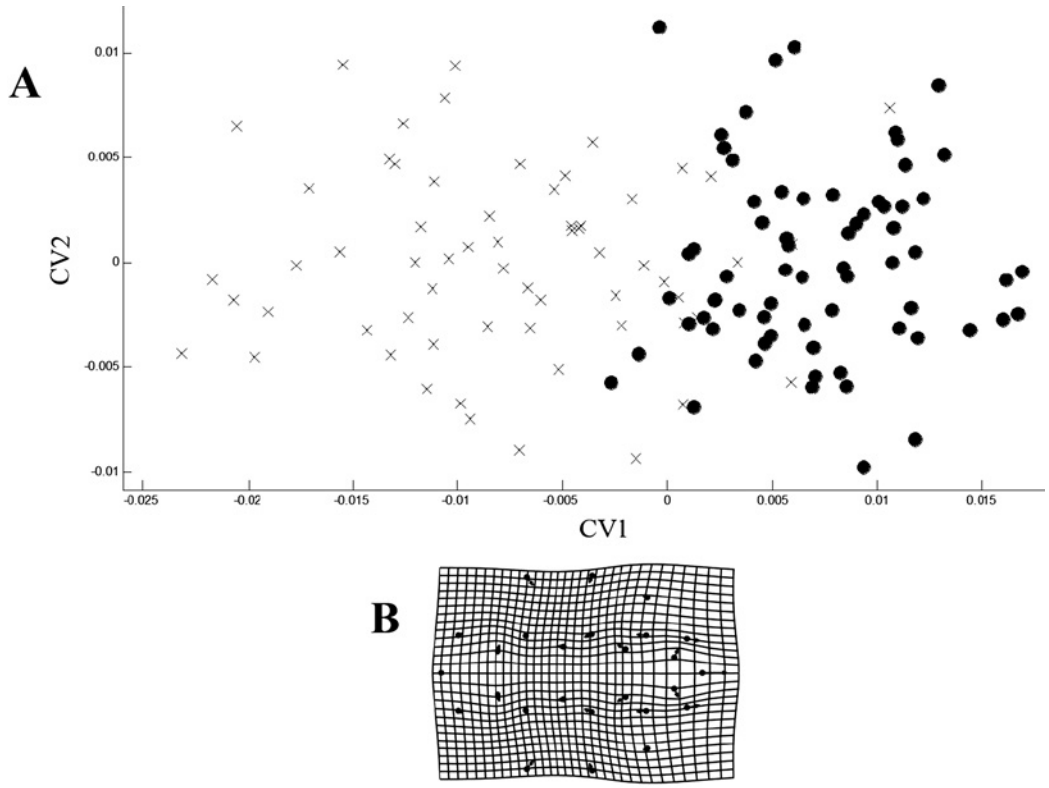

Fig. 2. Canonical plot for carapacial shape. Each carapace is represented by a symbol: × – Sumatra, ● – Borneo (A). The first axis accounts for 79.05% of the total between group variation. The thin plate spline diagram shows a change in carapacial shape along the first axis (in direction of arrows) (B).

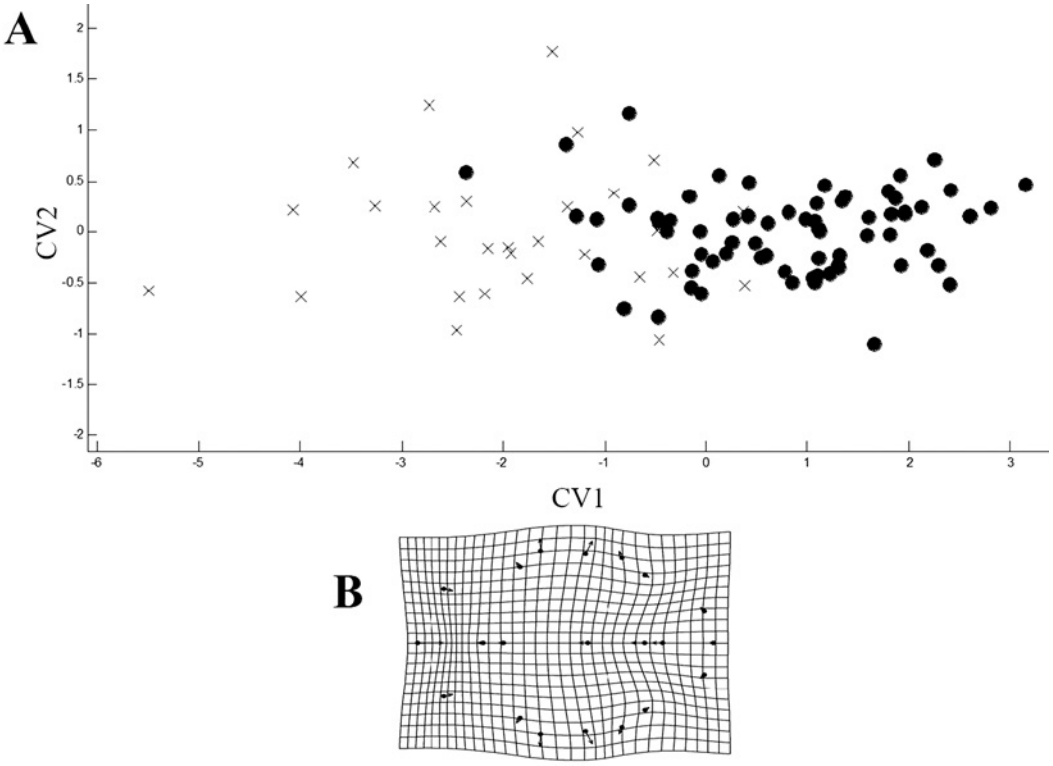

Fig. 3. Canonical plot for plastral shape. Each plastron is represented by a symbol: × – Sumatra, ● – Borneo (A). The first axis accounts for 55.25% of the total between group variation. The thin plate spline diagram shows a change in plastral shape along the first axis (in direction of arrows) (B).

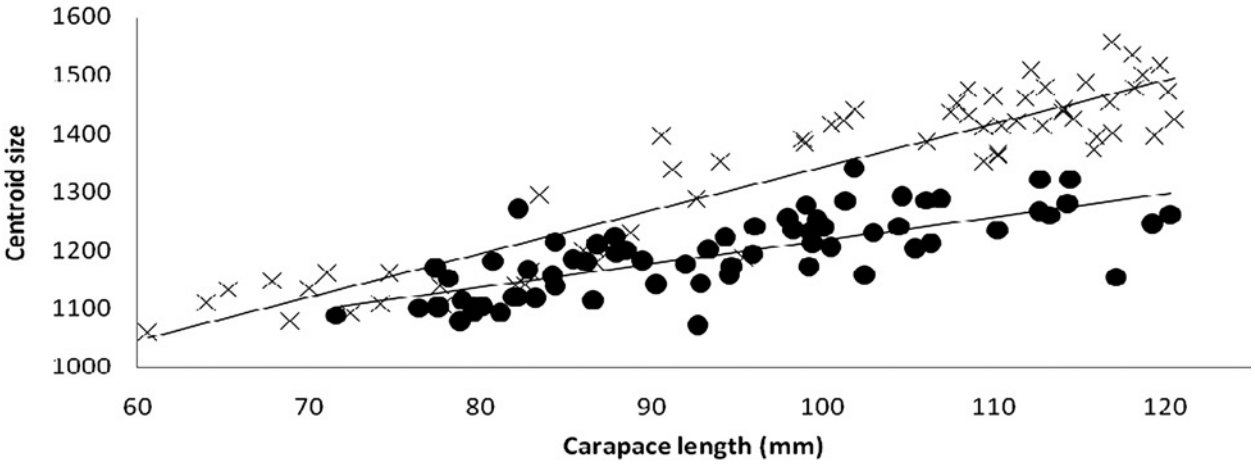

Fig. 4. Correlation of carapacial length and centroid size. × – Sumatra, ● – Borneo

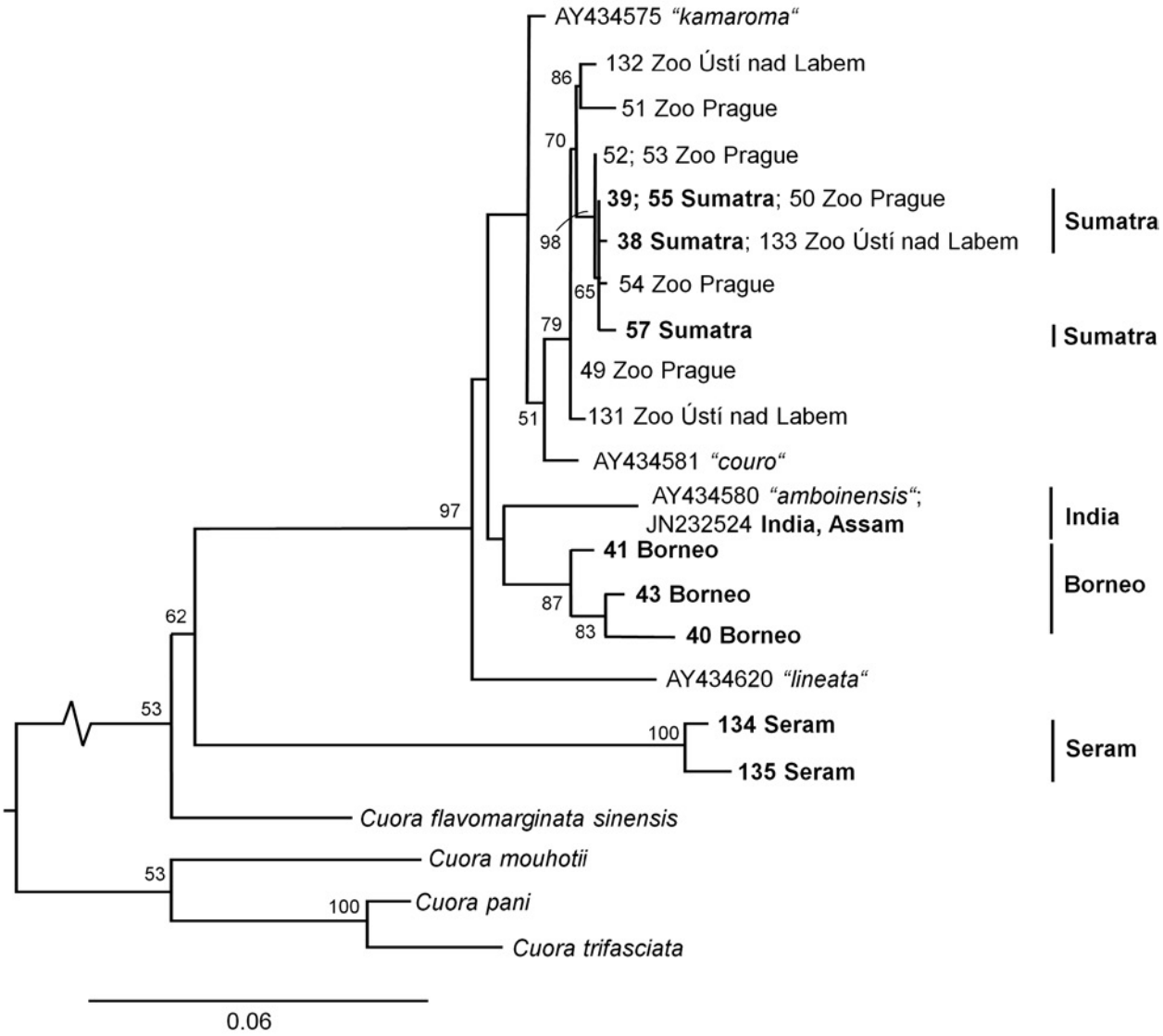

Figure 5. Phylogenetic tree by maximum likelihood (ML) method based on alignment of the complete cytochrome *b* gene (1140 bp). Numbers at branches are bootstrap values > 50. Samples sequenced in this study are labelled with sample numbers and are in bold, remaining samples were sequenced by previous 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 "*limeata*"	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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