Mechanismy karyotypové evoluce u štírů
(Arachnida: Scorpiones)

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Dizertační práce

PŘÍRODOVĚDECKÁ
FAKULTA
Univerzita Karlova

Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Zoologie

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Mechanismy karyotypové evoluce u štírů (Arachnida: Scorpiones)

Mechanisms of the karyotype evolution in scorpions (Arachnida: Scorpiones)

DIZERTAČNÍ PRÁCE

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Praha, 2019

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PODĚKOVÁNÍ

Tato práce by nevznikla nebýt pomoci, podpory a spolupráce rodiny, přátel a kolegů.

Předně bych ráda poděkovala své **rodině**, která mě vždy plně podporovala bez ohledu na to, na jakou cestu jsem se vydala. Vážím si toho, že nikdy nezpochybnila má rozhodnutí, ať sebevíc bláznivě mohla znít. Bez lásky, podpory a zázemí bych nikdy nedošla tam, kde jsem.

Děkuji mému školiteli, **Františkovi Šťáhlavskému**, za mnohaletou spolupráci, podporu a veškerou důvěru ve mě vloženou.

Velké poděkování patří **Jirkovi Šmídovi**, který mě před lety vzal pod svá fylogenetická křídla, stal se mým mentorem a pomohl mi se posunout v mnoha ohledech vpřed.

Děkuji **Frantovi Kovaříkovi**, že mi v průběhu let předal spousty cenných znalostí ze světa štírů, jenž mi ukázal i z jiné perspektivy.

Děkuji kolegům ze Zoologického oddělení Národního Muzea, jmenovitě **Radkovi Šandovi**, **Jiřímu Moravcovi** a **Petrovi Dolejšovi**, za velmi přínosnou spolupráci.

Ráda bych poděkovala **všem kolegům**, kteří mi věnovali svůj čas, pomohli jakoukoliv radou či podnětnou diskuzí. Nebylo vás málo! V tomto ohledu patří zvláště pak velké poděkování **Sašovi Semberovi**, **Věře Opatové**, a **Petrovi Nguyenovi**.

Děkuji **Pavlovi Justovi**, **Ann Pospíšilové**, **Ladťovi Soukupovi**, **Wojtovi a Anežce Millerovým**, kteří se během let aktivně podíleli na zachování mého psychického zdraví s humorem sobě vlastním.

Moc bych chtěla poděkovat **Aleně Hoškové**, **Jindřišce Peterkové** a **Markétě Hejné**, že společně vytvářejí na katedře zoologie oázu klidu a útočiště, kde se člověku dostane odpovědi na každou otázku a podpory za každé situace. Díky, že nám fandíte a podporujete nás na naší cestě.

Děkuji všem **tuzemských i zahraničním kolegům**, kteří přispěli sběrem materiálu pro naše cytogenetické analýzy a bez který by nebyl náš dataset tak bohatý. Současně také děkuji všem **štírům**, kteří obětovali svůj život na oltář vědy.

Velké DĚKUJI patří **Jenovi**, mému muži, který se mnou sdílí veškeré radosti i strasti a je mi bezmeznou oporou.

Finanční podpora: Tato práce by nevznikla za finanční podpory Grantové agentury Univerzity Karlovy v Praze (projekty č. 1350214 a 1324217) a programu STARS.

PROHLÁŠENÍ O PODÍLU NA PUBLIKACÍCH

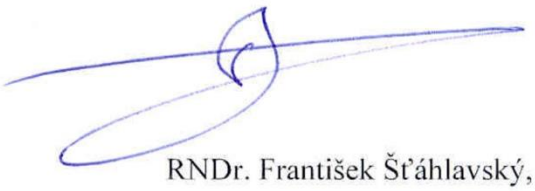
I. Šťáhlavský, F., Nguyen, P., Sadílek, D., **Štundlová, J.**, Just, P., Haddad, C. R., Koç, H., Ranawana, K.B., Stockmann, M., Yağmur, E.A., Kovařík, F. Evolutionary dynamics of rDNA clusters on chromosomes of buthid scorpions (Chelicerata: Arachnida). *Rukopis*.

Podíl J. Štundlové: zpracování živého materiálu, příprava chromozomových preparátů, resyntéza 18S rDNA sondy, rDNA-FISH, úprava rukopisu.

RNDr. František Šťáhlavský, Ph.D.

II. Just, P., Šťáhlavský, F., Kovařík, F., **Štundlová, J**. Tracking the trends of karyotype differentiation in the phylogenetic context in *Gint* Kovařík et al., 2013, a scorpion group endemic to the Horn of Africa (Scorpiones: Buthidae). *Rukopis*.

Podíl J. Štundlové: zpracování živého materiálu, příprava chromozomových preparátů, výroba 18S rDNA sondy, TTAGG FISH, editace sekvencí, fylogenetické analýzy, příprava obrazových tabulí a schémat, sepisování a úprava rukopisu (spolu s P. Just). Plánovaná korespondence s editorem a recenzenty.



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III. Kovařík, F., Košulič, O., Šťáhlavský, F., **Plíšková, J.**, Dongkhamfu, W., Wongprom, P., 2015. Two new species of *Euscorpiops* Vachon, 1980 from Thailand and Myanmar (Scorpiones: Euscorpiidae: Scorpiopinae). *Annales Zoologici* 65, 109–122. (IF_{2015}= 1.136)

Podíl J. Štundlové: příprava chromozomových preparátů, analýza karyotypu.

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IV. **Plíšková, J.**, Kovařík, F., Košulič, O., Šťáhlavský, F., 2016. Description of a new species of *Heterometrus* Ehrenberg, 1828 (Scorpiones: Scorpionidae) from Thailand with remarks about the utilization of cytogenetic data in taxonomy of the genus. *Annales Zoologici* 66, 467–476. (IF_{2016}= 0.699)

Podíl J. Štundlové: příprava chromozomových preparátů, analýza karyotypu, tvorba obrazové tabule, sepsání a úprava rukopisu.

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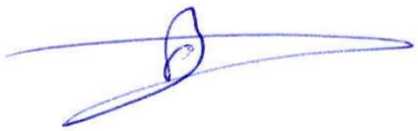
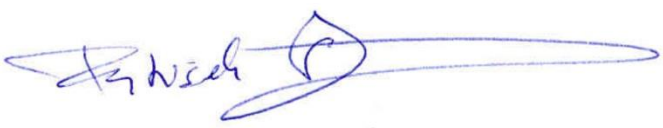
Podíl J. Štundlové: příprava chromozomových preparátů, výroba 18S rDNA sondy, analýza karyotypu, tvorba obrazové tabule, úprava rukopisu.



RNDr. František Šťáhlavský, Ph.D.

VI. **Štundlová, J.**, Šmíd, J., Nguyen, P., Šťáhlavský, F., 2019. Cryptic diversity and dynamic chromosome evolution in Alpine scorpions (Euscorpiidae: *Euscorpius*). *Molecular Phylogenetics and Evolution* 134, 152–163. ($IF_{2018/2019}$= 3.992)

Podíl J. Štundlové: terénní sběry, laboratorní práce: zpracování živého materiálu, příprava preparátů, 18S rDNA FISH, fotodokumentace, analýza karyotypu, PCR a příprava genových fragmentů na sekvenace, editace sekvencí, tvorba veškerých obrazových tabulí a schémat. Sepsání a úprava rukopisu (společně s J. Šmídem). Korespondence s editorem a recenzenty.



RNDr. František Šťáhlavský, Ph.D.

ABSTRAKT

Štíři představují starobylou skupinu pavoukovců, která od dob siluru kolonizovala rozmanitou škálu terestrických prostředí. Navzdory dlouhé evoluční historii a ekologické rozmanitosti je pro tyto živočichy příznačná morfologická stáze. Uniformní morfologie štírů nicméně může maskovat skutečnou diverzitu současných linií. Studium cytogenetických znaků tak pomůže prohloubit naše znalosti o rozmanitosti recentních taxonů štírů a současně přinést nový vhled do procesů stojících v pozadí karyotypových změn u této skupiny pavoukovců. Předmětem předkládané dizertační práce je studium rozmanitosti a dynamiky karyotypů vybraných zástupců štírů s holokinetickými a monocentrickými chromozomy. Komparativní cytogenetické analýzy více než 110 druhů z pěti čeledí zahrnovaly nejen standardních cytogenetické techniky, ale i mapování genu pro 18S ribozomální RNA a telomerických (TTAGG)*n* repetic metodou fluorescenční *in situ* hybridizace (FISH). Pro účely objasnění fundamentálních strukturních mechanismů stojících v pozadí diferenciace karyotypu u studovaných skupin byl kladen důraz na propojení cytogenetických a sekvenčních dat analyzovaných druhů. Znalost genetické struktury a příbuzenských vztahů studovaných taxonů sehrála nezastupitelnou roli jednak při interpretaci chromozomální polymorfismu, resp. polytypie, jednak pro navržení hlavních trendů uplatňujících se v reorganizaci štířích karyotypů. Předkládané výsledky demonstrují, že štíři se vyznačují nebývalou chromozomální variabilitou na různých strukturních úrovních. Detekované rozdíly naznačují různou míru zapojení makro- i mikrostrukturních změn při diferenciaci karyotypů v rámci jednotlivých evolučních linií štírů.

Klíčová slova: karyotypová diferenciace, holokinetické chromozomy, multivalentní asociace, chromozomální přestavby, telomerické (TTAGG)n repetice

ABSTRACT

Scorpions represent an ancient group of arachnids that have colonized a diverse range of terrestrial environments since Silurian times. Despite their long evolutionary history and ecological diversity, these animals are characterized by morphological stasis. However, the uniform morphology of extant scorpions may hide the true diversity of contemporary lineages. The study of cytogenetic traits thus has the potential to deepen our knowledge of the diversity of contemporary scorpion taxa and bring new insight into the processes underlying karyotypic changes in this group of arachnids. The subject of this thesis is to examine the diversity and dynamics of karyotypes of selected scorpion groups with holokinetic and monocentric chromosomes. Comparative cytogenetic analysis, including more than 110 species from five families, was based on standard cytogenetic techniques and mapping of the gene for 18S ribosomal RNA and telomeric (TTAGG)n repeats by fluorescence *in situ* hybridization (FISH). To elucidate the fundamental structural mechanisms underlying karyotype differentiation in the studied groups, the emphasis was placed on the interconnection of cytogenetic and sequence data of the analyzed species. The knowledge of the genetic structure and phylogenetic relationships of studied taxa played a crucial role in the interpretation of observed chromosomal polymorphism or polytypism. Moreover, this knowledge enabled us to propose the main trends involved in the reorganization of scorpion karyotypes. The present results demonstrate that scorpions exhibit exceptional chromosomal variability at different structural levels. The detected differences within individual scorpion lineages indicate a distinct degree of involvement of macro- and microstructural changes in karyotype differentiation.

Key words: karyotype differentiation, holokinetic chromosomes, multivalent association, chromosome rearrangements, telomeric (TTAGG)n repeat

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1. SEZNAM PUBLIKACÍ

I. Šťáhlavský, F., Nguyen, P., Sadílek, D., **Štundlová, J.**, Just, P., Haddad, C. R., Koç, H., Ranawana, K.B., Stockmann, M., Yağmur, E.A., Kovařík, F. Evolutionary dynamics of rDNA clusters on chromosomes of buthid scorpions (Chelicerata: Arachnida). *Rukopis*.

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Součástí výsledků dizertační práce jsou i nepublikovaná data pro *Pandinus sensu lato*, která jsou přiložena formou obrazových tabulí v sekci Přílohy (Obr. 1–5). V průběhu řešení dizertační práce jsem se také spoluautorsky podílela na několika taxonomických publikacích, jejichž součástí jsou popsané karyotypové charakteristiky u studovaných druhů štírů. Tyto publikace nejsou přímo zahrnuty do této dizertační práce, nicméně v hlavním textu jsou označeny podtržením příslušné reference.

2. ÚVOD

Štíři (Scorpiones) představují starobylý řád klepítkatců (Chelicerata) s doloženým fosilním záznamem již z období spodního siluru (438.5–433.4 Ma) (Dunlop 2010; Dunlop & Selden 2013). Jedná se o evolučně úspěšnou, celosvětově rozšířenou skupinu pavoukovců (Arachnida), která osídlila rozličná terestrická prostředí, včetně těch s extrémními klimatickými podmínkami (Polis 1990). Štíři představují s více než 2 400 druhy 5. nejpočetnější řád pavoukovců (Coddington et al. 2004; Rein 2019), který v současnosti zahrnuje 18 čeledí. Vyšší klasifikace štírů nicméně zůstává prozatím nevyřešena, a to i navzdory implementaci moderních metod s využitím transkriptomatických dat. Výstupy fylogenomických studií poukázaly na spornost monofylie některých čeledí i problematiku nejistých vzájemných příbuzenských vztahů řady evolučních linií (Santibáñez-López et al. 2019; Sharma et al. 2015). Navzdory dlouhé evoluční historii a ekologické rozmanitosti se štíři vyznačují nebývale uniformní morfologií. Recentní skupiny štírů se v tělním plánu téměř neliší od svých silurních předchůdců (Coddington et al. 2004). Není tedy překvapivé, že delimitace štířích taxonů zakládající se výhradně na morfologických znacích je nevyhnutelně provázena řadou komplikací. Konzervativní vnější morfologie štírů může maskovat jednak skutečnou diverzitu, jednak komplexní evoluční historii druhů (např. Bryson et al. 2018; Miller et al. 2014). V důsledku toho se studium evoluce a diverzity této skupiny pavoukovců v současné době zaměřuje na implementaci moderních molekulárních metod s důrazem na integrativní přístup (viz Bryson et al. 2013a,b; Ojanguren-Affilastro et al. 2016, 2017; Talal et al. 2015). V průběhu poslední dekády zaznamenal výrazný posun i cytogenetický výzkum štírů, díky čemuž se daří získávat hlubší povědomí o uspořádání genomu studovaných taxonů (např. Mattos et al. 2018; Ubinski et al. 2018). Evoluční historie druhů nemusí být vtisknuta pouze do genetické rozmanitosti současných linií, ale může se projevit také v reorganizaci karyotypu. Studium cytogenetických znaků tak může prohloubit naše znalosti o diverzitě štírů a současně přinést nový vhled do procesů stojících v pozadí karyotypových změn. Přímé propojení studia genetické a karyotypové rozmanitosti současných linií v kontextu jejich geografického rozšíření nicméně u štírů stále chybí.

Současné poznatky o cytogenetice štírů vycházejí z úzce zaměřených studií odlišného charakteru. Zásluhou těchto prací máme u tohoto řádu k dispozici základní znalosti o spermatogenezi (např. Sokolow 1913), stavbě synaptonemálního komplexu (např. Shanahan & Hayman 1990), achiasmatické meióze samců (např. Almeida et al. 2019) či karyotypové rozmanitosti druhů (např. Shanahan 1989a,b; Schneider et al. 2009a,b). Pro štíry je

charakteristické široké rozpětí počtů chromozomů – od 2*n* = 5 u *Tityus bahiensis* (Buthidae) (Schneider et al. 2009a) po 2*n* = 186 u *Chaerilus stockmannorum* (Chaerilidae) (Kovařík et al. 2018a). Současně byla u celé řady druhů identifikována přítomnost chromozomálního polymorfismu, který je zde spjatý s výskytem strukturních heterozygotů (např. Mattos et al. 2013; Shanahan 1989a,b; Schneider et al. 2009a,b). Řád štíři zahrnuje evoluční linie, jejichž chromozomy vykazují odlišnou distribuci kinetochoru. U většiny cytogeneticky studovaných čeledí byl doložen výskyt monocentrických chromozomů, jenž jsou charakteristické lokalizovanou centromerou. Naopak přítomnost holokinetických chromozomů vyznačujících se difúzním kinetochorem je příznačná pro jedinou evoluční linii štírů, čeleď Buthidae (Shanahan 1989a). Unikátní kombinace cytogenetických rysů činí štíry atraktivní skupinou pro výzkum rozličných aspektů týkajících se diverzity a diferenciace karyotypu. Výskyt evolučních linií jak s monocentrickými, tak holokinetickými chromozomy současně umožňuje porovnat cytogenetická specifika těchto dvou odlišných systémů.

Předkládaná dizertační práce si klade za cíl rozšířit současné poznatky o cytogenetice štírů a objasnit hlavní mechanismy diferenciace karyotypu u vybraných zástupců štírů s holokinetickými a monocentrickými chromozomy. U více než 110 druhů byla zkoumána karyotypová rozmanitost na různých strukturních úrovních za pomoci standardních i molekulárně cytogenetických přístupů. Získaná karyologická data byla dále u vybraných skupin (i) interpretována v kontextu fylogenetických vztahu studovaných druhů; (ii) využita pro taxonomické účely.

Dizertační práce je členěna do dvou hlavních kapitol, jenž přinášejí shrnující informace o karyotypové diverzitě, chromozomálním polymorfismu, distribuci genů pro ribozomolní RNA a telomerického (TTAGG)*n* motivu, a o využití cytogenetických znaků v taxonomii u skupin štírů s holokinetickými (kapitola 4.1) a monocentrickými chromozomy (kapitola 4.2).

3. CÍLE DIZERTAČNÍ PRÁCE

Předkládaná dizertační práce se zabývá studiem karyotypové diverzity a dynamiky uspořádání genomu u zástupců hlavních evolučních linií štírů, jejichž chromozomy se odlišují lokalizací kinetochoru [tj. holokinetické a monocentrické chromozomy]. Výzkum cytogenetických aspektů u štírů s holokinetickými chromozomy zahrnoval analýzu druhů náležejících do čeledi Buthidae, která jakožto jediná v rámci celého řádu vykazuje tento typ chromozomů. Pro objasnění cytogenetických aspektů u štírů s monocentrickými chromozomy byli vybráni zástupci náležející do tří čeledí obývajících různé biogeografické oblasti: Euscorpiidae, Hormuridae a Scorpionidae. Pro předkládanou dizertační práci se podařilo shromáždit unikátní komparativní materiál více než 110 druhů, jenž byl využit nejen pro standardní a molekulárně cytogenetické analýzy, ale v některých případech i pro molekulárně fylogenetické a morfologické analýzy. Hlavním cílem předkládané práce bylo rozšířit současné cytogenetické poznání štírů a pokusit se objasnit hlavní trendy karyotypové diferenciace u evolučních linií s holokinetickými a monocentrickými chromozomy. V průběhu řešení této práce jsem se zaměřila na několik dílčích cílů:

(***i***) Získat detailní informace o karyotypu studovaných druhů pomocí standardních a/nebo molekulárně cytogenetických metod, a takto získaná data využít pro komparativní karyotypové analýzy s důrazem na detekci chromozomálního polymorfismu či polytypie (**Článek I**, **Článek II**, **Článek VI**, **Příloha Obr. 1–5**)

(***ii***) Pomocí integrace cytogenetických a genetických dat objasnit hlavní trendy karyotypové diferenciace u kandidátních skupin štírů (**Článek II**, **Článek V**, **Příloha Obr. 1–5**)

(***iii***) Cytogenetická data využít pro taxonomické účely (**Článek III**, **Článek IV**, **Článek V**)

(***iv***) Získaná data využít pro identifikaci hlavních mechanismů podílejících se na diferenciaci karyotypu příznačných pro skupiny s holokinetickými a monocentrickými chromozomy.

4. VÝSLEDKY PRÁCE V KONTEXTU SOUČASNÝCH POZNATKŮ

4.1 Cytogenetické aspekty čeledi Buthidae s holokinetickými chromozomy

V rámci řádu štíři je dosud evidována jediná linie s holokinetickými chromozomy, a to čeleď Buthidae (např. Shanahan 1989a). Význačnost této skupiny ve srovnání s ostatními evolučními liniemi štírů nicméně netkví pouze ve výskytu tohoto typu chromozomů, ale také v její fylogenetické pozici, druhové rozmanitosti, či významné toxicitě jejích zástupců. Dle recentních fylogenomických analýz tvoří Buthidae společně s Pseudochactidae a Chaerilidae časně se větvící linii celého řádu, "parvorder" Buthida (Sharma et al. 2015, 2018). Pseudochactidae a Chaerilidae reprezentují co do počtu druhů a rozšíření poměrně malé čeledi štírů (Rein 2019). Buthidae naproti tomu představuje s 91 rody a 1150 druhy největší a druhově nejrozmanitější čeleď celého řádu (Rein 2019), která současně jako jediná pronikla, vyjma Antarktidy, na všechny světové kontinenty (Sissom 1990). Pouze nepatrný zlomek štířích druhů se vyznačuje silnou toxicitou, jenž u člověka vyvolává životu ohrožující komplikace, nicméně až na nepatrné výjimky náleží tyto druhy právě do čeledi Buthidae (shrnuto ve Ward et al. 2018). Zásluhou všech výše zmíněných atributů představují buthidní štíři atraktivní skupinu, která stojí ve srovnání s ostatními čeleděmi štírů v popředí jak veřejného, tak vědeckého zájmu. Tomu také nasvědčuje široká škála studií zabývající se u studovaných druhů například genetickou rozmanitostí (např. Sousa et al. 2011; Yamashita & Roads 2013), fylogeografií (např. Habel et al. 2012; Shi et al. 2013), toxicitou (např. Borges et al. 2006; Inceoglu et al. 2001), a v neposlední řadě také karyotypovou diferenciací (např. Piza 1944; Shanahan 1989a; Sadílek et al. 2015). Není tudíž překvapivé, že 60 % všech karyologicky analyzovaných druhů štírů náleží právě do čeledi Buthidae (viz Schneider et al. 2019). Nicméně vzhledem k druhové rozmanitosti této evoluční linie tyto druhy pokrývají pouze necelých 8 % celkové diverzity čeledi. Kontrastní je také samotná prozkoumanost jednotlivých biogeografických regionů. Cytogenetickým studiím dominují druhy neotropického regionu, zatímco jiné geografické oblasti zůstávají z velké části opomenuty.

4.1.1 Karyotypová diverzita evolučních linií

Současné poznatky o karyotypové diverzitě čeledi Buthidae vycházejí z dostupných dat, které byly dosud publikovány pro 87 druhů z 34 rodů (shrnuto v Příloha Tab. 1). Výsledky předkládané práce tyto znalosti zásadně rozšiřují, jelikož se podařilo shromáždit nové karyologické údaje pro bezmála jednou tolik taxonů (Příloha Tab. 1). Celkově se podařilo

získat či rozšířit informace o cytogenetických znacích pro dalších 81 taxonů náležejících do 23 rodů, které pocházejí z různých geografických oblastí napříč kontinenty, vyjma Austrálie (**Článek I a II**). V průběhu řešení této práce se také podařilo získat úplně první cytogenetická data pro několik rodů této čeledi, konkrétně pro rody *Apistobuthus*, *Grosphus*, *Orthochirus*, *Thaicharmus* (**Článek I**), *Buthoscorpio* a *Charmus* (Kovařík et al. 2016a).

Diploidní počty chromozomů dosahují u buthidních štírů relativně nízkých hodnost a pohybují se v úzkém rozpětí od $2n = 5$ u *Tityus bahiensis* (Schneider et al. 2009a) po $2n = 36$ u *Barbaracurus somalicus* (Kovařík et al. 2018b). Obdobné výsledky byly získány i vlastní analýzou studovaných druhů, kdy nejnižší diploidní počet chromozomů $2n = 9$ byl zaznamenám u druhu *Isometrus thwaitesi* (Kovařík et al. 2016a, **Článek I**), naopak nejvyšší $2n = 45$ u *Gint gubanensis* (**Článek II**). Přestože se na základě rozsahu $2n$ mohou karyotypy buthidních štírů zprvu jevit jako poměrně diverzifikované, z kompilace všech cytogenetických dat je patrné, že počty chromozomů u převážné většiny druhů oscilují kolem hodnoty $2n = 20$ (fig. 3, **Článek I**; Příloha Tab. 1).

Při srovnání karyotypové diverzity je patrné, že se jednotlivé rody čeledi Buthidae mezi sebou v tomto aspektu nápadně liší. Větší měrou jsou zde však zastoupeny linie, které vykazují na mezidruhové úrovni neměnné či mírně variabilní počty chromozomů (Příloha Tab. 1). Rody, u nichž se počet chromozomů jeví jako stabilní, jsou svým výskytem vázány na různé geografické oblasti, nicméně většina z nich tvoří typický element fauny Afriky a Blízkého východu, např. *Androctonus* [$2n = 24$] (Chovet et al. 1971; Moustafa et al. 2005; Sadílek et al. 2015; **Článek I**), *Buthus* [$2n = 22$] (Guénin 1961; **Článek I**), *Microbuthus* $2n = 26$ (Lowe et al. 2018), *Leiurus* [$2n = 22$] (Qumsiyeh et al. 2013; Šťáhlavský et al. 2014; **Článek I**), či *Compsobuthus* [$2n = 22$] (Kovařík et al. 2016b; Šťáhlavský et al. 2014; **Článek I**). Ve srovnáním s těmito skupinami naopak nápadně kontrastují rody, které vykazují výraznou variabilitu počtu chromozomů. Řadí se mezi ně například afrotropické rody *Uroplectes* [$2n =$ 16–28] (Kovařík et al. 2016c; Newlands & Martindale 1980; **Článek I**), *Gint* [$2n = 18$–45] (Kovařík et al. 2013a; Kovařík et al. 2018c; **Článek II**), či neotropický rod *Tityus* [$2n = 5$–32] (např. Mattos et al. 2013, 2018; Ojanguren-Affilastro et al. 2017; Schneider et al. 2009a; **Článek I**).

Z výše uvedených údajů se může zdát, že u rodů se stabilním počtem chromozomů karyotyp nepodléhá zjevným strukturním změnám, a naopak u rodů s proměnlivými počty chromozomů jsou tyto změny časté. Nicméně bližší poznání cytogenetických aspektů buthidních štírů pomocí standardních i molekulárně cytogenetických metod odhalilo přítomnost strukturních změn u taxonů jak s labilním, tak se stabilním karyotypem (viz podkapitola 4.1.2).

4.1.2 Chromozomální polymorfismus související se strukturními přestavbami

Znalosti o chromozomálním polymorfismu u buthidních štírů mají i přes intenzivní studium této skupiny fragmentární charakter. Karyologické údaje většiny dosud studovaných druhů jsou zpravidla založeny na nízkém počtu analyzovaných jedinců (nejčastěji 1–3), což často neumožňuje získat potřebné informace jak o přítomnosti, tak o samotném rozsahu chromozomálního polymorfismu v přirozených populacích (viz Příloha Tab. 1). Hlubší vhled do této problematiky poskytují studie zaměřené na populační cytogenetiku vybraných zástupců čeledi Buthidae [např. *Lychas* (Shanahan 1989a), *Tityus* (např. Adilardi et al. 2016; Mattos et al. 2018)]. Nicméně i zde Shanahan (1989a) poukazuje na četný výskyt málopočetných izolovaných populací, z nichž nebylo možné karyotypovat více než jednotlivé exempláře. Cytogenetická data zahrnutá v této dizertační práci se rovněž zakládají na omezeném množství analyzovaných jedinců stejného druhu. Buthidní štíři vykazují největší druhovou rozmanitost v tropických a subtropických oblastech (Fet et al. 2000). Ve spojitosti s nízkou populační hustotou a sezónalitou studovaných druhů je tudíž velice náročné z těchto vzdálených regionů získat živý materiál v dostatečném množství pro účely populační cytogenetiky. Nicméně i přes tato omezení poskytují předkládané cytogenetické výsledky nové dílčí poznatky týkající se chromozomálního polymorfismu u buthidních štírů (**Článek I a II**).

Z dosud publikovaných cytogenetických dat je patrné, že karyotypy buthidních štírů mají zvýšenou toleranci k strukturním změnám. Rozličné evoluční linie napříč čeledí vykazují určitý stupeň polymorfismu, který úzce souvisí s přítomností chromozomálních přestaveb v heterozygotním stavu [např. *Lychas* (Shanahan 1989a), *Tityus* (Mattos et al. 2018; Schneider et al. 2009a), *Jaguajir* (Mattos et al. 2013), *Somalicharmus* (Kovařík et al. 2016e); shrnuto v Příloha Tab. 1]. Fenomén vysoké četnosti strukturních přestaveb v karyotypu buthidních štírů podporují i karyologická data zahrnutá v této práci, kdy téměř polovina všech analyzovaných druhů vykazovala v samčí profázi I přítomnost heterozygotních chromozomálních asociací (Příloha Tab. 1, **Článek I a II**). Uvedený podíl může být navíc podhodnocen, jelikož pro 26 druhů se nepodařilo získat informace o samčí meióze. Nicméně u některých z těchto případů byly pozorovány v mitotické metafázi velikostní disproporce mezi homologickými chromozomálními segmenty nesoucími lokusy 18S rDNA, což u konkrétních jedinců nepřímo informuje o výskytu heterozygotních reciprokých translokací (např. *Hottentotta hottentotta* a *H. saulcyi* fig S1 Q, T **Článek I**).

Multivalentní meiotické řetězce u buthidních štírů mohou dosahovat různého stupně složitosti, a to v závislosti na mechanismu jejich vzniku a na celkovém počtu chromozomálních

elementů účastnících se strukturních přestaveb. Utváření heterozygotních chromozomálních asociací je u buthidních štírů zpravidla výsledkem dvou hlavních strukturních mechanismů, reciprokých translokací a chromozomálních fúzí/rozpadů (Mattos et al. 2013; Schneider et al. 2009a; Shanahan 1989a). Reciproké translokace, při nichž dochází k vzájemné výměně úseků mezi nehomologickými chromozomy, se u této skupiny štírů vyskytují hojně a pravděpodobně se jedná o nejčastější typ makrostrukturních změn, se kterým se zde můžeme setkat. To naznačují jak výstupy publikovaných studií (např. Shanahan 1989a; Ubinski et al. 2018), tak námi získaná data (**Článek I a II**). V důsledku jednoduché reciproké translokace vzniká heterozygotní konfigurace, která sestává ze čtyř chromozomálních elementů a tvoří v post-pachytene a metafázi I typický křížový útvar, tzv. kvadrivalent (viz fig. 2a **Článek II**). V souhrnu informací z literatury a zde prezentovaných výsledků je patrné, že se jedná o nejčastěji pozorovaný typ multivalentu v rámci celé čeledi (Příloha Tab. 1). U této evoluční linie štírů není ovšem výjimkou ani výskyt heterozygotů pro vícečetné reciproké translokace, které mohou zahrnovat od šesti [např. *Gint amoudensis* 2*n* = 36 – 15II + VI] (**Článek II**) až po 14 chromozomálních elementů [*Hottentotta flavidulus* 2*n* = 24 – 5II + XIV] (**Článek I**). Z literatury je ovšem znám i ojedinělý případ, kdy multivalentní řetězec sestával u neotropického druhu *Jaguajir agamemnon* z rekordních 28 elementů, které současně představovaly všechny chromozomy diploidní sady [2*n* = 28 – XXVIII] (Mattos et al. 2013). Samotná přítomnost reciprokých translokací nevyvolává změny na úrovni počtu chromozomů. Tento typ chromozomálních přestaveb by tudíž mohl představovat hlavní hybnou sílu v strukturní reorganizaci genomu u buthidních taxonů se stabilním 2*n*. Tento předpoklad zčásti podporují výstupy cytogenetických analýz u rodů *Androctonus* [2*n* = 24] a *Jaguajir* [2*n* = 28], u nichž byly zjištěny akumulace odlišných typů heterozygotních asociací pro reciproké translokace jak mezi různými druhy, tak mezi jedinci stejného druhu (Mattos et al. 2013; Ubinski et al. 2018; **Článek I**).

Dalším typem přestaveb, který se významně podílí na výskytu chromozomálního polymorfismu u buthidních štírů, jsou chromozomální fúze/rozpady. Nejčastěji pozorovanou heterozygotní formaci tohoto typu představuje napříč buthidními taxony tzv. trivalent, který vzniká v důsledku jednoduché fúze či rozpadu (viz fig. 3k **Článek II**). Jedinci heterozygotní pro vícečetné fúze byli také detekováni, např. u zástupců rodů *Gint* (fig. 3i, k **Článek II**), *Ananteris* či *Tityus* (Adilardi et al. 2016; Mattos et al. 2013; Schneider et al. 2009a). Multivalentní řetězce zahrnující nejvýše šest chromozomálních elementů zde nicméně nedosahují takové složitosti jako v případě reciprokých translokací. Strukturní přestavby typu fúze/rozpadu způsobují zřetelné variace v karyotypové makrostruktuře způsobující změny v

počtu chromozomů. Není tedy překvapivé, že buthidní linie vyznačující se mimořádně variabilními počty chromozomů mají značné zastoupení jedinců heterozygotních pro jednoduché fúze/rozpady nebo vícečetné fúze. To se jmenovitě týká zástupců rodů *Gint* [2*n* = 18–45] (fig. 5 **Článek II**) a *Tityus* [2*n* = 5–32] (Adilardi et al. 2016; Mattos et al. 2013; Schneider et al. 2009a). Vysokou frekvenci změn v reorganizaci genomu u příslušných rodů nicméně dokládá nejen přítomnost heterozygotních fúzí/rozpadů, ale i reciprokých translokací. Oba typy strukturních přestaveb se zde na mezipopulační i vnitropopulační úrovni akumulují s různou frekvencí a v různých kombinacích. Navíc u druhů rodu *Tityus* byl hojně sledován fenomén intraindividuální variability heterozygotních asociací (Mattos et al. 2018; Schneider et al. 2009a; Příloha Tab. 1).

Chromozomální polymorfismus související s přítomností heterozygotních chromozomálních přestaveb je bezesporu jedním z nejvýraznějších cytogenetických specifik čeledi Buthidae. Sledovaný fenomén v populacích buthidních druhů nicméně nebyl dosud interpretován v kontextu genetické rozmanitosti a příbuzenských vztahů studovaných jedinců. Naše integrativní studie, jež propojuje cytogenetická a sekvenční data zástupců rodu *Gint*, je tak vůbec první svého druhu v rámci štírů s holokinetickými chromozomy (**Článek II**). Kompilace dat z cytogenetických a molekulárně fylogenetických analýz nám umožnila: (i) ověřit druhovou i příbuzenskou identitu polymorfních jedinců; (ii) odhalit, že výrazné mezi- i vnitrodruhové chromozomální rozdíly nápadně kontrastují s nízkou úrovní genetické variability studovaných druhů; (iii) navrhnout hlavní trendy karyotypové diferenciace v kontextu příbuzenských vztahů (**Článek II**). Tato pilotní studie by mohla představovat jakýsi odrazový můstek pro budoucí cytogenetické práce, které by si kladly za cíl prohloubit znalosti o genetické variabilitě chromozomálně polymorfních populací buthidních štírů např. pomocí analýzy SNP (single-nucleotide polymorphism), která se u štírů již využívá (např. Bryson et al. 2016, 2018).

Přítomnost strukturních heterozygotů v populacích buthidních štírů přirozeně otevírá řadu otázek týkajících se procesu vzniku, akumulace a fixace chromozomálních změn, a především pak jejich role v evoluci druhů. Objasnění těchto dílčích aspektů nicméně v současnosti ztěžuje nejen nedostatek informací o celkovém rozsahu polymorfismu v přirozených populacích, ale také neznalost genetické struktury, ekologie a populační biologie studovaných druhů. Dalším důležitým aspektem je beze sporu také absence fundamentálních znalostí o cytogenetice samic. Prodloužený reprodukční cyklus komplikuje u samic studium meiotických fází, v důsledku čehož panují dohady jak ohledně přítomnosti chiasmat, tak ohledně výskytu multivalentních asociací (Adilardi et al. 2015; Shanahan 1989a).

Samotný výskyt a udržení heterozygotních asociací v populacích buthidních štírů bezesporu umožňuje či ovlivňuje řada odlišných faktorů. Za klíčové z nich se považují: (i) cytogenetické vlastnosti skupiny [tj. holokinetické chromozomy, absence chiasmat u samců, vytvoření účinného mechanismu pro bezchybnou segregaci všech chromozomů v průběhu meiózy]; (ii) nízká schopnost disperze štírů a s tím související náchylnost populací k prostorové izolaci a inbreedingu (Schneider et al. 2009a; Shanahan 1989a). Ačkoliv izolace a nízká populační hustota signalizují, že k šíření nových chromozomálních variant zde může docházet především vlivem genetického driftu, předpokládá se naopak, že akumulace heterozygotních chromozomálních přestaveb v populacích není náhodná a mohou být nositelem evoluční výhody a mít adaptivní význam (Mattos et al. 2018; Schneider et al. 2009a; Shanahan 1989a). Recentně byla přítomnost multivalentních asociací u samců druhu *Tityus confluens* dokonce dávána do souvislosti s existencí kryptických pohlavních chromozomů (Adilardi et al. 2016). Všichni samci studované populace *T. confluens* byli strukturní heterozygoti, samice byly naopak vyhodnoceny jako homozygotní, a to na základě měření mitotických metafází, jež neodhalily mezi jednotlivými chromozomy zjevné velikostní disproporce. Na základě těchto informací Adilardi et al. 2016 usuzují, že u *T. confluens* existuje kryptický pohlavní systém XX/XY, přičemž chromozomální heterozygozita je zde vázaná na heterogametické pohlaví, kde multivalent pravděpodobně představuje zfúzovaný Y chromozom s autozomem.

4.1.3 Chromozomální lokalizace a distribuce rDNA

Holokinetické chromozomy jsou v důsledku absence lokalizované centromery morfologicky uniformní a zpravidla vykazují pouze menší či větší velikostní rozdíly. Charakterizace jednotlivých chromozomů v karyotypu a stejně tak identifikace jejich případného zapojení v strukturních přestavbách tak není na základě konvenčního barvení Giemsou možná. V posledních deseti letech se tudíž v cytogenetice štírů uplatňuje mapování specifických chromozomálních markerů za účelem identifikace homologických chromozomálních oblastí a odhalení potenciální variability (např. Almeida et al. 2017; Schneider et al. 2009a; Ubinski et al. 2018). V současnosti je u této skupiny pavoukovců široce využívaný pouze jediný marker, a to oblast nukleolárního organizátoru (NOR), jenž obsahuje tandemově uspořádané repetice genů pro ribozomální RNA (45S = 28S, 5.8S a 18S). Lokalizace NOR byla u buthidních štírů zprvu identifikována pomocí techniky stříbření (např. Mattos et al. 2013; Schneider et al. 2009a). Tato metoda nicméně umožňuje detekovat jen oblasti NOR aktivně přepisované v předešlé interfázi (Miller et al. 1976). U štírů byly touto metodou získány pozitivní výsledky

výhradně na mitotických metafázích, které nemohou odhalit případné zapojení chromozomů nesoucích NOR v multivalentních asociacích (např. Mattos et al. 2013; Schneider et al. 2009a). Následná implementace rDNA-FISH za využití specifických sond tak umožnila nejen identifikovat veškeré lokusy rDNA (resp. potenciální oblasti NOR) v genomu, ale také získat informace o párování chromozomů nesoucích rDNA v profázi I (Mattos et al. 2014).

Chromozomální mapování oblastí rDNA, ať již pomocí stříbření či rDNA-FISH, bylo dosud provedeno u 28 buthidních druhů náležejících do 9 rodů (Příloha Tab. 1). Prvotním zjištěním bylo, že navzdory vysoké četnosti strukturních chromozomálních změn v karyotypu buthidních štírů, počet i lokalizace rDNA lokusů napříč analyzovanými druhy vykazovaly pozoruhodnou stabilitu (Mattos et al. 2013; Ojanguren-Affilastro et al. 2017). Úseky rDNA byly u analyzovaných jedinců zpravidla identifikovány v terminální oblasti dvou homologických chromozomů, resp. dvou homologických chromozomálních segmentů v případě strukturních heterozygotů, a to v úzké asociaci s blokem konstitutivního heterochromatinu (Adilardi et al. 2015; Mattos et al. 2013; Schneider et al. 2009a; Schneider & Cella 2010). Terminální umístění rDNA je příznačným cytogenetický rysem pro řadu organismů s holokinetickými chromozomy (viz Grozeva et al. 2014; da Silva et al. 2010; Vanzela et al. 1998). Na základě těchto poznatků Heckmann et al. (2011) navrhli, že tato specifická chromozomální lokalizace by mohla být funkčním požadavkem pro zajištění stability a správné segregace holokinetických chromozomů v průběhu buněčného dělení. U Buthidae by tudíž terminálně umístěné lokusy rDNA mohly být pozitivně selektovány pro funkční stabilitu, kolokalizovaný heterochromatin by pak mohl hrát důležitou roli pro zachování integrity a zamezení zlomů v příslušných oblastech (Mattos et al. 2014).

S rostoucím počtem studií byly postupně u buthidních štírů odhaleny i ojedinělé případy, jež vykazovaly buď vyšší počty rDNA lokusů [2 páry u *Tityus obscurus* a *T. uruguayensis* (Almeida et al. 2017; Ojanguren-Affilastro et al. 2017)], či odlišnou chromozomální pozici rDNA [intesticiální u *Androctonus* (Sadílek et al. 2015)]. U některých druhů byl odhalen i polymorfismus v lokalizaci a/nebo počtu rDNA, jenž byl připisován strukturním přestavbám typu chromozomálních translokací [např. *Jaguajir rochai* (Mattos et al. 2013)] či fúzí [*T. bahiensis* (Schneider et al. 2009a), *T. confluens* (Adilardi et al. 2016)].

Vyjma rodu *Androctonus*, všechny buthidní druhy, u nichž bylo provedeno chromozomální mapování rDNA, pocházely výlučně z neotropické oblasti (Příloha Tab. 1). Širší znalosti o fenotypu rDNA v karyotypu druhů obývajících jiné biogeografické regiony tudíž nebyly do současné doby k dispozici. Otázkou tedy zůstává, zda identifikovaný terminálně umístěný jeden pár rDNA představuje sdílený cytogenetický charakter pro čeleď Buthidae, nebo se jedná

o znak příznačný pouze pro americké druhy. V rámci našich srovnávacích studiích jsme se proto zaměřili na analýzu distribuce rDNA u široké plejády druhů náležejících do rozličných evolučních linií čeledi Buthidae a pocházejících z různých biogeografických regionů (**Článek I** a **II**). Předkládané studie přinášejí řadu zásadních poznatků ohledně fenotypu rDNA u čeledi Buthidae. Ve srovnání s prvotními náznaky stability chromozomální lokalizace rDNA, naše data naopak poukazují na existující variabilitu umístění těchto oblastí v karyotypu buthidních štírů. Specifická lokalizace rDNA u studovaných taxonů navíc vykazuje pozitivní fylogenetický signál (fig. 3 **Článek I**). Na základě získaných výsledků se zdá, že intersticiální umístění rDNA by mohlo představovat sdílený cytogenetický rys pro blízce příbuzné rody náležející do morfologické skupiny zvané "*Buthus* group" (fig. 3 **Článek I**). Tento znak sdílejí i některé druhy afrotropického rodu *Gint* (fig. 5 **Článek II**), jenž dle našich předběžných výstupů multigenové molekulární fylogeneze do této skupiny příbuzensky náleží (fylogeneze čeledi Buthidae v přípravě). Pokud by se potvrdila příbuzenská příslušnost *Gint* k "*Buthus* group", jednalo by se v rámci této evoluční linie o nebývale variabilní skupinu nejen v makrostruktuře karyotypu, ale také co do počtu a distribuce rDNA (fig. 5 **Článek II**). Terminální lokalizace rDNA je naopak příznačná pro evoluční linii Buthidae zahrnující blízce příbuzné rody, které utvářejí pět různých morfologických skupin a pocházejí z rozličných biogeografických regionů – od neotropické oblasti ("*Tityus* group") přes afrotropickou ("*Uroplectes* group"), až po indomalajskou oblast ("*Thaicharmus* group", "*Isometrus* group") (fig. 3 **Článek I**).

Odlišné pozice lokusů rDNA, jenž byly identifikovány v rámci některých skupin, poukazují na zjevné změny v reorganizaci karyotypu u příslušných druhů. Například terminální pozice rDNA, jenž byla pozorována u *Hottentotta flavidulus* a *Buthacus macrocentrus* náležejících do "*Buthus* group", byla pravděpodobně způsobena rozpadem příslušného chromozomu v blízkosti rDNA klastru. Tomu napovídá nejen malá velikost chromozomálního páru nesoucího rDNA, ale také vyšší počet chromozomů ve srovnání s blízce příbuznými druhy (tab. 2 **Článek I**).

Ačkoliv se hlavní evoluční linie Buthidae vzájemně liší chromozomální lokalizací rDNA, počet rDNA mají v základním stavu totožný, tj. jeden pár v diploidní sadě. Ve výjimečných případech jsme identifikovali v každé z těchto linií také zmnožené oblasti rDNA, což pravděpodobně reflektuje pro Buthidae odvozený stav. Zvýšený počet oblastí rDNA se pohyboval od 3 (např. *Gint amoudensis*, fig. 3b,d **Článek II**) až po 7 (*Reddyanus ceylonensis*, fig. 2B **Článek I**). Zvýšení počtu rDNA klastrů pravděpodobně nevzniklo u buthidních štírů pomocí totožného mechanismu. V rámci "*Buthus* group" jsme evidovali jediný druh s vyšším

počtem těchto chromozomálních oblastí. U *Orthochirus glabrifrons* došlo ke vzniku dvou párů rDNA v intersticiální oblasti pravděpodobně za pomoci inverze, v jejímž důsledku byla část původního rDNA klastru přemístěna do jiné oblasti téhož chromozomu (fig. 1U **Článek I**). Větší podíl druhů majících zmnožené úseky rDNA v karyotypu byl zastoupen u buthidních skupin s terminálně umístěnými rDNA (fig. 3 **Článek I**). Získaná data dále naznačují, že zde není přítomna pozitivní korelace mezi multiplikací lokusů rDNA a zvyšujícím se počtem chromozomů. Lokusy rDNA se tak v karyotypu buthidních druhů mohly rozšířit na nové chromozomální oblasti za pomoci mechanismů, v jejichž důsledku nedochází ke změně 2*n*. U jiných organismů byla disperze rDNA oblastí v genomu dávána do souvislosti s výskytem ektopické rekombinace či s transpozicí repetitivních oblastí zprostředkovanou transpozibilními elementy (TEs) (např. Datson & Murray 2006; Ferretti et al. 2019; Nguyen et al. 2010; Schmid et al. 2017). Není vyloučeno, že se tyto mechanismy mohou uplatňovat v dynamice lokusů rDNA i v genomu u Buthidae. Nicméně v současnosti nedisponujeme dalšími daty, jež by pomohly objasnit pozadí multiplikace a mobility oblastí rDNA v karyotypu buthidních štírů. Tento aspekt tak prozatím zůstává předmětem dalšího zkoumání.

4.1.4 Chromozomální lokalizace a distribuce telomerického (TTAGG)*n* motivu

Znalost o distribuci telomerických (TTAGG)*n* repetic v genomech buthidních štírů není příliš široká, ačkoliv mapování tohoto cytogenetického markeru může odhalit případné kryptické změny v reorganizaci chromozomů i u taxonů se stabilním karyotypem (např. Rovatsos et al. 2015). Lokalizace telomerické sekvence byla analyzována pomocí FISH pouze u zlomku všech dosud cytogeneticky studovaných buthidních druhů. Příslušné údaje byly k dnešnímu dni publikovány výhradně pro zástupce neotropických rodů *Ischnotelson*, *Jaguajir*, *Physoctonus*, *Rhopalurus* (Ubinski et al. 2018), *Tityus* (Adilardi et al. 2016; Mattos et al. 2018; Ojanguren-Affilastro et al. 2017) a *Zabius* (Adilardi et al. 2015). Dostupná data tak byla v rámci dizertační práce rozšířena o mapování (TTAGG)*n* repetic na chromozomech zástupců afrotropického rodu *Gint*, jenž se v rámci čeledi Buthidae řadí mezi taxony s mimořádně rozmanitou karyotypovou makrostrukturou (**Článek II**).

Z kompilace veškerých dat o chromozomální distribuci (TTAGG)*n* u buthidních štírů je patrné, že analyzované druhy bez ohledu na odlišnou četnost strukturních přestaveb mají telomerické repetice lokalizované výhradně na koncích chromozomů, bez sebemenších náznaků přítomnosti intersticiálních telomerických sekvencí (ITS) (viz Adilardi et al. 2015, 2016; Ojanguren-Affilastro et al. 2017; Mattos et al. 2018; Ubinski et al. 2018; **Článek II**).

Oblasti ITS nebyly překvapivě identifikovány ani u jedinců heterozygotních pro jednoduché či vícečetné chromozomální fúze (Adilardi et al. 2016; Almeida et al. 2017; fig. 3b **Článek II)**. Na základě absence ITS u heterozygotů pro fúze u *Tityus confluens* Adilardi et al. (2016) usuzují, že k chromozomálním zlomům zde pravděpodobně dochází v subtelomerických oblastech, v rámci nichž může dojít buď ke kompletní ztrátě telomerických repetic nebo k jejich parciální redukci v důsledku molekulární eroze na úroveň, již není možné vizualizovat pomocí FISH. Analogický mechanismus ztrát (TTAGG)*n* repetic při fúzním procesu byl navržen i pro *Tityus obscurus* (Almeida et al. 2017) a pravděpodobně tento proces nastal i u *Gint dabakalo* (**Článek II**). ITS sestávající z (TTAGG)*n* motivu se pravděpodobně zachovávají u evolučních linií členovců s holokinetickými chromozomy velice vzácně. Tyto specifické oblasti, jež patrně představují pozůstatky chromozomálních fúzí, byly dosud identifikovány pouze u nočního motýla *Orgiya antiqua* (Rego & Marec 2003) a sladkovodních ploštic rodu *Belostoma* (Chirino et al. 2017).

Vyjma absence ITS u produktů chromozomální fúze, mapování (TTAGG)*n* sekvencí na chromozomech buthidních štírů dále odhalilo, že u heterozygotů pro chromozomální fragmentace vykazovaly příslušné chromozomální elementy v oblasti zlomu již dobře patrné terminální telomerické signály (Almeida et al. 2017; fig. 4e **Článek II)**. U buthidních štírů tak pravděpodobně existuje efektivní systém stabilizace fragmentovaných chromozomů v oblastech zlomů. V takovýchto případech může být rekonstituce telomer zprostředkována telomerázou zajišťující *de novo* syntézu telomerických sekvencí (Pennaneach et al. 2006; Vermeesch & Price 1994), případně bez jejího zapojení za pomoci mechanismu zvaného "telomere capture", při němž dochází k přemístění telomer z nepoškozených chromozomů na fragmentované chromozomální oblasti (Meltzer et al. 1993). U Buthidae by se tak mohlo jednat o prvně zmíněný příklad, jelikož u různých organismů s holokinetickými chromozomy, např. u rostliny *Luzula elegans* (Jankowska et al. 2015) či červce *Planococcus lilacinus* (Mohan et al. 2011), byla obnova telomer u fragmentovaných chromozomů indukována právě aktivitou telomerázy.

4.1.5 Využití cytogenetických znaků v taxonomii

Zástupci čeledi Buthidae vykazují buď poměrně konzervativní nebo naopak vysoce polymorfní karyotypy (viz Příloha Tab. 1**,** podkapitola 4.1.1). Cytogenetické znaky tak nemohou nalézt u této skupiny štírů všeobecně přímé uplatnění v delimitaci blízce příbuzných taxonů či v odhalení kryptických linií. Mohou tvořit ovšem podpůrné charakteristiky v taxonomických

studiích, které při vymezení druhů využívají různé metodické přístupy. Takto byly například na základě výstupů morfologických, sekvenčních a cytogenetických analýz delimitovány sesterské druhy *Tityus curupi* [2*n* = 31–32] a *T. uruguayensis* [2*n* = 31] (Ojanguren-Affilastro et al. 2017). V jiné taxonomické studii analogický integrativní přístup naopak přispěl k synonymizaci *T. ythieri* s *T. magnimanus* (Kovařík et al. 2009). Jedná se nicméně o dosud jediné studie tohoto druhu v rámci Buthidae. Naopak jsou zde hojně zastoupeny práce, které propojují tradiční taxonomii s čistě cytogenetickými přístupy za účelem zvýšení informativní hodnoty studovaných exemplářů (např. Kovařík et al. 2018a,b, 2019). Na takto orientované studie se jako jediná v současné době aktivně zaměřuje laboratoř vedoucího předkládané dizertační práce, a to v úzké spolupráci s předními odborníky na taxonomii štírů. V průběhu řešení této dizertační práce se našemu pracovnímu týmu takto podařilo získat cytogenetické údaje pro: (i) vzácné druhy buthidních štírů [např. *Charmus laneus*, *Buthoscorpio sarasinorum* (Kovařík et al. 2016a)]; (ii) typový materiál nově popisovaných druhů [např. *Parabuthus kajibu* (Kovařík et al. 2016c), *Compsobuthus eritreaensis* (Kovařík et al. 2016b)]; (iii) druhy obývající specifickou biogeografickou oblast [např. štíři Srí Lanky (Kovařík et al. 2016a)]. Tato mezioborová kooperace umožnila nejen významně rozšířit znalosti o karyotypové rozmanitosti čeledi Buthidae (Příloha Tab. 1), ale také získat početný materiál pro komparativní molekulárně cytogenetické studie (**Článek I a II**), a v neposlední řadě vybudovat rozsáhlou DNA banku pro navazující molekulárně fylogenetické studie (*Gint* **Článek II**; rekonstrukce fylogeneze čeledi Buthidae v přípravě).

4.2 Cytogenetické aspekty čeledí štírů s monocentrickými chromozomy

V rámci řádu štíři převládají evoluční linie mající chromozomy s lokalizovanou centromerou. Výskyt monocentrických chromozomů byl k dnešnímu dni evidován u 9 čeledí (Příloha Tab. 2). Nicméně tři z nich, Chactidae, Hormuridae a Vaejovidae, nejsou dle recentních fylogenomických analýz považovány za monofyletické (Santibáñez-López et al. 2019). Mezi skupiny štírů mající monocentrické chromozomy se pravděpodobně řadí i čeleď Chaerilidae, která je geneticky blízce příbuzná k čeledi Buthidae, jež má holokinetické chromozomy (Sharma et al. 2015, 2018). Příslušné cytotaxonomické práce u Chaerilidae přinášejí informace o diploidním počtu chromozomů studovaných druhů, neobsahují však zmínku, o jaký typ chromozomů se jedná (viz Kovařík et al. 2014, 2015c, 2018a). Nicméně existují zde určité cytogenetické charakteristiky, které naznačují příslušnost čeledi Chaerilidae ke skupinám štírů s monocentrickými chromozomy, jako např. vysoké počty chromozomů a výrazně

diferencované karyotypy [2*n* = 76–186] (podkapitola 4.2.1, Příloha Tab. 2), či identifikace zjevných konstrikcí na chromozomech v profázi I u *Chaerilus majkusi* a *C. tichyi* naznačující přítomnost lokalizované centromery (Kovařík et al. 2018a). S jistou dávkou opatrnosti jsou tak cytogenetická data pro Chaerilidae zahrnuta do této kapitoly.

Z přehledu recentní literatury je patrné, že skupiny štírů s monocentrickými chromozomy se pohybují na okraji cytogenetického zájmu, ačkoliv dohromady představují celou polovinu diverzity štírů. Studie komparativního charakteru s využitím molekulárně cytogenetických technik a fylogenetických přístupů zde v zásadě chybí. Výsledky předkládané dizertační práce tak zásadním způsobem rozšiřují poznání o cytogenetických aspektech skupin štírů s monocentrickými chromozomy.

4.2.1 Karyotypová diverzita

Dosud bylo cytogeneticky charakterizováno 55 druhů štírů z 9 různých čeledí (Příloha Tab. 2). Tato data byla v průběhu dizertační práce rozšířena o deskripci cytogenetických znaků pro dalších 37 druhů náležejících do 4 čeledí (**Článek III**, **IV**, **V**, **VI**, Příloha Obr. 1–5). Díky kompilaci veškerých dat tak máme k dispozici základní karyologické údaje u 10 evolučních linií pocházejících z různých biogeografických regionů (Příloha Tab. 2). Štíři s monocentrickými chromozomy obecně vykazují nebývale rozmanitou karyotypovou makrostrukturu. Počty chromozomů zde dosahují vyšších hodnot a jejich rozpětí je nebývale široké – od 2*n* = 29 u *Urodacus manicatus* (Urodacidae) (Shanahan 1989b) až po 2*n* = 186 u *Chaerilus stockmannorum* (Chaerilidae) (Kovařík et al. 2018a). Tento trend byl potvrzen i analýzou vlastních dat, kdy nejnižší detekovaný počet chromozomů 2*n* = 46 byl popsán např. u druhu *Alpiscorpius germanus* (Euscorpiidae) (**Článek IV**), naopak nejvyšší 2*n* = 168 u *Pandinoides hawkeri* (Scorpionidae) (Příloha Obr. 2f). Patrné rozdíly ve 2*n* nejsou ovšem přítomny jen na úrovni vyšších taxonomických jednotek, ale především mezi blízce příbuznými druhy (Příloha Tab. 2). Z veškerých cytogenetických údajů vyplývá, že napříč rozličnými evolučními liniemi štírů s monocentrickými chromozomy dominují skupiny, které mají na mezidruhové úrovni mimořádně diverzifikované karyotypy. Řadí se mezi ně např. evropský rod *Alpiscorpius* (Euscorpiidae) [2*n* = 46–92] (Kovařík et al. 2019b; **Článek VI**), afrotropický *Hadogenes* (Hormuridae) [2*n* = 48–113] (Newlands & Cantrell 1985; **Článek V**), asijský *Chaerilus* (Chaerilidae) [2*n* = 76–186] (Kovařík et al. 2018a), či australský *Urodacus* (Urodacidae) [2*n* = 29–175] (Shanahan 1989b). Nízká mezidruhová karyotypová variabilita je naopak příznačná pro dílčí afrotropické rody *Pandinus sensu lato* (Scorpionidae) (Příloha Obr.

1–5). Výjimečnou situaci v rámci štírů s monocentrickými chromozomy pak tvoří tři druhy neotropického rodu *Brachistosternus* (Bothriuridae), které sdílejí identickou karyotypovou makrostrukturu 2*n* = 46 (Adilardi et al. 2013; Rodríguez-Gil et al. 2009).

4.2.2 Chromozomální polymorfismus a polytypie

Odhalení přítomnosti a rozsahu chromozomální variability u štířích taxonů s monocentrickými chromozomy mohou provázet analogické obtíže jako u buthidních druhů s holokinetickými chromozomy (viz podkapitola 4.1.2). Údaje z literatury nicméně ukazují, že vnitrodruhová chromozomální variabilita se hojně vyskytuje i u těchto evolučních linií štírů (Příloha Tab. 2). Ta zde může být spjata s přítomností strukturních heterozygotů v populacích [dále jako polymorfismus] nebo s identifikací odlišných geograficky vymezených karyomorf [dále jako polytypie].

Přítomnost chromozomálního polymorfismu souvisejícího s výskytem strukturních heterozygotů byla zaznamenána u 13 druhů náležejících do 6 čeledí (Příloha Tab. 2). Většina těchto cytogenetických údajů se nicméně zakládá na limitovaném počtu analyzovaných exemplářů, v důsledku čehož nelze usuzovat jak frekvenci výskytu heterozygotních jedinců v populacích, tak potenciální rozsah polymorfismu v rámci studovaných druhů. Nejpodrobnější údaje tohoto charakteru poskytla Shanahan (1989b) pro vysoce polymorfní druh *Urodacus manicatus*, u něhož pomocí cytogenetické analýzy bezmála 160 jedinců odhalila na mezi- i vnitropopulační úrovni různé stupně chromozomální variability. Z kompilace všech cytogenetických údajů pro skupiny štírů s monocentrickými chromozomy je patrné, že identifikované heterozygotní chromozomální asociace vznikají primárně v důsledku následujících typů strukturních přestaveb: (i) centrické (resp. Robertsonovské) fúze/rozpady; (ii) reciproké translokace; (iii) inverze; (viz Shanahan 1989b; Schneider et al. 2009b; **Článek III**, **IV** a **VI**). V karyotypu strukturních heterozygotů byl nejčastěji detekován trivalent, jenž vzniká v důsledku jednoduchých centrických (resp. Robertsonovských) fúzí či rozpadů (pro *U. manicatus* viz Shanahan (1989b), ostatní shrnuto v Příloha Tab. 2). Napříč studovanými druhy nebyl ovšem výjimkou ani výskyt heterozygotů pro komplikovanější multivalentní konfigurace pocházející z vícenásobných fúzí. Tyto heterozygotní řetězce sestávaly ze čtyř [např. *Pandinoides duffmackayi* 2*n* = 108 – 52II + IV, Příloha Obr. 2d] až po třináct chromozomálních elementů [*Heterometrus minotaurus* 2*n* = 54 – 19II + III + XIII, fig. 23 **Článek IV**). Častý výskyt heterozygotů pro fúze či rozpady naznačuje, že diferenciace karyotypu u štírů s monocentrickými chromozomy může probíhat přednostně skrze tento typ makrostrukturních

přestaveb. Tomuto trendu by napovídaly často nápadné rozdíly v počtu chromozomů i u blízce příbuzných druhů (Příloha Tab. 2).

Reciproké translokace se pravděpodobně nepodílejí na změnách v reorganizaci chromozomů v takové míře jako výše zmíněné fúze/rozpady. Heterozygotní asociace informující o přítomnosti tohoto typu přestaveb byly dosud evidovány u tří druhů, *Alpiscorpius omikron* (Euscorpiidae) [2*n* = 58 – 28II + IV] (Kovařík et al. 2019b; **Článek VI**) *Bothriurus araguayae* (Bothriuridae) [2*n* = 42 – 19II + IV] (Schneider et al. 2009b) a *Heterometrus yaleensis* (Scorpionidae) [2*n* = 99 – 46II + III + IV] (Kovařík et al. 2019c). Ve všech případech byli příslušní jedinci strukturními heterozygoty pro jednoduchou reciprokou translokaci zahrnující čtyři chromozomální elementy. Dosud tak u štírů s monocentrickými chromozomy neexistuje jediná zmínka o výskytu komplikovaných multivalentních asociací plynoucích z kumulativních translokačních událostí.

Vyjma interchromozomálních změn vedoucím ke vzniku strukturních heterozygotů byla v karyotypu štírů s monocentrickými chromozomy doložena i přítomnost inverzí. Tento typ intrachromozomálních přestaveb byl dosud evidován v heterozygotním stavu pouze v rámci jediné populace druhu *U. manicatus* (Shanahan 1989b; Shanahan & Hayman 1990). Pericentrické inverze byly u analyzovaných jedinců identifikovány na základě heterozygozity C-pruhů a přítomnosti smyček na chromozomech v metafázi I (Shanahan 1989b), zatímco výskyt paracentrických inverzí byl odhalen za pomoci analýzy synaptonemálního komplexu (Shanahan & Hayman 1990).

V rámci druhů štírů s monocentrickými chromozomy se mimo přítomnosti heterozygotních asociací můžeme setkat i s výskytem geograficky odlišných karyomorf. Náznaky existence polytypie byly dříve zaznamenány například u *Brachistosternus pentheri* (Bothriuridae) [2*n* = 42, 46] (Giacomozzi 1977; Rodríguez-Gil et al. 2009), *Heterometrus fulvipes* (Scorpionidae) [2*n* = 86, 88] (Venkatanarasimhiah & Rajasekarasetty 1964a), či u *Urodacus armatus* (Urodacidae) [2*n* = 124, 144] (Shanahan 1989b). Tyto poznatky nicméně poukazují na fakt, že z výhradně cytogenetických dat nelze usuzovat, zda sledovaný jev odráží u analyzovaných exemplářů vnitrodruhovou variabilitu či naopak již mezidruhové rozdíly; zvláště pak v případě taxonů vykazujících nízkou úroveň morfologické diferenciace. Úskalí interpretace identifikované karyotypové variability nastínila i Shanahan (1989b) ve své studii odhalující mimořádný stupeň chromozomální variability u rodu *Urodacus*. Shanahan (1989b) předpokládala, že detekované vnitrodruhové karyotypové rozdíly mohou u *U. novaehollandiae* a *U. manicatus* reflektovat existenci druhových komplexů. Nicméně pro ověření těchto předpokladů, např. pomocí analýzy DNA, nebyly v té době u štírů etablovány vhodné

metodické nástroje. Možnou existenci druhového komplexu u karyotypově rozmanitého druhu *Urodacus yaschenkoi* [2n = 94, 114, 116] (Shanahan 1989b) podporují i nezávislé výstupy recentní studie zaměřené na genetickou diverzitu tohoto druhu (Luna-Ramirez et al. 2017). Propojení těchto dvou odlišných přístupů při zkoumání skryté rozmanitosti a populační struktury u štírů nicméně do současné doby chybělo, ačkoliv integrativní studie tohoto charakteru mohou sehrát klíčovou roli jak v interpretaci sledované karyotypové variability, tak ve zhodnocení rozsahu zkoumané diverzity. Vysoký potenciál kombinace cytogenetických a multilokusových molekulárních dat při řešení těchto specifických otázek demonstruje naše integrativní studie zaměřená na výzkum štírů z oblasti Alp (**Článek VI**). V rámci studovaných druhů jsme zásluhou extenzivního vzorkování objevili přítomnost 10 karyotypových ras vykazujících specifickou geografickou distribuci (fig. 1 **Článek VI**). Analýza sekvenčních dat nám současně umožnila prozkoumat genetickou divergenci karyotypově odlišných populací, rekonstruovat vzájemné příbuzenské vztahy a predikovat stáří těchto linií (figs 3, 4 **Článek VI**). Výsledky této studie poukazují nejen na význam integrativního přístupu, ale také na nezbytnost rozsáhlého vzorkování při studiu karyotypové variability u druhů štírů, jejichž populace jsou v důsledku nízké vagility a stenotopie senzitivní na prostorovou izolaci (**Článek VI**).

4.2.3 Chromozomální lokalizace a distribuce rDNA

Informace týkající se distribuce rDNA nejsou pro druhy štírů s monocentrickými chromozomy téměř k dispozici. První a svého času také jediná data tohoto druhu přinášela studie Schneider et al. (2009b), v rámci níž byla metodou stříbření analyzována chromozomální lokalizace NOR u dvou blízce příbuzných druhů rodu *Bothriurus* (Bothriuridae). U obou studovaných druhů byly shodně identifikovány tři páry chromozomů s terminálně umístěnými NOR (Schneider et al. 2009b). V průběhu řešení dizertační práce se podařilo získat ucelená data a současně také první informace o fenotypu rDNA identifikovaného pomocí rDNA-FISH pro tři různé čeledi štírů: Euscorpiidae (**Článek VI**), Hormuridae (**Článek V**) a Scorpionidae (Příloha Obr. 2). Analyzované druhy bez ohledu na fylogenetickou příslušnost sdílely totožný počet lokusů rDNA, tj. jeden pár na diploidní sadu. Dva páry rDNA detekované u *Pandinurus exitialis* byly tak jedinou evidovanou výjimkou (Příloha Obr. 2k). Ve srovnání se stabilním počtem lokusů rDNA existuje v rámci studovaných taxonomických skupin variabilita jak ve velikosti a morfologii chromozomu nesoucího rDNA, tak v samotné chromozomální lokalizaci úseků rDNA. Pro druhy rodu *Alpiscorpius* jsou příznačné subterminálně umístěné lokusy rDNA, ať

již na krátkých (*p*) či dlouhých (*q*) chromozomálních raménkách (fig. 2 **Článek VI**). Naopak v karyotypu zástupců *Pandinus s.l.* převládá terminální lokalizace úseků rDNA na *p* raménkách (Příloha Obr. 2, 5).

Rozdíly v distribuci rDNA, které byly identifikovány mezi blízce příbuznými populacemi alpských štírů rodu *Alpiscorpius*, byly připisovány především vlivu strukturních přestaveb, jež současně sehrály významnou roli v diferenciaci samotné karyotypové makrostruktury studovaných taxonů: (i) chromozomální fúze/rozpady, v jejichž důsledku došlo ke změně v morfologii zúčastněných chromozomů nesoucích rDNA, bez dopadu na chromozomální umístění úseků rDNA; (ii) paracentrické inverze zahrnující oblast rDNA, v jejichž důsledku došlo ke změně lokace příslušného chromozomálního segmentu ze subterminální na terminální pozici v rámci *q* raménka (**Článek VI**).

Zástupci rodu *Hadogenes*, vykazující diverzifikované karyotypy podobně jako *Alpiscorpius*, se na mezidruhové úrovni lišily jak ve velikosti chromozomálního páru nesoucího rDNA, tak i v chromozomální lokalizaci lokusů rDNA (fig. 1b,e,h **Článek V**). Specifická lokalizace úseků rDNA u studovaných druhů posloužila jako podpůrný cytotaxonomický znak. Nicméně vzhledem k limitovanému datasetu a absenci znalostí příbuzenských vztahů studovaných druhů nebylo možné zjevné rozdíly v distribuci rDNA spolehlivě interpretovat (**Článek V**).

Jednotlivé rody *Pandinus s.l.*, jež jsou oproti *Alpiscorpius* a *Hadogenes* méně rozmanité svoji karyotypovou makrostrukturou, vykazují nepatrné rozdíly i na úrovni chromozomální lokalizace rDNA (Příloha Obr. 2). V karyotypu zkoumaných druhů rodů *Pandinops* [2*n* = 166–168] a *Pandiborellius* [2*n* = 101–102] si pravděpodobně chromozomální pár nesoucí rDNA zachoval vlastní integritu (Příloha Obr. 2e-f, g-h). Naopak druhy rodu *Pandinoides* [2n = 108–109] vykazují odlišnou chromozomální pozici rDNA (Příloha Obr. 2c-d), jež mohla nastat v důsledku pericentrické inverze. Ačkoliv jsme detekovali rozdílnou distribuci rDNA i u druhů rodu *Pandinus sensu stricto*, získaná data nelze interpretovat vzhledem k tomu, že na základě rekonstrukce příbuzenských vztahů netvoří tento rod monofylum. V karyotypu zástupců rodu *Pandinurus* pokrývají úseky rDNA stereotypně *p* raménka akrocentrického či submetacentrického páru chromozomů (Příloha Obr. 2i–t). Pouze u čtyř druhů byla detekována přítomnost velikostní heteromorfie terminálních oblastí rDNA mezi homologickými chromozomy (Příloha Obr. 2m,q,r,t). Velikostní heteromorfie ribozomálních genů mezi homologickými chromozomy byly detekovány u rozličných organismů (např. Amaro-Ghilardi et al. 2007; Mandrioli et al. 1999; Orosová et al. 2010; Sember et al. 2015), včetně pavoukovců (např. Hiřman et al. 2018; Šťáhlavský et al. 2018). Vzhledem k limitovanému množství

analyzovaných exemplářů nicméně nemáme pro druhy rodu *Pandinurus* k dispozici dostatek informací, jež by naznačovaly mechanismus odpovědný za pozorované velikostní změny úseků rDNA u homologických chromozomů.

Souhrnné výstupy naznačují, že mapování rDNA u štírů s monocentrickými chromozomy může představovat užitečný nástroj pro identifikaci skryté karyotypové variability a odhalení přítomnosti chromozomálních přestaveb. Specifický počet a chromozomální lokalizace lokusů rDNA může navíc sloužit jako vhodný mezidruhový cytotaxonomický znak (**Článek V a VI**).

4.2.4 Chromozomální lokalizace a distribuce telomerického (TTAGG)*n* motivu

První mapování telomerických sekvencí v karyotypu štírů s monocentrickými chromozomy bylo provedeno u druhu *Heterometrus spinifer* v rámci studie zaměřené na evoluční původ TTAGG motivu u členovců (Vítková et al. 2005). Ucelené komparativní údaje o distribuci telomerického (TTAGG)*n* motivu v karyotypech štírů s monocentrickými chromozomy pak přinášejí jako první naše cytogenetické studie zaměřené na analýzu druhů rodu *Hadogenes* (Hormuridae) (**Článek V**) a *Pandinus s.l.* (Scorpionidae) (Příloha Obr. 3). Tyto studie poskytují unikátní informace o specifické distribuci telomerických sekvencí u taxonů pocházejících z odlišných evolučních linií štírů. U zástupců rodu *Hadogenes* jsme ukázali, že ačkoliv vykazují výrazné mezidruhové rozdíly v karyotypové makrostruktuře i chromozomální lokaci rDNA, které poukazují na rozsáhlé změny v reorganizaci genomu, telomerické repetice vykazují naopak stabilní distribuci a jsou zde lokalizované výhradně na koncích chromozomů (**Článek V**). Telomerické oblasti u chromozomů účastnících se přestaveb u *Hadogenes* tak mohly být v průběhu diferenciace karyotypu zcela eliminovány, či mohlo dojít sekundárně ke ztrátě ITS nebo redukci počtu kopií na úroveň, již není možné cytogeneticky detekovat. Odlišný fenomén distribuce (TTAGG)*n* repetic na chromozomech štírů s monocentrickými chromozomy nicméně odhalila extenzivní analýza 19 druhů *Pandinus s.l.*. Získané výsledky zde poukázaly na celou řadu zajímavých aspektů týkající se chromozomální lokalizace telomerických sekvencí (Příloha Obr. 3). (TTAGG)*n* sekvence byly napříč analyzovanými druhy identifikovány v: (i) terminálních oblastech ve větším počtu kopií; (ii) centromerických oblastech metacentrických nebo submetacentrických chromozomů; (iii) intesticiálních oblastech metacentrických, submetacentrických nebo akrocentrických chromozomů; (iv) subterminálních oblastech akrocentrických chromozomů; (v) centromerických oblastech strukturních heterozygotů (Příloha Obr. 3, shrnuto v Obr. 5). Jedna či libovolná kombinace výše uvedených specifických distribucí ITS byly zaznamenány u všech druhů *Pandinus s.l.*,

vyjma *Pandinops turieli*. Tento druh vykazoval karyotyp s výhradně terminální distribucí telomerických sekvencí, které se ovšem na některých chromozomech nacházely ve větším počtu kopií (Příloha Obr. 3e). Zjištěná chromozomální lokalizace telomerických sekvencí napříč *Pandinus s.l.* poukazuje na zapojení několika různých procesů souvisejících se změnami distribuce a akumulace (TTAGG)*n* repetic u příslušných druhů. ITS v centromerických oblastech mohly vzniknout z pozůstatků původních inaktivovaných terminálních telomer po Robertsonovských fúzích (Slijepcevic 1998). Přítomnost tohoto typu přestaveb naznačuje skutečnost, že větší zastoupení chromozomů s centromerickými ITS vykazují druhy, které mají ve srovnání s blízce příbuznými taxony nižší počet chromozomů (Příloha Obr. 5). Nejlépe je toto názorné u druhu *Pandinus viatoris*, který má v rámci *Pandinus s.l.* nejnižší počet chromozomů [2*n* = 93–94] a současně i největší podíl centromerických ITS (Příloha Obr. 3b). Pravděpodobný vznik centromerických ITS vlivem fúzí je dobře patrný na přítomných strukturních heterozygotech u *Pandiborellius igdu*, *Pandinoides cavimanus* a *P. duffmackayi*, jenž vykazují zřetelné bloky ITS ve fúzních místech (Příloha Obr. 3b,c,d,h). Naopak absence ITS oblastí u trivalentu druhu *Pandinus imperator* naznačuje, že studovaný jedinec může být strukturním heterozygotem nikoliv pro fúzi, ale chromozomální rozpad (Příloha Obr. 3a). Jinou možností také může být, že detekovaný trivalent je produktem fúze, nicméně telomerické oblasti se tohoto procesu neúčastnily a byly již na jeho počátku eliminovány. Komplikovanější situaci pak představují jedinci *P. viatoris* heterozygotní pro vícečetné fúze. Propojení jednotlivých chromozomálních elementů uvnitř heptavalentu a samotná distribuce ITS signalizují, že se zde pravděpodobně uplatňují i tandemové fúze (Příloha Obr. 3b). Tento typ fúzí by v karyotypu příslušného druhu mohl být zodpovědný i za vznik ITS detekovaných mimo centromerickou oblast u tří bivalentů (Příloha Obr. 3b). U dalších detekovaných typů ITS, tj. subterminálních u *Pandinurus* a intersticiálních u *Pandinoides*, již není možné s větší mírou jistoty určit jejich původ. Subterminální ITS umístěné na jediném páru (pravděpodobně) akrocentrických chromozomů byly identifikovány u všech analyzovaných druhů rodu *Pandinurus* (vyjma *P. trailini*) (Příloha Obr. 3i-t). Dle velikostních proporcí je možné usuzovat, že se pravděpodobně jedná napříč druhy o tentýž chromozom, jenž by mohl představovat cytogenetický charakter příznačný pro tuto skupinu.

Získané výsledky TTAGG FISH poukazují na potenciál využití tohoto cytogenetického markeru u skupin štírů s monocentrickými chromozomy pro odhalení mezidruhových rozdílů a odlišných aspektů chromozomálních změn.

4.2.5 Využití cytogenetických znaků v taxonomii

Štíři s monocentrickými chromozomy obecně vykazují mezidruhové rozdíly již na úrovni karyotypové makrostruktury (např. Kovařík et al. 2018a; Schneider et al. 2009b; Příloha Tab. 2). Zásluhou toho mají cytogenetické znaky u těchto linií značný potenciál jak v odhalení kryptických linií (**Článek VI**), tak i v přímém využití v integrativní taxonomii (Kovařík et al. 2018a; **Článek V**). Newlands & Cantrell (1985) použili ke stanovení taxonomického statusu u 14 zástupců rodu *Hadogenes* (Hormuridae) tři nezávislé přístupy; morfologickou analýzu, popis karyotypu a elektroforetické vyšetření proteinů obsažených v jedu. Vysoce diferencovaná karyotypová makrostruktura i specifické složení proteinů u zkoumaných taxonů napomohly k vyřešení některých sporných otázek týkajících se taxonomické validity u morfologicky identických či velice podobných jedinců (Newlands & Cantrell 1985). Na tuto práci jsme navázali vlastní cytotaxonomickou analýzou pro další tři druhy rodu *Hadogenes* (**Článek V**). Výsledky naší komparativní analýzy byly v souladu s dřívějšími zjištěními Newlands & Cantrell (1985). Morfologicky komplikovaný rod *Hadogenes* vykazuje na mezidruhové úrovni mimořádně diferencované karyotypy. Zdá se tedy, že cytogenetické charakteristiky zde mohou představovat podpůrný nástroj při vymezení morfologicky indiferentních blízce příbuzných taxonů (**Článek V**).

Nicméně ne vždy lze původní cytogenetické záznamy využít v řešení současných taxonomických otázek. Skrytá úskalí využití dříve publikovaných karyologických dat v cytotaxonomii rodu *Heterometrus* (Scorpionidae) jsme diskutovali ve studii zaměřené na deskripci morfologických a karyotypových charakteristik holotypu nově popsaného druhu *Heterometrus minotaurus* (**Článek IV**). Zásadní komplikace v tomto ohledu způsobuje absence faunistických údajů a informací o depozitu analyzovaného materiálu, jež by umožnily případné přezkoumání druhové příslušnosti studovaných exemplářů. V kontextu rozsáhlých změn týkajících se taxonomického statusu a vymezení druhů rodu *Heterometrus* tak není možné identifikovat, jakému taxonu známé cytogenetické charakteristiky v současnosti náleží (**Článek IV**).

V průběhu řešení projektu vznikly také taxonomické práce kombinující tradiční morfologii a cytogenetické charakteristiky u vzácných exemplářů štírů, které by nebylo možné získat pro účely komparativní analýzy (Kovařík et al. 2013b; **Článek III**). Díky tomu jsme získali první cytogenetické údaje pro typový materiál dvou druhů asijského rodu *Euscorpiops* (Kovařík et al. 2013b; **Článek III**) a rozšířili tak informace týkající se diverzity karyotypu u štírů s

monocentrickými chromozomy. Tyto údaje mohou posloužit jako odrazový můstek pro případné navazující studie zabývající diverzitou této skupiny.

Integrativní studie kombinující molekulárně cytogenetická a sekvenční data mohou u štírů s monocentrickými chromozomy poukázat na existenci skryté druhové diverzity (**Článek VI**) nebo na zjevné rozpory v současné systematice (Příloha Obr. 4). Tyto výstupy pak mohou tvořit informační podklad pro navazující taxonomické revize studovaných skupin (viz *Alpiscorpius* Kovařík et al. 2019b). Studie zaměřená na chromozomální a genetickou diverzitu štírů obývajících Alpy odhalila u studovaných druhů existenci geneticky divergentních karyotypových ras, jež vykazovaly specifickou geografickou distribuci (**Článek VI**). V navazující taxonomické revizi bylo ověřeno, podobně jako v předešlých studiích (viz Gantenbein et al. 2000; Scherabon et al. 2000), že alpští štíři postrádají zjevné morfologické odlišnosti. Detekované linie tak představují kryptické druhy, jež jsou primárně definovány geografickou distribucí, cytogenetickými a genetickými znaky (Kovařík et al. 2019b). V důsledku taxonomických změn je tak na území Alp recentně rozeznáváno z původních tří 10 druhů rodu *Alpiscorpius*. V případě *Pandinus s.l.* poukázaly výstupy molekulárně fylogenetických a cytogenetických analýz na to, že rod *Pandinus sensu stricto*, jenž je zastoupen v analýze druhy *P. imperator*, *P. viatoris* a *P. dictator*, není zjevně monofyletický a vyžaduje bližší zkoumání (Příloha Obr. 4).

5. ODBORNÉ PUBLIKACE ZAHRNUTÉ DO DIZERTAČNÍ PRÁCE

I. Šťáhlavský, F., Nguyen, P., Sadílek, D., **Štundlová, J.**, Just, P., Haddad, C. R., Koç, H., Ranawana, K.B., Stockmann, M., Yağmur, E.A., Kovařík, F. Evolutionary dynamics of rDNA clusters on chromosomes of buthid scorpions (Chelicerata: Arachnida). *Rukopis*.

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ČLÁNEK I

Evolutionary dynamics of rDNA clusters on chromosomes of buthid scorpions (Chelicerata: Arachnida)

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Abstract

We examined distribution of major ribosomal DNAs (rDNAs) on holokinetic chromosomes of 75 species belonging to 19 genera of scorpions from family Buthidae using fluorescence in situ hybridization (FISH). Our analysis revealed differences between two main evolutionary lineages within the family. The genera belonging to "*Buthus* group" with proposed Laurasian origin possess one pair of rDNAs mainly in interstitial position with the only exception in some *Hottentotta* and *Buthacus* species with terminal location, possibly as the effect of chromosome fissions. All the remaining buthid "groups" possess rDNAs strictly in terminal position. However the number of the signals may increase from ancestral one pair of rDNA loci up to seven loci in *Reddyanus ceylonensis*. Despite of the differences in evolutionary dynamics of rDNA clusters between *Buthus* and the other "groups" we found high amount of reciprocal translocations and presence of multivalent associations during meiosis in majority of observed genera. This phenomenon seems to be typical feature common for the whole family Buthidae.

Key words: 18S rDNA, FISH, holokinetic chromosome, reciprocal translocation, karyotype evolution

Introduction

Scorpions are a distinctive chelicerate order representing one of the first lineages of arachnids to colonize terrestrial habitats (Dunlop & Penney 2012). Even today they represent successful group of predators in many terrestrial habitats worldwide. With more than 2400 extant species (Rein 2019) they constitute one of smaller arachnid orders with yet unresolved relationship to the remaining arachnid lineages (see Sharma et al. 2014) as well as among scorpion families (see Sharma et al. 2015). Despite of that it is evident that the family Buthidae represents unique and successful deep evolutionary branch of scorpions (Sharma et al. 2015) with more than 1150 described species classified into 96 genera (Rein 2019). It is the only family of scorpions where the holokinetic chromosomes evolved (Melters et al. 2012). This type of chromosome organization has evolved independently several times in chelicerata as it was documented also in some mites (Wrensch et al. 1994) and two families of spiders (Král et al. 2006). It was proposed that holokinetic nature of buthid chromosomes may allow for more frequent fusions and fissions of chromosomes and thus underlie high intraspecific karyotype variation observed within some species (e.g. *Tityus bahiensis* with 2n=5-19) (Schneider et al. 2009) and presence of odd diploid chromosome numbers (see Schneider et al. 2019). The intensive fragmentation of the holokinetic chromosomes may produce karyotypes with more than hundred chromosomes in different organisms (e.g. Cook 2000, Lukhtanov 2015). However, the scorpions of the family Buthidae have relatively low number of chromosomes. Chromosome numbers of buthids range between 2n=5 and 2n=36 (see discussion for ambiguous reports of higher number of chromosomes) with the modal chromosome number of 2n=24 (mean and median 19) (see Schneider et al. 2019).

Although the buthids represent the best cytogenetically explored family of scorpions our understanding of the dynamics and mechanisms of the chromosomal change is still far from being comprehensive (e.g. Schneider et al. 2009, Mattos et al. 2013, Sadílek et al. 2015, Almeida et al. 2017, Ubinski et al. 2018). Partially it is because holokinetic chromosomes lack any morphological landmarks as well as due to absence of any standard cytogenetic banding techniques suitable for identification of homologous chromosomes in invertebrates. That is why fluorescence *in situ* hybridization (FISH) techniques could potentially considerably advance study of the chromosomal evolution in scorpions. For example, rDNA clusters, i.e. arrays of genes for major ribosomal RNAs, 18S, 5.8S, and 28S rRNA, proved to represent important and useful marker for the reconstruction of karyotype evolution or detection of concealed karyotype variation (e.g. Nguyen et al. 2009, Cabral de Mello et al. 2011, Panzera et al. 2012, Svojanovská et al. 2016). This marker has been used up to now in seven buthid genera and the results show

a pattern consistent for different geographic regions. While the South American species of the *Ischnotelson*, *Jaguajir*, *Physoctonus*, *Rhopalurus*, *Tityus*, and *Zabius* genera contain always terminally located NORs on one (Schneider et al., 2009b, Mattos et al., 2013, 2018, Adilardi et al., 2014, 2015, Ubinski et al., 2018) or exceptionally on two (Almeida et al. 2017, Ojanguren-Affilastro et al. 2017a) pairs of chromosomes, the genus *Androctonus* from northern Africa and western Asia contain only one pair of NORs localised interstitially on the largest chromosome pair (Sadílek et al. 2015). This considerably different location of the scorpion NORs may reflect different evolutionary history of analyzed genera (Sadílek et al. 2015). However, we are still missing the information on the distribution of NORs in majority of buthid genera.

It should be noted that the higher-level phylogeny of the family Buthidae is still not well resolved and it is mainly based on morphological characters (e.g. Fet et al. 2005). Fet et al. (2003) conducted the first attempt to reconstruct phylogenetic relationships mainly between the genera of the Palearctic biogeographic region, as they sampled 17 genera using a single sequence of the mitochondrial gene for 16S rRNA. Subsequent phylogenetic analysis of various buthid genera included more genes and focused on species from Neotropical region (Ojanguren-Affilastro et al. 2017b, Esposito et al. 2018). However, more frequently the molecular analyses focussed on phylogeography of specific groups of species or regions within the family Buthidae (e.g. Teruel et al. 2006, Sousa et al. 2012, Yamashita & Rhoads 2013, Suranse et al. 2016, Graham et al. 2019) and thus did not enhance our understandig of the relationships within the whole family. That is why we still have only limited knowledge about the phylogeny of this scorpion group.

In the present study we examined number and distribution of 18S rDNA clusters using FISH in 75 species belonging to 19 buthid genera. Our sampling covers all the main evolutionary lineages within this family as recognised by Fet et al. (2013, 2015) and Esposito et al. (2018). Our study brings the first comprehensive information about the evolutionary dynamics of rDNA clusters beyond the subfamily Centruroidinae and the first analysis of the karyotype evolution in phylogenetic context of the whole family.

Materials and Methods

Taxon sampling

The details on the material included in this study are summarized in Table 1. The selection of the genera covers all the six morphological groups recognized by Fet et al. (2005) (*Ananteris,*

Buthus, *Charmus*, *Isometrus*, *Tityus* and *Uroplectes*) and the majority of genera included in the molecular phylogenetic analyses of the family Buthidae (Fet et al. 2003, Ojanguren-Affilastro et al. 2017b, Esposito et al. 2018).

Chromosome preparation and karyotype analysis

We used the "plate spreading" technique for chromosome preparations following Šťáhlavský & Král (2004). The gonads were dissected and hypotonized in 0.075 M KCl solution for 20 min., fixed in glacial acetic acid: methanol (1: 3) for 20 min., and macerated piece by piece in a drop of 60% acetic acid on a microscope slide,. The chromosome slides used for basic karytype characterization of the analyzed species were stained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 20 min.

FISH detection of 18S rDNA

The 18S rDNA probe for FISH was prepared according to Forman et al. (2013). Briefly, a fragment of the 18S rRNA gene was amplified by PCR from genomic DNA of spider *Dysdera erythrina* (Walckenaer, 1802) (Dysderidae) with forward 5′-CGAGCGCTTTTATTAGACCA-3′ and reverse 5′-GGTTCACCTACGGAAACCTT-3′ primers. The 18S rDNA fragment (app. 1000 bp) was then labelled with biotin-14-dUTP by nick translation using the Nick Translation Kit (Abbott Molecular). We carried out FISH with labelled probe as described by Fuková et al. (2005). Hybridization was performed overnight and biotin was detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs Inc.). To amplify a signal we used biotinylated antistreptavidin and Cy3-conjugated streptavidin (Vector Labs Inc.). The preparations were counterstained and mounted using Fluoroshield with DAPI (4′,6-diamidino-2-phenylindole; Sigma).

Documentation and image analysis

Preparations were observed in an Olympus IX81 microscope and documented with an ORCA-AG monochromatic camera (Hamamatsu). In case of FISH, the images were pseudocoloured (red for Cy3 and blue for DAPI) and superimposed with Cell^R software (Olympus). The Giemsa stained chromosomes were documented using the same microscope in a DIC mode. The relative diploid set length (DSL) and the relative position of the 18S rDNA signal were measured following Sadílek et al. (2015) using the plug-in Levan for ImageJ (Sakamoto & Zacaro 2009) in ten postpachytene or mitotic metaphases nuclei.

Results

"*Ananteris* group"

We analysed four *Lychas* species belonging to "*Ananteris* group".

Lychas. The diploid numbers of chromosomes ranged from 2n = 10 (*L. obsti* and *L. mucronatus*) (Figs 1a-c) to 2n = 16 (*L. krali*) (Figs S1a). The chromosomes gradually decreased in length in karyotypes of all species except *L. scutilus* (2n=14) in which distinct long pair of chromosomes was observed (Fig. S1b) Meiotic multivalent associations were observed in two species, namely a hexavalent in *L. mucronatus* (Fig. 1c) and an octavalent in *L. obsti* from Tanzania (Fig. 1b). *Lychas* spp. showed differences in number of 18S rDNA clusters, with one pair detected in *L. obsti* (Figs 1b, c) and *L. scutilus* (Fig. 1a) and two pairs in *L. krali* (Fig. S1a) and *L. mucronatus* (Fig. 1c). Despite the differences in number of rDNA loci, all clusters were localized in the terminal region of the chromosomes in all species examined. The rDNA-bearing chromosome pairs differed in length among *Lychas* spp. (Table 2). Also, the species having multiple rDNA loci exhibited two rDNA-bearing chromosome pairs of distinct length (Table 2). The rDNA-bearing chromosomes involved in multivalent were detected only in *L. obsti* from Tanzania (Fig. 1a).

"*Buthus* group"

We examined 38 species belonging to nine genera in this group.

Androctonus. Eight analyzed species of this genus showed a stable number of chromosomes, 2n=24 (Figs 1d-f, S1c-h) and a typical extra-large chromosome pair in their karyotypes (Table 2). We identified meiotic multivalent associations in *A. amoreuxi* (hexavalent in one male and octavalent in another male) (Fig. 1e) and *A. gonneti* (hexavalent in one male) (Fig. 1f). The numbers as well as the position of rDNA clusters were stable across the species. The one pair of 18S rDNA was localized on the longest pair of chromosomes on approximately one quarter of its length (Figs 1d, f, S1c-h, Table 2). Only two males of *A. amoreuxi* showed rDNA-bearing chromosomes, which differed in length, associated in hexavalent (Fig. 1e) and octavalent (not shown), respectively.

Apistobuthus. The only analyzed species *A. pterygocercus* has 2n=22 (Fig. 1g) and its chromosomes gradually decreased in length (Table 2). We did not identify meiotic multivalent associations in this species. The 18S rDNA clusters were situated in terminal position on a single pair of chromosomes in *A. pterygocercus* (Fig. 1g).

Buthacus. We analysed three species of this genus. The karyotype of *B. stockmanni* (2n=20) was already reported by Kovařík et al. (2016b) and we examined the number and position of

18S rDNA in the same specimen. The chromosomes of *B. macrocentrus* (2n=28) (Fig. 1i) and *B. nigroaculeatus* (2n=26) (Fig. S1i) were analysed for the first time and in both species chromosomes gradually decrease in length (Table 2). All species of this genus displayed one pair of 18S rDNA clusters. The main difference between species was position of these clusters. They were placed close to the middle of the large chromosomes in *B. stockmanni* (Fig. 1h) and *B. nigroaculeatus* (Fig. S1i) (Table 2). Whereas the 18S rDNA clusters was situated in terminal position of the small chromosomes in *B. macrocentrus* (Fig. 1i).

Buthus. All four analyzed species of this genus had 2n=22 usually with one extra-large pair of chromosomes (Figs 1j, k, S1j, k) (Table 2). We observed only bivalents during postpachytene in all species but *B. awashensis*. In the latter we found multivalent composed from ten chromosomes during postpachytene and two of them with different length bore the 18S rDNA clusters (Fig. 1k). All other species had one pair of the 18S rDNA clusters localized always on the longest chromosome pair. Except for *B. awashensis* rDNA was situated approximately in one third of the chromosome length in all species (Figs 1j, S1k, l). In the *B. awashensis* multivalent one cluster was detected close to the centre of shorter chromosome (Fig. 1k).

Compsobuthus. We analysed six species of this genus (Figs 1l-n, S1l-o). The karyotypes of *C. matthiesseni* (2n=22) and *C. erithreaensis* (2n=22) were already reported by Šťáhlavský et al. (2014) and Kovařík et al. (2016b). We examined the same specimens for number and position of 18S rDNA. All other analysed species also possessed 2n=22 and chromosomes gradually decrease in length (Table 2). We identified meiotic multivalent associations in *C. maindroni* (quadrivalent in one male) (Fig. 1l) and *C. mattiesseni* (octavalent in one male) (Fig. 1m). All examined species had a single pair of rDNA clusters. Except for *C. acutecarinatus* the 18S rDNA signal was detected on the longest chromosomes in interstitial position close to the middle of the chromosomes in all species under study (Figs 1l, m, S1l-o). In *C. acutecarinatus* we identified the signal of 18S rDNA in subterminal position of the shorter chromosomes in comparison to the remaining species (Fig. 1n, Table 2). In the male of *C. mattiesseni* with octavalent the 18S rDNA clusters were born by two chromosomes of different length involved in the multivalent (Fig. 1m).

Hottentotta. We analysed nine species of the genus *Hottentotta* (Table 2). The diploid chromosome numbers ranged from 14 (*H. arenaceus*, *H. salcuyi*, *H. soisai* Figs S1p, s, t) to 24 (*H. flavidulus*; Fig. 1p). In majority of examined species the length of chromosomes gradually decreased in length (Table 2). The exceptions were only one extra-long and two extra-short chromosomes in karyotype of *H. ugandensis* (Fig. S1u) and three extra-long chromosomes in karyotype of *Hottentotta* sp. (Fig. S1v). Unfortunately, we observed meiosis only in five

analysed species (*H. conspersus*, *H. flavidulus*, *H. minusalta*, *H. sousai*, *Hottentotta* sp.). We identified meiotic multivalent associations in *H. flavidulus* (14 chromosomes involved; Fig. 1p) and *Hottentotta* sp. (hexavalent; Fig. S1v). We observed one pair of 18S rDNA signal in all observed species. However, we found differences in position of this cluster on chromosomes. The 18S rDNA cluster was detected in interstitial position in species with 2n=14 and 16 (*H. arenaceus*, *H. conspersus*, *H. hottentotta*, *H. minusalta*, *H. saulcyi*, *H. sousa*) (Figs 1o, S1p-t), while it was terminal in species with 2n=22 and 24 (*H. ugandensis*, *H. flavidulus*, *Hottentotta* sp.) (Figs 1p, S1u, v). The chromosomes bearing rDNA differed in size between species. They were usually long with the exception of *Hottentotta* sp. from Pakistan. In this species the 18S rDNA was localized on one of the shortest chromosomes (Table 2).

Leiurus. We analysed three species of the genus *Leiurus*. The karyotype of *L. abdullahbayrami* (2n=22) was already reported by Šťáhlavský et al. (2014). We examined the number and position of 18S rDNA in the same specimen (Figs 1q, S1w). The other analysed species also possessed 2n=22 (Figs 1r, S1x). We identified one pair of the signal of 18S rDNA in interstitial position in all species. The signal was localised on the longest pair of chromosomes in *L. abdullahbayrami* and *Leiurus* sp. (Table 1). The 18S rDNA cluster is localised on the chromosomes of only slightly different length in *L. hebraeus* (Fig. 1r, Table 2). Unfortunately, we obtained only mitotic metaphases in this species. Thus we had no information about multivalent association during meiosis in this species that could have explained different length of chromosomes with rDNA clusters.

Mesobuthus. We analysed only *M. eupeus* (2n=22) (Fig. 1s). In this species chromosomes gradually decreased in length. During observed meiosis we identified only bivalents in this species (Fig. 1s). The position of one pair of 18S rDNA clusters was close to the centre of the longest chromosomes (Fig. 1s).

Orthochirus. Three *Orthochirus* species were analysed. We identified 2n=24 in *O. afar* (Fig. 1t) with one extra-large pair of chromosomes and 2n=22 with gradually decreasing chromosomes in *O. glabrifrons* (Fig. 1u) and *O. innesi* (Fig. S1y). We did not find any meiotic multivalent associations in examined material. We observed one pair of 18S rDNA signals in the interstitial position on the longest chromosomes in all examined species. Interestingly we detected also one additional signal close to the centre on both chromosomes bearing 18S rDNA in both males of *O. glabrifrons* from different localities (Fig. 1u).

"*Charmus* group"

Thaicharmus. We analysed only one species *Thaicharmus* sp. from Vietnam belonging to this evolutionary lineage. The karyotype of this species contained 16 chromosomes that gradually decrease in length (Fig. 1v). We found only bivalents during postpachytene and one pair of the signals of 18S rDNA in terminal position of chromosome pair No. 5.

"*Isometrus* group"

We examined position and number of 18S rDNA clusters in one species of the genus *Isomentrus* and five species of the genus *Reddyanus* belonging to this group.

Isomentrus. The karyotype of *I. thwaitesi* (2n = 8) was already reported by Kovařík et al. (2016d). The analysis of meiosis identified presence of the quadrivalent in this male. In this sample we localised one pair of the 18S rDNA clusters in terminal position of the shortest chromosomes (Fig. 1w, Table 2).

Reddyanus. The karyotypes of three *Reddyanus* species from Sri Lanka were based on the mitosis and already reported by Kovařík et al. (2016d). These species had 2n=15-17 and chromosomes gradually decreased in length (Figs 2a-c, S2a, b, Table 2). Two additionally analysed species from Thailand had 2n=14 and 2n=11. In *Reddyanus* sp. 1 (2n=14) chromosomes gradually decreased in length (Fig. 1y) whereas *Reddyanus* sp. 2 (2n=11) has one extra-large chromosome (Fig. 1x, Table 2). The analysis of the meiosis of the same males used by Kovařík et al. (2016d) disclosed the occurrence of the multivalent association in *R. basilicus* (2n=15, one trivalent) (Fig. S2b) and in *R. loebli* (2n=17, chain of 11 or 13 chromosomes) (Figs 2d, c), whereas both analysed males of *R. ceylonensis* contained only bivalents during meiosis (Figs 2a, b). *Reddyanus* sp. 1 (2n=14) possesed only bivalents (Fig. 2y), whereas in *Reddyanus* sp. 2 (2n=11) nine chromosomes formed multivalent during postpachytene (Fig. 1x). We identified only terminal signals of 18S rDNA in all analysed species. However, we found conspicuous variability in number of signals among species. *Reddyanus* sp. 2 had only one pair of 18S rDNA clusters (Fig. 1x). The rest species showed increasing number of 18S rDNA clusters up to four in *R. basilicus*, *R. loebli*, and *R*. sp. 1 (Figs 1y, 2c, d S2a, b), six in one male of *R. ceylonensis* (Fig. 2a), or even seven as observed in one male of *R. ceylonensis* (Fig. 2b). Interestingly one pair of the signal was localised on multivalents in both analysed males of *R. loebli* (Figs 2c, d) and we observed one of the signals detected on both ends of a single chromosome pair being heterozygous in *R. ceylonensis* (Fig. 2b).

"*Uroplectes* group"

In this group, we examined position and number of 18S rDNA clusters in two species of the *Grosphus*, eight species of the genus *Parabuthus* and three species of the genus *Uroplectes*.

Grosphus. The chromosomes of *G.* cf. *darainensis* (2n=14) gradually decreased in length (Fig. 2e), whereas the karyotype of *Grosphus* sp. (2n=13) contained one extra short chromosome. Interestingly, the karyotype of the latter species contained not only one pair of bivalents and octavalent but also one univalent during meiotic prometaphase (Fig. 2f). *G.* cf. *darainensis* contained one quadrivalent and five bivalents during the same phases of meiosis. Both analyzed *Grosphus* spp. had only one pair of 18S rDNA clusters. They were identified in terminal position in *Grosphus* sp. (Fig. 2f), whereas in *G.* cf. *darainensis* the cluster was localized in subterminal position (Fig. 2e). Both species also clearly differed in length of chromosomes bearing rDNA. In *G.* cf. *darainensis* rDNA loci were located on the chromosomes of medium size, while rDNA was situated on the longest and one of the shortest chromosomes of the octavalent in *Grosphus* sp. (Fig. 2f)

Parabuthus. The diploid numbers of *P. abyssinicus* (2n=16), *P. kajibu* (2n=18) and *P. pallidus* (2n=20) were based on the mitotic complements and were already reported by Kovařík et al. (2016c). We examined meiosis and distribution of 18S rDNA in the same specimens (Figs 2g, h, i). The numbers of chromosomes in the rest of species under study were as follows: *P. mossambicensis* and *P. planicauda* had 2n=18 (Figs S2c, d), while *P. capensis*, *P. grabrimanus*, and *P. raudus* had 2n=20 (Figs S2e-g). The length of chromosomes gradually decreased in all species examined (Table 2). From all examined species, we identified multivalent chromosome association, namely quadrivalent, only in meiotic prophase I of *P. pallidus* (Fig. 2i). All species had only one pair of 18S rDNA clusters localized always terminally. The analysed species differed only in length of chromosomes carrying 18S rDNA signal. It varied from the longest chromosomes in *P. capensis*, *P. grabrimanus*, and *P. planicauda* to the one of the shorter chromosomes in *P. kajibu*, *P. mossambicensis*, and *P. raudus* (Table 2). In *P. pallidus*, the only species with chromosome multivalent we identified signal of 18S rDNA probe on chromosomes of similar length involved in the multivalent (Fig. 2i).

Uroplectes. The diploid numbers of analysed *Uroplectes* species were 16 in *U. formosus* (Fig. 2j), 26 in *U. otjimbinguensis* (Fig. 2k,l), and 28 in *U. emiliae* (Fig. 2m). Interestingly, we identified one extra-large pair of chromosomes in all species examined except for *U. otjimbinguensis*. In a single male of the latter only one large chromosome was observed. All the other chromosomes gradually decreased in length. We observed multivalent association during meiotic prophase I only in *U. otjimbinguensis* and two analysed males differed in number of chromosomes involved in a chain. Octavalent was observed in one male (Fig. 2k)

whereas the multivalent was formed by ten chromosomes in the second male (Fig. 2l). We detected only terminal signals of 18S rDNA in all analysed species. However, we identified variability in number of 18S rDNA cluster within this genus. Species *U. emiliae* and *U. formosus* had only one pair of rDNA loci, on the chromosomes of medium size (Table 2). In contrast male of *U. otjimbinguensis* with octavalent had two pairs of 18S rDNA clusters (Fig. 2k), while the second analyzed male of this species with decavalent contained three pairs of 18S rDNA clusters (Fig. 2l). One pair of these clusters was always localized on chromosomes of different length belonging to the multivalent association and the rest of signals were located on chromosome pairs.

"*Tityus* group"

We examined distribution of 18S rDNA clusters in two species of the genus *Centruroides*, three species of the genus *Heteroctenus* and eight species of the genus *Tityus* in this group.

Centruroides. *C. gracilis* (2n=24) (Fig 2n) as well as *C. baracoae* (2n=26) (Fig. 2o) contained one longer pair of the chromosomes while the other pairs gradually decreased in length (Table 2). In *C. baracoae* we found only bivalents during postpachytene whereas the analyzed male of *C. gracilis* contained one quadrivalent in meiosis (Fig. 2o). In both species we identified one pair of the signals for 18S rDNA in terminal position on the chromosomes of medium size (Table 2).

Heteroctenus. *H. abudi* (2n=16) (Fig. 2p) and *H. priceps* (2n=16) (Fig. S2h) contained chromosomes gradually decreasing in length whereas *H. garridoi* (2n=23) (Fig. S2i) had one extra-large chromosome in its karyotype. In this species we observed one trivalent in postpachytene nuclei whereas both previous species had only bivalents in this meiotic stage. We identified one pair of the 18S rDNA cluster localised in terminal position on the chromosomes of medium size in all species (Table 2).

Tityus. Analysed species of this genus showed the highest interspecific variability of diploid chromosome number that ranged from 2n=6 in *T. smithii* (Fig. 2q) to 2n=32 in *T. neibae* (Fig. 2r). Except *T. mana* (2n=20) (Fig. S2J), *T. elii* (2n = 22) (Figs S2k,l) with two and *T. neibae* (2n = 32) (Fig. 2r) with one extra-large chromosomes, all chromosomes gradually decreased in length in remaining species under study (Table 2). We identified also high frequency of multivalent associations in this genus in meiosis. Number of chromosomes in a multivalent chain was three in *T. naibae* (Fig. 2r), four in one male of *T. ecuadoriensis* (2n=24; Fig. 2s), five in *T. crassimanus* (2n=29; Fig. S2m), six in *T. mana* and *T. neibae*) (Figs S2j, 2r), or eight in *T. elii* (Figs S2k, l). Whereas 18S rDNA was localized terminally in all analysed species, the

number of signals varied. One pair of 18S rDNA was found in *T. crassimanus*, *T. ecuadoriensis*, *T. elii*, *T. fuhrmanni* (2n=22), *T. mana* and *T. naibae* (Figs 2s,t, S2j-o). Increase of number of signals to two pairs in *T. graffini* (2n=22; Fig. 2t) and three pairs in *T. smithii* (Fig. 2q) was observed. Interestingly no signal was observed on chromosomes involved in multivalents in this genus.

Discussion

Our results conform the basic cytogenetic characteristics of the family Buthidae. We identified lower number of chromosomes (2n=6-32, average 20, median 22), holokinetic organization, achiasmatic meiosis in males, absence of the morphologically differentiated sex chromosomes, and high frequency (about one third) of multivalent association in the first meiotic division of males of all groups. The 18S rDNA loci were detected in all species examined and, apart from a few cases, distribution of this marker showed strong phylogenetic pattern supporting the split of the Laurasian "*Buthus* group" from the other lineages, which diversified in Gondwana (Fet et al. 2003) (Table 2, Fig. 3).

Karyotype variability of buthid scorpions

It is assumed that karyotypes of organisms with holokinetic chromosomes mainly change via fissions and fusions of chromosomes because absence of localized centromere decreases risk of formation of dicentric or acentric fragments (e.g. Melters et al. 2012). The fragmentation of chromosomes may increase the chromosome number up to the 2n=ca448-452 reported in butterfly *Polyommatus atlanticus* (Elwes, 1906) without polyploidization (Lukhtanov 2015). The fragmentation of chromosomes may also lead to the distinct intrapopulation variability (e.g. Sadílek et al. 2013). However many holokinetic organisms do not increase karyotype diversity at all and their karyotypes are rather uniform (Melters et al. 2012, Van't Hof et al. 2013, Sadílek et al. 2015). The family Buthidae comprises taxa with both conserved and diverse karyotypes. The most analysed genus *Tityus* represents the lineage with high level of karyotypic diversity, which results from frequent fission/fussion events. *T. bahiansis* in particular shows high intraspecific variation in chromosome numbers range from 2n=5 to 2n=19 (Schneider et al. 2009b), while the intraspecific variability within the other species is usually much smaller e.g. *Tityus obscurus* (2n=11-16) (Almeida et al. 2017), *Tityus paraqguayensis* (2n=16-18) (Mattos et al. 2018), *Tityus metuendus* (2n=15 and16), *Tityus neglectus* (2n=26 and 27) (Mattos et al. 2013), *Reddyanus basilicus* (2n=15 and16) (Kovařík et al. 2016d), *Lychas marmoreus* (2n=12, 14 and 15) (Shanahan 1989), *Ananteris balzanii* (2n=12 and14) (Mattos et al. 2013). The

heterozygous fissions/fusions may also lead to the odd diploid number of chromosomes and conspicuous differences in their size which is evident for example in *Reddyanus basilicus* (2n=15), *Charmus laneus* (2n=9) (Kovařík et al. 2016d), *Tityus confluensis* (2n=13) (Mattos et al. 2013), and *Heteroctenus garridoi* (present study).

It should be noted that the cytogenetic analyses of almost all scorpions are usually based on very limited material, very often a single population, with the only exception being few *Tityus* and *Lychas* species (Shanahan 1989, Mattos et al. 2013, Almeida et al., 2017, Mattos et al. 2018, Ubinski et al. 2018). In some cases the observed differences may in fact reflect inter- rather than intraspecific variability due to yet undescribed diversity of some species complexes frequently documented by DNA analysis (e.g. Sousa et al. 2011, 2012). Despite of the expected diversity of buthid karyotypes it seems that the diploid chromosome number is rather constant especially in genera *Androctonus* (2n=24, 11 species), *Compsobuthus* (2n=22, 6 species), and *Leiurus* (2n=22, 4 species) (see Sadílek et al. 2015, Schneider et al. 2016, present study). The majority of the other genera then show only small interspecific differences. Furthermore, based on our results we can dispute high chromosome number of chromosomes previously reported in *Parabuthus mossambicensis* (2n=36), *Uroplectes carinatus*, (2n=48) (Newlands & Martindale 1980), and *Buthus occitanus* (2n=44 and 56) (Carnoy 1885, Makino 1956). This data is not well documented and may be an artefact due to the low quality of the chromosomes obtained as mentioned by Newlands & Martindale (1980) or an incorrect assessment and misinterpretation of some meiotic stages.

Multivalent associations in buthid scorpions

As mentioned above karyotypes of many buthids possess very consistent diploid chromosome numbers and the fissions or fusions were not observed very frequently. It may be due to troubles with identification of homologies, which is limited by use of standard cytogenetic techniques. In meiosis, these chromosomal rearrangements may sometimes be detected in the heterozygous state as trivalents observed already in *Heteroctenus garridoi* (Fig. S2i, present study), *Ischnotelson guanambiensis* (Ubinski et al. 2018), *Reddyanus basilicus* (Fig. S2b, present study), *Somalicharmus whitmanae* (Kovařík et al. 2016e), and six species of the genus *Tityus* (Piza 1950, Schneider et al. 2009, Mattos et al. 2013, Almeida et al. 2017, Ojanguren-Affilastro et al. 2017a, Mattos et al. 2018). More conspicuous effect on differentiation of karyotypes within the family Buthidae is evident from reciprocal translocations. Although this type of chromosome rearrangement does not affect chromosome numbers, it may however considerably change the size of the chromosomes (e.g. Kovařík et al. 2015, see Fig. 1x of

Reddyanus sp. 2, present study). All that at no or low fitness cost due to the holokinetic nature of the buthid chromosomes, which allow proper segregation of both fusion and fission products as reported e.g. in *Tityus bahiensis* (Schneider et al. 2009).

Number and location of rDNA clusters in buthid scorpions

The number and position of NORs has been so far described only in 33 buthid species belonging to nine genera. Only 25 of them have been detected using FISH technique. Furthermore almost all analysed genera (*Ananteris*, *Ischnotelson*, *Jaguajir*, *Physoctonus*, *Rhopalurus*, *Tityus*, *Troglorhopalurus*, and *Zabius*) are distributed only in South America whereas the other continents were represented only by *Androctonus* spp. from Northern Africa and Middle East (Sadílek et al. 2015). Analyzed South American species possess only one or two pairs of chromosomes bearing rDNA clusters in terminal position (Schneider et al., 2009, Schneider and Cella 2010; Mattos et al. 2013, Adilardi et al. 2014, 2015, Almeida et al. 2017, Ojanguren-Affilastro et al. 2017a, Mattos et al. 2018, Ubinski et al. 2018), whereas one pair of rDNA clusters is localized in an interstitial position in four *Androctonus* species (Sadílek et al. 2015). Our analysis of the position and number of major rDNA clusters in 19 buthid genera supports differences between representatives of "*Buthus* group" and the remaining main evolutionary lineages of the family Buthidae (Fig. 3).

The "*Buthus* group" genera possess one pair of rDNA clusters with the only exception of double signal on one chromosome pair in *Buthacus macrocentrus* (Fig. 3). It is evident that the interstitial position of rDNA loci localised on large chromosome pair is the ancestral state in this lineage. The exceptions such as some *Hottentotta* and *Buthacus* species with terminal location may be explained as the effect of chromosome fission close to the rDNA cluster rather than inversions as the rDNA clusters are localised on the shorter (or even the shortest) chromosomes and the diploid numbers of chromosomes are increased in comparison to other species (Table 2).

In the remaining lineages ("*Ananteris*", "*Charmus*", "*Isometrus*", "*Tityus*" and "*Uroplectes*" groups) rDNA was detected only in terminal position (Fig. 3). The number of the signals may, however, increase up to seven. Three distinct rDNA loci previously reported in *Rhopalurus rochai* male suggested that rDNA clusters can be mobile in buthids (Mattos et al. 2013). This notion is supported by intraspecific variability in *Reddyanus ceylonensis* (six and seven) in this study. The increase of the number of rDNA loci was observed also in other arachnids, namely up to seven loci in the harvestmen *Rhampsinitus leighi* (Šťáhlavský et al. 2018), ten loci in the harvestmen *Parapurcellia amatola* (Svojanovská et al. 2016), or even 19

loci in a spider *Wadicosa fidelis* (Forman et al. 2013) (all the numbers are mentioned for the diploid nucleus). However multiplication of rDNA clusters seems to be exceptional in arachnids and the ancestral state of the NORs number is probably one pair in this class (Forman et al. 2013).

In conclusion, our study provides unprecedented insight into karyotype evolution of buthid scorpions and revealed dynamic evolution of rDNA distribution in their six main evolutionary lineages. It is evident that modes of karyotype change differ between the Laurasian "*Buthus* group" and the remaining buthid lineages originating from Gondwana. The interstitial position and presence of one pair of rDNA clusters is typical and probably ancestral state for the "*Buthus* group". The change to terminal position is probably effect of chromosomal fragmentation close to the rDNA clusters in this group. In contrast the terminal position is typical and ancestral for remaining buthid groups and independent increase in rDNA loci can be found in almost all of them.

Acknowledgements

The study was supported by Grant Agency of the Charles University (GA UK 1324217) and grants of Ministry of Education, Youth and Sports of the Czech Republic no. SVV 260 434/2018. Microscopy was performed in the Laboratory of Confocal and Fluorescence Microscopy co-financed by European Regional Development Fund and the state budget of the Czech Republic. Project no. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515.

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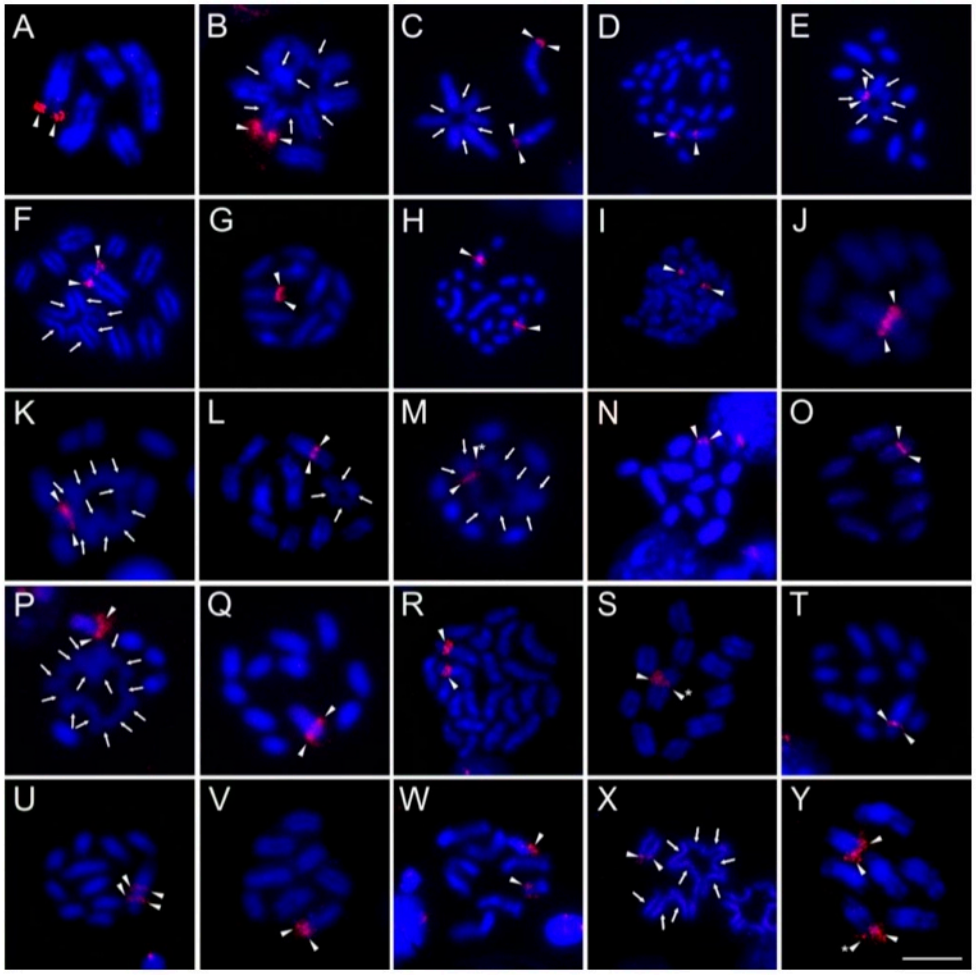
641



642

643 **Figure 1.** The location of 18S rDNA clusters (red signal) (arrowheads) in mitotic metaphases
644 (**D, H, I, R, W**) and in meiotic postpachytene (**A-C, E-G, J-Q, S-V, X**) of *Ananteris*, *Buthus*,
645 *Charmus* and part of *Isometrus* groups. The signals of the lower intensity (the heterozygous
646 size) are marked with asterisk. The chromosomes in multivalent association are marked with
647 arrows. The chromosomes are counterstained with DAPI (blue). (**A**) *Lychas obsti* from Ethiopia
648 (2n=10, 5II), (**B**) *L. obsti* from Tanzania (2n=10, 1II+CVIII), (**C**) *L. mucronatus* (2n=10,
649 2II+CVI), (**D**) *Androctonus amoreuxi* (2n=24), (**E**) *A. amoreuxi* (2n=24 9II+CVI), (**F**) *A.*
650 *gonneti* (2n=24, 9II+CVI), (**G**) *Apistobuthus pterygocercus* (2n=22, 11II),(**H**) *Buthacus*
651 *stockmanni* (2n=20), (**I**) *B. macrocentrus* (2n=28), (**J**) *B. maroccanus* (2n=22, 11II), (**K**) *Buthus*
652 *awashensis* (2n=22, 6II+CX), (**L**) *Compsobuthus maindroni* (2n=22,9II+CIV), (**M**) *C.*
653 *matthiesseni* (2n=22, 7II+CVIII), (**N**) *C. acutecarinatus* (2n=22, 11II), (**O**) *Hottentotta*
654 *conspersum* (2n=16, 8II), (**P**) *H. flavidulus* (2n=24, 5II+CXIV), (**Q**) *Leiurus abdullahbayrami*
655 (2n=22, 11II), (**R**) *L. hebraeus* (2n=22), (**S**) *Mesobuthus eupeus* (2n=22, 11II), (**T**) *Orthochirus*
656 *afar* (2n=24, 12II), (**U**) *O. glabrifrins* (2n=22, 11II), (**V**) *Thaicharmus* sp. (2n=16, 8II), (**W**)
657 *Isometrus thwaitesi* (2n=8), (**Y**) *Reddyanus* sp. 1 (2n=14, 7II). Bar = 10 μm.

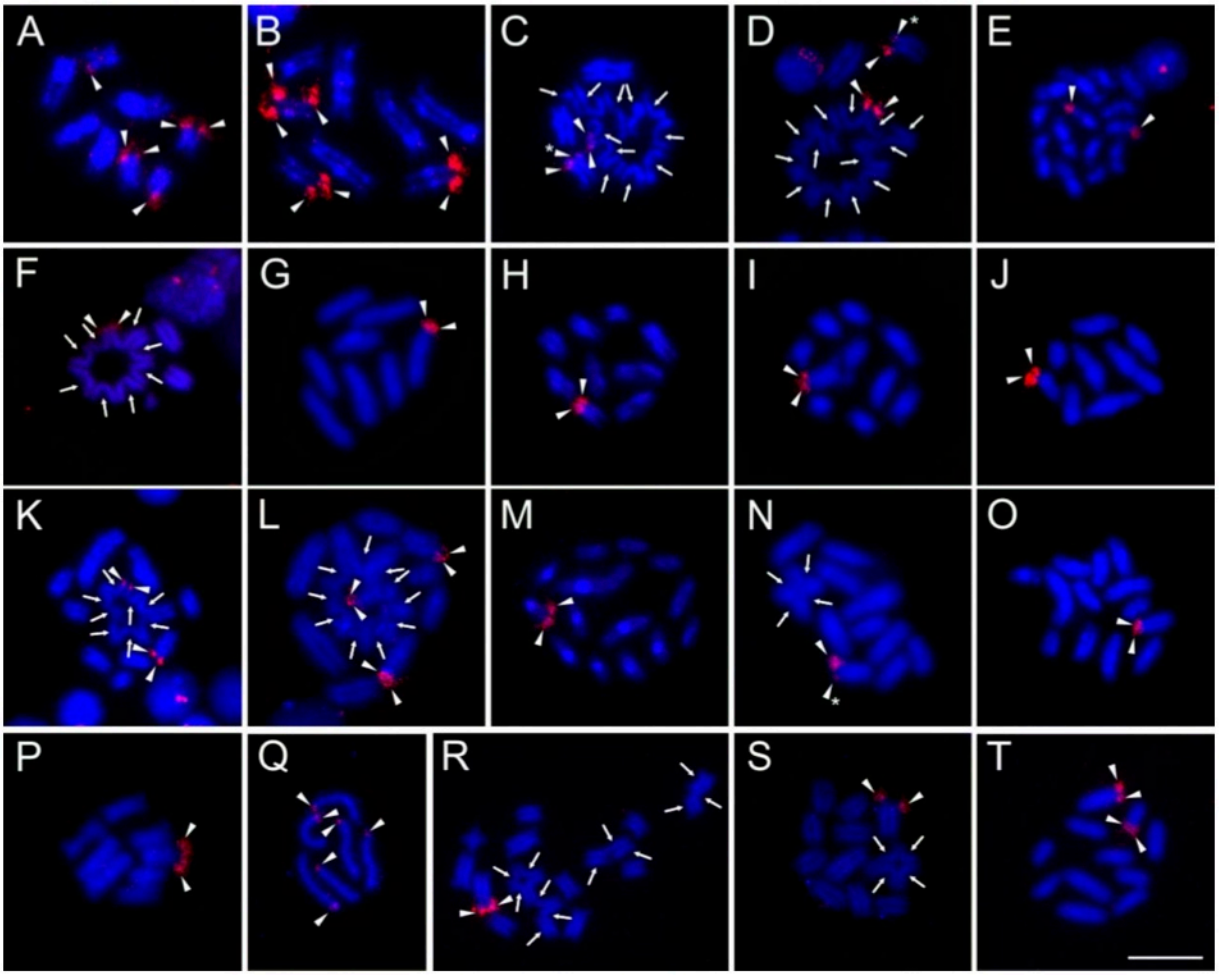


Figure 2. The location of 18S rDNA clusters (red signal) (arrowheads) in mitotic metaphases (**E**) and in meiotic postpachytene (**A-D, F-T**) of part of *Isometrus*, *Uroplectes* and *Tityus* groups. The signals of the lower intensity (the heterozygous size) are marked with asterisk. The chromosomes in multivalent association are marked with arrows. The chromosomes are counterstained with DAPI (blue). (**A**) *Reddyanus ceylonensis* (2n=16, 8II), six signals, (**B**) *R. ceylonensis* (2n=16, 8II), seven signals, (**C**) *R. loebli* (2n=17, 3II+CXI), (**D**) *R. loebli* (2n=17, 2II+CXIII), (**E**) *Grosphus cf. darainensis* (2n=14), (**F**) *Grosphus* sp. (2n=13, 2II+CVIII+I), (**G**) *Parabuthus abyssinicus* (2n=16, 8II), (**H**) *P. kajibu* (2n=18, 9II), (**I**) *P. pallidus* (2n=20, 8II+CIV), (**J**) *Uroplectes formosus* (2n=16, 8II), (**K**) *U. otjimbinguensis* (9II+CVIII), four signals, (**L**) *U. otjimbinguensis* (9II+CX), six signals, (**M**) *U. emiliae* (2n=28, 14II), (**N**) *Centruroides gracilis* (2n=24, 10II+CIV), (**O**) *C. baracoae* (2n=26, 13II), (**P**) *Heteroctenus abudi* (2n=16, 8II), (**Q**) *Tityus smithii* (2n=6, 3II), (**R**) *T. neibae* (2n=32, 10II+CIII+CIII+CVI), (**S**) *T. ecuadoriensis* (2n=24, 10II+CIV), (**T**) *T. gaffini* (2n=22, 11II). Bar = 10 μm.

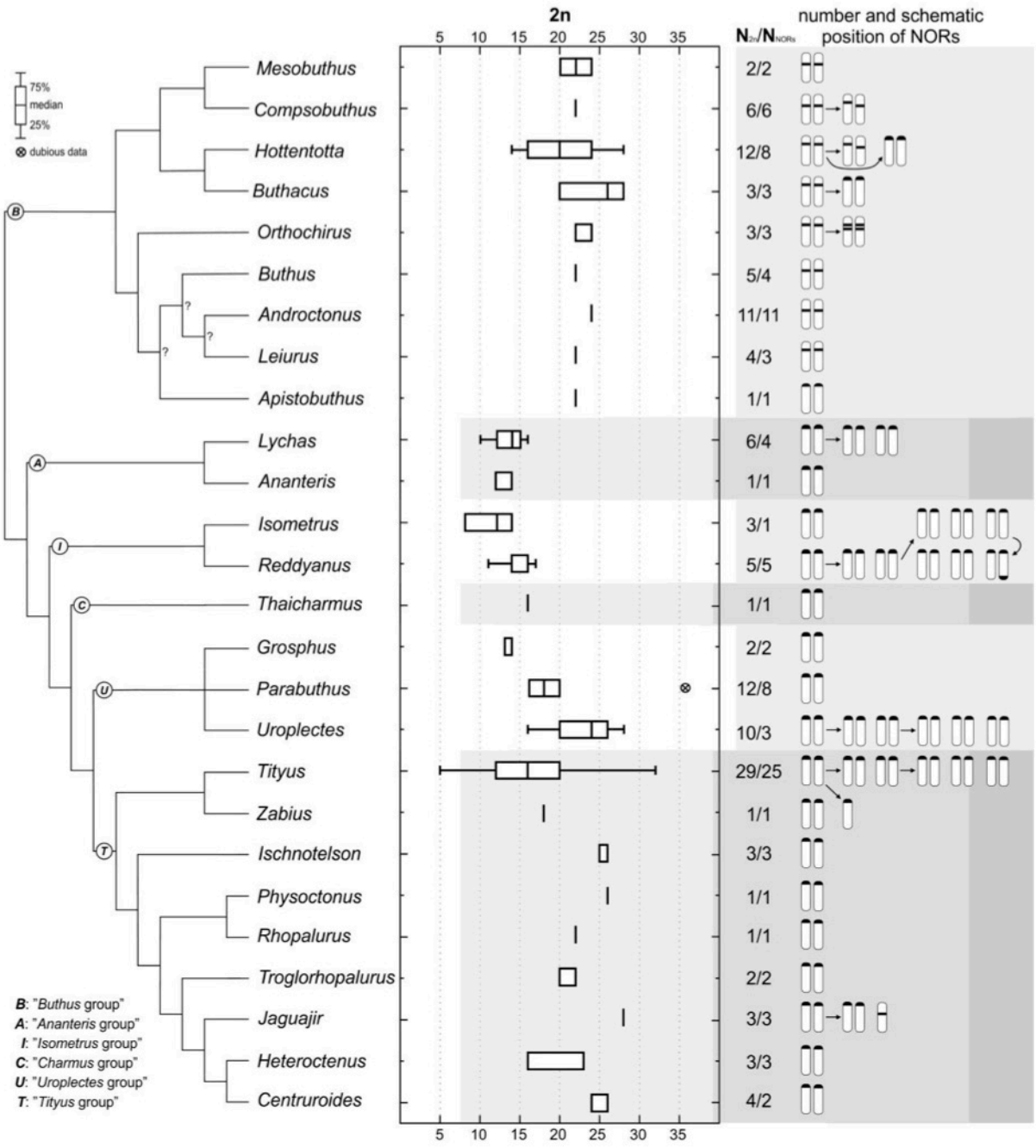


Figure 3. Summary of the 2n variability and number and position of NORs in buthid genera with proposed phylogeny. Idiograms of NOR-bearing chromosomes show number and location (terminal or interstitial without closer specification) of rDNA clusters in diploid set. Phylogeny according Fet et al. (2003, 2005) and Esposito et al. (2017). $\mathbf{N}_{2n}$=number of karyotyped species, $\mathbf{N}_{NORs}$=number of species with known number and position of NORs or rDNAs, ?=low supported topology.

682 **Table 1.** List of buthid scorpion species used in the present study.

species	N	country	GPS
***Ananteris*group**			
Lychas kraliKovarík, 1995	2m	Thailand	18.543, 98.579
L. mucronatus (Fabricius. 1798)	1m	Thailand	?
L. obsti Kraepelin, 1913	1m	Tanzania	-6.895, 39.207
	1m	Ethiopia	4.967, 36.483
L. scutilus C. L. Koch, 1845	2m	Thailand	8.239, 98.915
***Buthus* group**			
Androctonus amoreuxi (Audouin,1826)	1m	Morocco	31.971, -3.407
	1m	Morocco	29.059, -8.853
	1m	Morocco	28.249, -9.333
	1m	Morocco	28.044, -10.850
A. bicolor Ehrenberg, 1828	1m	Israel	?
A. bourdoni	1m	Morocco	?
	1m	Morocco	29.825, -9.636
A. gonneti Vachon, 1948	1m	Morocco	28.526, -11.289
	1m	Morocco	28.546, -10.993
A. liouvillei (Pallary, 1924)	1m	Morocco	32.081, -1.227
	1m	Morocco	?
A.mauritanicus (Pocock, 1902)	1m	Morocco	29.752, -9.777
A. sergenti Vachon, 1948	1m	Morocco	30.073, -8.462
	1m	Morocco	31.219, -7.412
*A. tenuissimus*Teruel, Kovarik & Turiel, 2013	2m	Egypt	?
Apistobuthus pterygocercus Finnegan, 1932	1m	Oman	22.491, 58.715
Buthacus macrocentrus (Ehrenberg, 1828)	3m	Turkey	36.947, 38.022
B. stockmanni Kovraik, Lowe & Stahlavsky, 2016	1m	Morocco	28.249, -9.333
Buthus atlantis Pocock, 1889	2m	Morocco	30.721, -9.840
B. awashensis Kovarik, 2011	1m	Ethiopia	?
B. berberensis Pocock, 1900	3m	Somaliland	10.600, 43.435
B. maroccanus Birula, 1903	1m	Morocco	33.928, -6.759
Compsobuthus acutecarinatus (Simon, 1882)	1m	Oman	17.256, 54.022
	1f	Oman	17.926, 55.564
C. arabicus Levy, Amitai & Shulov, 1973	1m	Oman	22.491, 58.715
C. maindroni (Kraepelin, 1900)	1m	Oman	22.813, 59.059
	2m	Oman	24.775, 55.915
C. matthiesseni (Birula, 1905)	2m, 1f	Turkey	37.002, 37.524
	1m	Turkey	37.002, 37.524
C. schmiedeknechti Vachon, 1949	1m	Turkey	36.885, 36.800
C. eritreaensis Kovařík, Lowe, Plíšková et Šťáhlavský, 2016	1m	Eritrea	15.616, 39.376
Hottentotta arenaceus (Purcell, 1902)	1m	Namibia	-27.037, 17.858
H. conspersus (Thorell, 1876)	1m	Namibia	-21.457, 15.079
H. flavidulus Teruel et Rein, 2010	1m	Pakistan	?
H. hottentotta (Fabricius, 1787)	1m	Mali	?
	1f	Benin	?
H. minusalta Vachon, 1959	3m	Afganistan	?
H. saulcyi (Simon, 1828)	1m	Turkey	37.499, 42.442
H. sousai Turiel, 2014	1m	Morocco	28.092, -10.889
*H. ugandensis*Kovarik, 2013	1f	Uganda	33.781, 1.661
Hottentotta sp.	1m	Pakistan	?
Leiurus abdullahbayrami Yagmur, Koc & Kunt, 2009	1m	Turkey	37.002, 37.524
	1m	Turkey	37.002, 37.524
L. hebraeus(Birula, 1908)	1m	Jordan	29.445, 35.168
Leiurus sp.	1m	Africa	?
Mesobuthus eupeus (C.L. Koch, 1839)	1m	Turkey	38.434, 35.251
	1m	Turkey	40.617, 43.091
Orthochirus afar Kovarik, Lowe & Stahlavsky, 2016	2m	Somaliland	10.600, 43.435
*Orthochirus glabrifrons (*Kraepelin, 1903)	1m	Oman	22.950, 57.673
	1m	Oman	22.813, 59.059
O. innesi Simon, 1910	1m	Morocco	32.107, -1.213
	1m	Morocco	28.516, -9.854

Table 1. ***continued***

species	N	country	GPS
	5m	Morocco	28.092, -10.889
	2m	Oman	18.828, 56.403
***Charmus* group**			
Thaicharmus sp.	1m	Vietnam	12.221, 109.1416
***Isometrus* group**			
Isometrus thwaitesi Pocock, 1897	1m	Sri Lanka	8.145, 80.851
Reddyanus basilicus (Karsch, 1879)	1m	Sri Lanka	7.334, 81.699
	1m	Sri Lanka	6.879, 81.723
R. ceylonensis Kovarik, Lowe, Ranawana, Hoferek, Jayarathne, Plíšková & Stahlavsky, 2016	1m	Sri Lanka	8.559, 79.947
	1m	Sri Lanka	8.559, 79.947
R. loebli (Vachon, 1982)	1m	Sri Lanka	8.807, 80.179
	1m	Sri Lanca	8.807, 80.179
Reddyanus sp. 1	1m	Vietnam	10.928, 107.849
Reddyanus sp. 2	3m	Thailand	?
***Uroplectes* group**			
Grosphus cf. darainensisLourenço, Goodman & Ramilijaona, 2004	1m	Madagascar	?
Grosphus sp.	1m	Madagascar	-18.947, 48.418
Parabuthus abyssinicus Pocock, 1901	1m	Ethiopia	9.010, 40.299
	1m	Eritrea	15.809, 38.471
P. capensis(Ehrenberg, 1831)	1 m	South Africa	-30.100, 17.600
*P. grabrimanus*Pocock, 1895	1m	Namibia	?
P. kajibu Kovařík, Lowe, Plíšková & Šťáhlavský, 2016	1m	Ethiopia	4.976, 36.515
P. mossambicensis(Peters, 1861)	1m	Mozambique	?
P. pallidus Pocock, 1895	1m	Ethiopia	4.891, 38.129
P. planicauda (Pocock, 1889)	1m	South Africa	-33.224, 20.412
	1m	South Africa	-33.338, 20.508
P. raudus(Simon, 1888)	1m	Namibia	?
Uroplectes emiliae (Werner, 1916)	1m		?
U. formosus Pocock, 1890	1m	South Africa	-32.568, 26.920
	1m	South Africa	-30.275, 30.609
U. otjimbinguensis (Karsch, 1879)	1m	Namibia	-24.524, 16.072
	2m	Namibia	?
***Tityus* group**			
Centruroides gracilis (Latreille, 1804)	1m	Cuba	22.896, -82.504
C. baracoae Armas, 1976	2m	Cuba	20.554, -74.734
Heteroctenus abudi Armas & Marcano Fondeur, 1987	1m	Dominican Rep.	18.363, -68.618
H. garridoi Armas, 1974	1m	Cuba	19.978, 74.915
H. princeps (Karsch, 1879)	3m	Dominican Rep.	18.355, -70.515
Tityus crassimanus (Thorell, 1876)	2m	Dominican Rep.	18.112, -71.617
T. ecuadoriensis Kraepelin, 1896	2m	Ecuador	-4.256, -79.219
	1m	Ecuador	-4.256, -79.219
T. elii (Armas & Marcano Fondeur, 1992)	1m	Dominican Rep.	19.006, -70.539
T. fuhrmanni Kraepelin, 1914	1m	Colombia	?
T. gaffini Lourenço, 2000	3m	Colombia	4.200, -73.600
*T. mana*Lourenço, 2012	1m	Fr. Guyana	5.746, -53.935
T. neibae Armas, 1999	1m	Dominican Rep.	18.581, -71.422
T. smithii Pocock, 1893	2m	Grenada	12.017, -61.750

683

684

685 **Table 2.** Cytogenetic data of studied buthid scorpions.

species	2n	Ppt config.	chrom. length	rDNA placing	number and 18S rDNA position	Fig.
***"Ananteris* group"**						
Lychas krali	16	8II	2x7.11-2x4.84	2. and 8. pair	4x T	S1A
L. mucronatus	10	2II+CVI	12.98-2x7.61	5., 6. and 9., 10. chrom.	4x T	1C
L. obsti	10	1II+CVIII	14.32-2x6.84	3. and 5. chrom. (CVIII)	2x T	1B
	10	5II	2x13.64-2x7.33	3. pair	2x T	1A
L. scutilus	14	7II	2x9.99, 2x7.44-2x5.88	6. pair	2x T	S1B
***"Buthus* group"**						
Androctonus amoreuxi	24	mit	2x7.76, 6.35-2.64	1. pair	2x 2.40	
	24	12II	2x7.28, 2x5.71, 2x4.65-2x2.92	1. pair	2x 2.49	1D
	24	9II+CVI	6.77, 6.04, 2x5.41, 4.79-2x2.88	1. and 5. chrom. (CVI)	1x 2.18, 1x 1.37	1E
	24	8II+CVIII	8.03, 6.21-2.86	1. and 7. chrom. (CVIII)	1x 2.93, 1x 1.34	
A. bicolor	24	12II	2x7.82, 2x6.29-2x2.59	1. pair	2x 2.72	S1C
A. bourdoni	24	12II	2x7.33, 2x5.03-2x3.06	1. pair	2x 2.55	S1D
	24	12II	2x7.18, 5.21-2.94	1. pair	2x 2.30	
A. gonneti	24	mit	2x6.64, 4.90-2.94	1. pair	2x 2.41	
	24	9II+CVI	2x6.60, 5.65-2.19	1. pair	2x 2.51	1F
A. liouvillei	24	mit	2x7.87, 2x6.00, 2x4.83-2x2.80	1. pair	2x 2.18	
	24	12II	2x7.39, 2x6.83, 2x5.13-2x2.58	1. pair	2x 2.39	S1E
A. mauritanicus	24	12II	2x7.05-2x2.83	1. pair	2x 2.42	S1F
A. sergenti	24	10II+CIV	2x8.31, 5.11-2.63	1. pair	2x 2.36	S1G
	24	12II	2x7.51, 2x5.36-2x2.98	1. pair	2x 2.16	
A. tenuissimus	24	mit	2x7.89, 6.21-3.06	1. pair	2x 2.76	S1H
Apistobuthus pterygocercus	22	11II	2x6.10-2x3.13	4. pair	2x T	1G
Buthacus macrocentrus	28	mit	2x5.68-2x1.68	13. pair	2x T	1I
B. nigroaculeatus	26	mit	2x5.40-2x2.64	4. pair	2x 1.39	S1I
B. stockmanni	20[1]	mit	2x13.41, 2x5.84-2x2.69	2. pair	2x 1.26	1H
Buthus atlantis	22	11II	2x6.93-2x2.86	1. pair	2x 2.01	S1J
B. awashensis	22	6II+CX	7.32-2.91	1. and 7. chrom. (CX)	1x 2.86, 1x 1.79	1K
B. berberensis	22	11II	2x7.5, 2x5.8 - 2x3.1	1. pair	2x 3.00	S1K
B. maroccanus	22	11II	2x7.8, 2x6.14 - 2x3.04	1. pair	2x 2.45	1J
Compsobuthus acutecarinatus	22	11II	2x6.23-2x3.46	5. pair	2x 9.25	1N
C. arabicus	22	mit	7.33-2.80	2. pair	2x 1.83	S1L
C. eritreaensis	22[3]	11II	6.70-2.81	2. pair	2x 1.68	S1M
C. maindroni	22	9II+CIV	2x6.58-2.55	2. pair	2x 1.82	1L
	22	11II	2x6.94-2x3.12	1. pair	2x 1.77	S1N
C. matthiesseni	22[2]	11II	2x6.09-2x3.24	1. pair	2x 1.91	
	22[2]	7II+CVIII	6.68-3.15	1. and 3. chrom (CVIII)	1x 1.59, 1x 1.12	1M
C. schmiedeknechti	22	11II	6.91-2.99	1. pair	2x 1.52	S1O
Hottentotta arenaceus	14	mit	9.56-5.09	7. and 8. chrom.	2x 2.58	S1P
H. conspersus	16	8II	2x7.90-2x4.48	3. pair	2x 2.46	1O
H. flavidulus	24	5II+CXIV	6.76-2.34	6. and 7. chrom.	2x T	1P
H. hottentotta	16	mit	9.44-3.94	1. and 6. chrom.	1x 2.05, 1x 2.37	S1Q
	16	mit	9.48-4.64	2. and 4. chrom.	1x 1.37, 1x 3.19	
H. minusalta	16	8II	2x7.82-2x4.00	2. pair	2x 1.80	S1R
H. saulcyi	14	mit	11.65-4.39	3. and 4. chrom.	1x 2.16, 1x 1.23	S1S
H. sousai	14	7II	2x8.02-2x6.55	3. pair	2x 2.72	S1T

Table 2. *continue*

species	2n	Ppt config.	chrom. length	rDNA placing	number and 18S rDNA position	Fig.
H. ugandensis	22	mit	2x7.54, 6.10-2x3.05, 2x2.28	5. pair	2x T	S1U
Hottentotta sp.	22	8II+CVI	7.03, 6.17, 5.72,2x5.60-2x2.88	12. and 13. chrom.	2x T	S1V
Leiurus abdullahbayrami	22[1)]	11II	2x6.09-2x3.24	1. pair	2x 3.66	1Q
	22[1)]	8II+CVI	2x5.99-2x3.18	1. pair	2x 3.75	S1W
L.hebraeus	22	mit	7.13-2.92	4. and 6. chrom.	1x 3.30, 1x 4.08	1R
Leiurus sp.	22	11II	2x5.50, 2x4.90-2x3.07	1. pair	2x 3.21	S1X
Mesobuthus eupeus	22	11II	2x6.69-2x2.92	1. pair	2x 1.55	1S
	22	11II	2x7.15-2x2.82	1. pair	2x 1.70	
Orthochirus afar	24	12II	2x7.02, 2x5.43-2x2.94	1. pair	2x 3.07	1T
O. glabrifrons	22	11II	2x7.72-2x3.21	1. pair	2x 3.19, 2x 1.15	1U
	22	11II	2x7.58-2x2.97	1. pair	2x3.09, 2x 1.50	
O. innesi	22	mit	2x7.60-2x2.97	1. pair	2x 2.59	
	22	11II	2x7.14-2x3.06	1. pair	2x 2.77	S1Y
	22	11II	2x7.66-2x3.06	1. pair	2x 3.17	
***"Charmus* group"**						
Thaicharmus sp.	16	8II	2x7.59-2x4.68	5. pair	2x T	1V
***"Isometrus* group"**						
Isometrus thwaitesi	8[4)]	2II+CIV	16.40-13.00, 2x6.30	4. pair	2x T	1W
Reddyanus basilicus	16[4)]	8II	8.00-4.60	6. and 8. pair	4x T	S2A
	15[4)]	7II+CIII	11.50, 8.00-4.60	12. - 15. chrom.	4x T	S2B
R. ceylonensis	16	8II	2x8.38-2x4.43	6. - 8. pair	6x T	2A
	16[4)]	8II	8.90-4.20	6. - 8. pair	7x T	2B
R. loebli	17	3II+CXI	7.65-4.22, 3.88	4., 14. - 16. chrom. (2x II + 2x CXI)	4x T	2D
	17[4)]	2II+CXIII	7.70-5.00, 3.50	2., 12. - 14. chrom. (2x II + 2x CXIII)	4x T	2C
Reddyanus sp. 1	14	7II	2x9.15-2x5.36	6. and 7. pair	4x T	1Y
Reddyanus sp. 2	11	1II+CIX	19.23, 10.11-4.18	8. and 9. chrom.	2x T	1X
***"Uroplectes* group"**						
Grosphus cf. *darainensis*	14	5II+IV	10.75-4.81	5. and 6. chrom.	2x7.45	2E
Grosphus sp.	13	2II+CVIII+I	11.15-2x6.42, 3.88	1. and 10. chrom. (CVIII)	2x T	2F
Parabuthus abyssinicus	16[5)]	8II	2x7.51-2x4.84	3. pair	2x T	2G
	16[5)]	8II	2x7.34-2x5.04	3. pair	2x T	
P. capensis	20	mit	8.13-2.88	1. and 2. chrom.	2x T	S2E
P. grabrimanus	20	10II	2x7.88-2x3.00	1. pair	2x T	S2F
P. kajibu	18[5)]	9II	2x7.17-2x3.81	8. pair	2x T	2H
P. mossambicensis	18	mit	8.31-3.31	14. and 16. chrom.	2x T	S2C
P. pallidus	20[5)]	8II+CIV	2x7.11-2x3.31	6. and 7. chrom. (CIV)	2x T	2I
P. planicauda	18	9II	2x7.51-2x3.31	1. pair	2x T	S2D
P. raudus	20	10II	2x6.64-2x3.59	9. pair	2x T	S2G
Uroplectes emiliae	28	14II	2x8.19, 2x5.31-2x2.47	7. pair	2x T	2M
U. formosus	16	8II	2x9.51, 2x7.28-2x4.09	7. pair	2x T	2J

Table 2. ***continue***

species	2n	Ppt config.	chrom. length	rDNA placing	number and 18S rDNA position	Fig.
U. otjimbinguensis	26	9II+CVIII	2x7.47, 5.69-2.30	11. and 18. chrom (CVIII), 16. and 17. chrom (II)	4x T	2K
	26	8II+CX	7.01, 5.66-2x2.41	2. and 16. chrom (CX), 13. and 14. chrom (II), 20. and 21. chrom (II)	6x T	2L
***"Tityus* group"**						
Centruroides gracilis	24	10II+CIV	2x6.05, 2x4.92-2x3.14	13. and 14. chrom.	2x T	2N
C. baracoae	26	13II	2x5.89, 2x4.67-2x2.58	4. pair	2x T	2O
Heteroctenus abudi	16	8II	2x8.03-2x2x4.38	4. pair	2x T	2P
H. garridoi	23	10II+CIII	7.25, 2x5.53-2.98	8. and 9. chrom.	2x T	S2I
H. princeps	16	8II	2x7.81-2x4.47	5. pair	2x T	S2H
Tityus crassimanus	29	12II+CV	5.56-1.86	8. and 9. chrom.	2x T	S2M
T. ecuadoriensis	24	12II	2x5.56-2x3.10	2. pair	2x T	S2N
	24	10II+CIV	2x5.48-2x3.12	1. pair	2x T	2S
T. elii	22	7II+CVIII	7.60, 5.96-2.64	10. and 11. chrom.	2x T	S2K+S2L
T. fuhrmanni	22	11II	2x5.69-2x3.29	5. pair	2x T	S2O
T. gaffini	22	11II	2x6.27-2x3.26	2. pair and 9. pair	4x T	2T
T. mana	20	7II+CVI	2x7.88, 2x6.48-2x3.88	15. and 16. chrom.	2x T	S2J
T. neibae	32	10II+CIII+CIII+CVI	5.81, 4.83-2x2.00	5. and 6. chrom.	2x T	2R
T. smithii	6	3II	2x18.44-2x15.04	1., 2. and 3. pairs	6x T	2Q

Abbreviation: Ppt config. - postpachytene configuration (after Mattos et al. 2013); mit - analysis based only on mitotic metaphases; T -terminal possition of NOR; upper index: 2n and length of chromosomes after: 1) Kovařík et al. 2016e, 2) Šťáhlavský et al. 2014, 3) Kovařík et al. 2016b, 4) Kovařík et al. 2016d, 5) Kovařík et al. 2016c.

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Supplementary files

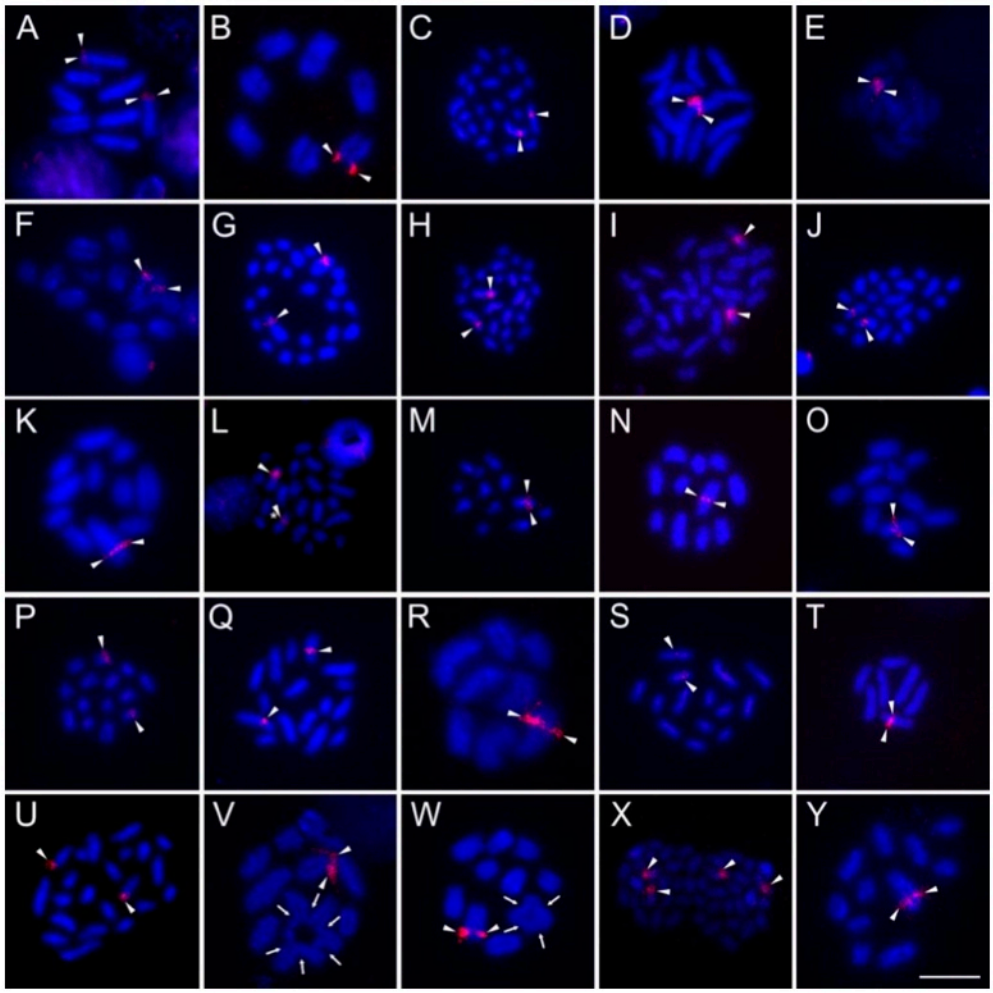

Figure S1. The location of 18S rDNA clusters (red signal) (arrowheads) in mitotic metaphases (**C, G-J, L, P, Q, S, U, X**) and in meiotic postpachytene (**C-F, I-O, R, T, V, W, Y**) of *Ananteris* and *Buthus* group. The signals of the lower intensity (the heterozygous size) are marked with asterisk. The chromosomes in multivalent association are marked with arrows. The chromosomes are counterstained with DAPI (blue). **(A)** *Lychas krali* (2n=16, 8II), (**B**) *L. scutilus* (2n=14, 7II), **(C)** *Androctonus bicolor* (2n=24), (**D**) *A. bourdoni* (2n=24, 12II), (**E**) *A. liouvillei* (2n=24, 12II), (**F**) *A. mauritanicus* (2n=24, 12II), (**G**) *A. sergeti* (2n=24), (**H**) *A. tenuissimus* (2n=24), (**I**) *Buthacus nigroaculeatus* (2n=26), (**J**) *Buthus atlantis* (2n=22), (**K**) *B. berberensis* (2n=22, 11II), (**L**) *Compsobuthus arabicus* (2n=22), (**M**) *C. eritreaensis* (2n=22, 11II), (**N**) *C. maindroni* (2n=22, 11II), (**O**) *C. schmiedeknechti* (2n=22, 11II), (**P**) *Hottentotta arenaceus* (2n=14), (**Q**) *H. hottentotta* (2n=16), (**R**) *H. minusalta* (2n=16, 8II), (**S**) *H. saulcyi* (2n=14), (**T**) *H. sousai* (2n=14, 7II), (**U**) *H. ugandensis* (2n=22), (**V**) *Hottentotta* sp. (2n=22, 8II+CVI), (**W**) *Leiurus abdullahbayrami* (2n=22, 8II+CVI), (**X**) *Leiurus* sp. (2n=22), (**Y**) *Orthochirus innesi* (2n=22, 11II). Bar = 10 μm.

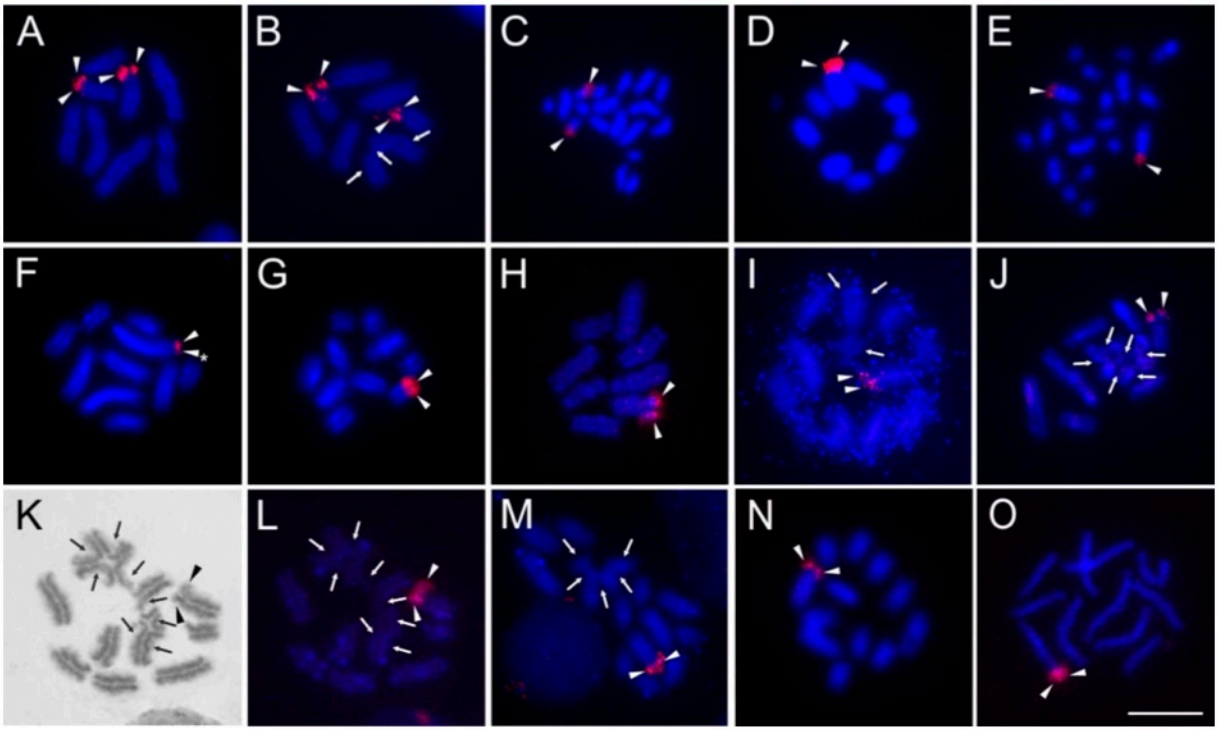

Figure S2. The location of 18S rDNA clusters (red signal) (arrowheads) in mitotic metaphases (**E**) and in meiotic postpachytene (**A-D, F-O**) of *Isometrus*, *Uroplectes* and *Tityus* groups. The signals of the lower intensity (the heterozygous size) are marked with asterisk. The chromosomes in multivalent association are marked with arrows. The chromosomes are counterstained with DAPI (blue). **(A)** *Reddyanus basilicus* (2n=16, 8II), **(B)** *R. basilicus* (2n=15, 7II+CIII), **(C)** *Parabuthus mossambicensis* (2n=18), (**D**) *P. planicauda* (2n=18, 9II), (**E**) *P. capensis* (2n=20), (**F**) *P. grabrimanus* (2n=20, 10II), (**G**) *P. raudus* (2n=20, 10II), (**H**) *Heteroctenus princeps* (2n=16, 8II), (**I**) *H. garridoi* (2n=23, 10II+CIII), (**J**) *Tityus mana* (2n=20, 7II+CVI), (**K**, **L**) *T. elii* (2n=22, 7II+CVIII), (**M**) *T. crassimanus* (2n=29, 12II+CV), (**N**) *T. ecuadoriensis* (2n=24, 12II), (**O**) *T. fuhrmanni* (2n=22, 11II). Bar = 10 μm.

ČLÁNEK II

Tracking the trends of karyotype differentiation in the phylogenetic context in *Gint* Kovařík et al., 2013, a scorpion group endemic to the Horn of Africa (Scorpiones: Buthidae)

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Abstract

In the present study, we employed an integrative approach, combining cytogenetic data and sequence-based phylogeny, to determine the principal mechanisms of karyotype differentiation in *Gint* Kovařík et al., 2013, a scorpion genus endemic to the Horn of Africa. We examined the chromosomal characteristics of six *Gint* species with emphasis on multivalent meiotic configurations, 18S rDNA and TTAGG distribution. Cytogenetic data were compared with genetic divergence/variation of the species studied based on analysis of 16S rRNA and COI gene markers. *Gint* exhibited substantial karyotype diversity [2n = 18–45] and a high incidence of chromosome heterozygosity. Meiotic chromosome chains formed by up to six elements were found in 85 % of the analyzed individuals, causing intraspecific chromosome variation in three species (*G. amoudensis*, *G. banfasae* and *G. dabakalo*). The number of 18S rDNA sites, two per 2n, appeared to be conserved across *Gint* spp. Only *G. amoudensis* showed up to three 18S rDNA sites in its genome. The rDNA chromosomal location and the appearance of rDNA bearing chromosome varied among species. Involvement of rDNA bearing chromosome in multivalent configurations was observed in three species. Overall, the cytogenetic findings indicated frequent and ongoing structural changes in the genome architecture in *Gint* spp., presumably through independent fusions, fissions and reciprocal translocations. This contrasted with the shallow genetic divergence of the species.

Keywords: holokinetic chromosomes, multivalent meiosis, cytotaxonomy, karyotype evolution, scorpions,

Introduction

Eukaryotic organisms, as a general rule, possess species-specific karyotypic features (e.g. number, morphology and size of chromosomes, copies and location of multigene families). However, karyotypes may differ considerably at the macro- or microstructural level even among closely related taxa (White 1969, Cohen and Roth 1970, O'Neill et al. 2001, Ruiz-Herrera et al. 2012, Nalepa et al. 2017, Štundlová et al. 2019). Such findings have contributed to the assumption that structural changes in genome architecture may be involved in evolutionary processes such as adaptation and speciation (Rieseberg 2001, Gorelick & Olson 2013, Feulner and De-Kayne 2017). They potentially contribute to the formation of post-zygotic barriers.

During the course of species evolution, the karyotype can be subjected to structural modifications which lead to alterations in number and/or architecture of chromosomes (King 1993, Schubert 2007). Such changes may arise from gain (i.e. insertion, duplication) or loss (i.e. deletion) of chromosome segments, or rearrangements within or between chromosomes (i.e. inversion, translocation, or fusion/fission) (Rieseberg 2001, Eichler and Sankoff 2003, Gross et al. 2009, Schubert and Lysak 2011). These events occur in both monocentric and holokinetic chromosomes (Luceño and Guerra 1996), however sometimes with differing impacts. This is, in particular, due to the distinct spatial distribution of the kinetochore, a protein complex that ensures the attachment of spindle microtubules to the chromosomes during cell division (Márquez-Corro et al. 2017). In monocentric chromosomes, with the kinetochore localized at a single site (i.e. centromere), some rearrangements produce unstable acentric or dicentric fragments that are aberrant and prone to elimination during cell division (Carrano and Heddle 1973, Dernburg 2001). On the contrary, in holokinetic chromosomes, with kinetochore proteins occurring diffusely along most of the chromosome length, such rearrangements generate chromosomal fragments that retain their ability to properly segregate (Hughes-Schrader and Schrader 1961, Dernburg et al. 2001, Maddox et al. 2004, Melters et al. 2012).

During the stages of karyotype differentiation, novel structural rearrangements may give rise to chromosomal polymorphism (e.g. reviewed for mammals by Dobigny et al. 2015). According to de Resende (2017) “Chromosomal variation can occur between different populations of the same species, between individuals of the same population, and even between different cells of the same tissue of the individual.”. Rearranged chromosomes (via translocations, fusions or fissions) occurring in a heterozygous state form multivalent chains or rings during the first meiotic metaphase (King 1993, Rieseberg 2001). The multivalent

formations may consist of varied numbers of chromosome elements, which may be autosomes, sex chromosomes, or both. The presence of complex chromosome configurations has been documented both in plants and animals (King 1993, Moscone et al. 2007, de Araújo et al. 2016). In arthropods, multiple examples of multivalents have been found in lepidopterans, termites, cockroaches, or scorpions (e.g. John and Lewis 1957, Luykx and Syren 1981, Šíchová et al. 2015, 2016, Plíšková et al. 2016, McClure et al. 2017). Despite this, the mechanisms regarding emergence, accumulation and fixation of heterozygous chromosomes remain obscure.

Scorpions are an ancient group of arachnids exhibiting distinctive cytogenetic features. They are distinguished by the occurrence of both types of chromosomes (i.e. monocentric and holokinetic), achiasmatic male meiosis, lack of recognizable sex chromosomes, and an extensive karyotype variability (2n = 5–186). Besides, scorpions also typically show a high incidence of chromosomal heterozygosity (Shanahan 1989a,b, Shanahan and Hayman 1990, Mattos et al. 2013, 2018). The most cytogenetically researched scorpion group, comprising almost 60 % of all analyzed species, is the family Buthidae (Schneider et al. 2019). Buthid scorpions represent an early-branching and most diverse scorpion lineage with worldwide distribution (Sharma et al. 2015, Rein 2019). Moreover, they are unique among other scorpion families in having holokinetic chromosomes (Schneider et al. 2019). Buthidae generally show a narrow range of chromosome counts (2n = 5–48) and a distinct pattern of karyotype diversity across the genera. Most genera studied exhibit a stable or a slightly variable number of chromosomes among their species [e.g. *Androctonus* spp. (2n = 24), *Microbuthus* spp. (2n = 26), *Jaguajir* spp. (2n = 28), *Anateris* spp. (2n = 12–14), *Reddyanus* spp. (2n = 15–17)] (Mattos et al. 2013, Sadílek et al. 2015, Kovařík et al. 2016a, Lowe et al. 2018, Ubinski et al. 2018). On the other hand, the genera with diversified karyotypes, such as *Babycurus* (2n = 16–30), *Parabuthus* (2n = 18–30) or *Tityus* (2n = 5–32) are characterized by remarkably polymorphic karyotypes including at the intraindividual level (Schneider et al. 2009, Mattos et al. 2013, 2018, Ojanguren-Affilastro et al. 2017).

In general, buthid scorpions exhibit an exceptionally high tolerance to structural changes at the chromosomal level. Male chromosome heterozygosity stemming from translocation and fusion/fission events has been observed across the buthid genera with a high frequency (Shanahan 1989a, Mattos et al. 2013, 2018, Adilardi et al. 2015, 2016). Among the species studied, the numbers of multivalent elements varied from commonly three (e.g. species) up to 28 (*Jaguajir agamemnon*). Even extreme cases in which the heterozygous chromosome chains comprised all the elements of the diploid set were detected in some buthid species (Mattos et al. 2013). Presence of intraspecific chromosomal heterozygosity in buthid males usually results

in a polymorphism in chromosome numbers, and/or in numbers and types of multivalent chains (Mattos et al. 2018). Although chromosomal heterozygosity is a well-documented phenomenon in buthids, integrative studies examining the correlations between chromosomal and genetic variation of the species studied are lacking. Moreover, *Tityus*, owing to its extraordinary degree of chromosomal polymorphism, is a much sought-after and, by far, most frequently studied scorpion group from the cytogenetic point of view. Thus, the knowledge of karyotype variation and mechanisms of its origin remain poorly understood in two thirds of the buthid genera.

Genus *Gint* Kovařík et al., 2013, with twelve species, represents a small group of buthid scorpions with a distribution restricted to the Horn of Africa (Kovařík and Lowe 2019). In taxonomic studies of the genus, the description of four species has been supplemented with information on the diploid numbers of chromosomes [*G. amoudensis* 2n = 35–36, *G. dabakalo* 2n = 23, *G. gaitako* 2n = 30, *G. maidensis* 2n = 34] (Kovařík et al. 2013, Kovařík and Mazuch 2015, Kovařík et al. 2018a). These findings have indicated the presence of both inter- and intraspecific chromosome variation in this scorpion group. In the species studied, the cytogenetic analyses were usually performed on mitotic metaphases, and, to date, there is no information on the appearance and configuration of *Gint* meiotic chromosomes. Meiotic chromosome configuration has been documented in only a single *G. gaitako* specimen, showing the association of four chromosomes forming a quadrivalent (IV) in metaphase I (Kovařík et al. 2013). However, it is not yet known whether the heterozygous chromosome chain is fixed in *G. gaitako* population, or if there is still undiscovered chromosome variability.

The present study follows up on pilot cytogenetic analyses and integrates cytogenetic approaches with sequence-based phylogenetic reconstruction in order to elucidate characteristics of karyotype differentiation in *Gint*. We investigated the meiotic chromosomes of six *Gint* species with emphasis on the variability in meiotic multivalent configurations, and distributions of 18S rDNA loci, and TTAGG telomeric repeats. This allowed us to determine the extent of *Gint* karyotype polymorphism and the types of structural rearrangements involved in generating karyotype variability. In the next step, we analyzed species genetic differentiation and reconstructed the phylogeny of *Gint* with the use of 16S rDNA and COI (Cytochrome c oxidase I) gene markers.

This integrated approach, combining cytogenetic and sequence-based data, enabled us to assess correlations between genetic and karyotype variability at inter- and intraspecific level and to gain insights into details of species differentiation in *Gint*.

Material and methods

Species sampling

We examined seven scorpion species belonging to *Gint*, an endemic genus of the Horn of Africa. In six species, we obtained a total of 22 specimens from 8 localities (Fig. 1) for integrative studies combining cytogenetic data, DNA barcoding and phylogenetic reconstruction. One specimen of *G. calviceps* for which cytogenetic analysis could not be performed was included in the phylogenetic reconstruction of the genus.

G. amoudensis Kovařík, Lowe, Just, Awale, Elmi & Šťáhlavský, 2018: Somaliland: Borama, Amoud University campus (09°56'49"N 43°13'23"E), 1 male; Laas Gel, 50 km NE Hargeisa (09°46'47"N 44°26'43"E), 3 males.

G. banfasae Kovařík & Lowe, 2019: Somaliland: Shanshade vill. (08°39'35"N 45°55'49"E), 5 males.

G. calviceps (Pocock, 1900): Somaliland: Toon village near Hargeisa (09°23'30"N 44°07'10"E), 1 male.

G. dabakalo Kovařík & Mazuch, 2015: Somaliland: Burao (09°31'51"N 45°33'15"E), 1 male; N of Burao, Togdheer (09°33'24"N 45°31'58"E), 2 males.

G. gaitako Kovařík, Lowe, Plíšková & Šťáhlavský, 2013: Ethiopia: Oromia State, Sidamo Province, Wachile (04°32'33"N 39°03'07"E), 6 males.

G. gubanensis Kovařík, Lowe, Just, Awale, Elmi & Šťáhlavský, 2018: Somaliland: Gerissa, N of Borama (10°36'01"N 43°26'07"E), 1 male.

G. maidensis Kovařík, Lowe, Just, Awale, Elmi & Šťáhlavský, 2018: Somaliland: Maid (11°00'03"N 47°06'30"E), 3 males.

Chromosome preparations and karyotype analysis

Chromosome slides were prepared from male gonads according to the procedure described by Plíšková et al. (2016). In brief, the dissected tissue was subjected to the following steps: (1) hypotonic treatment (0.075 M KCl) for 20 min, (2) fixation in a methanol:acetic acid solution (3:1) for 30 min, (3) maceration in a drop of 60% acetic acid, (4) spreading of the cell suspension on slides on a heating histological plate at 45°C. The chromosome preparations for standard karyotype analyses were stained with 5% Giemsa solution in Sörensen phosphate buffer (pH 6.8) (20 min). Unstained slides selected for the FISH technique were dehydrated with an ethanol series (70, 80 and 96%; 1 min each), air-dried and stored at -20°C.

Fluorescence *in situ* hybridization with 18S rDNA and telomeric probes

We examined the topology of the 18S rRNA sites and the telomeric motif, (TTAGG)n, in all individuals with a distinct karyotype macrostructure. The unlabeled 18S rDNA gene probe was prepared by the polymerase chain reaction (PCR) using the genomic DNA of the scorpion species *Euscorpius sicanus* (Koch, 1837) (GenBank accession number MG761815). PCR was performed with primers 18S-Gal forward (5'-CGAGCGCTTTTATTAGACCA-3') and 18S-Gal reverse (5'-GGTTCACCTACGGAAACCTT-3') according to Fuková et al. (2005). The probe was labelled with biotin-14-dUTP by nick translation using a kit (Nick Translation Kit, Abbott Molecular). The telomeric probe (TTAGG)n was prepared by non-template PCR using primers 5'-TAGGTTAGGTTAGGTTAGGT-3' and 5'-CTAACCTAACCTAACCTAAC-3' and labelled with biotin-14-dUTP as described by Sahara et al. (1999).

The FISH protocol for both of the probes was identical and followed Forman et al. (2013). Briefly, chromosome preparations were pretreated with RNase A (100 µg/ml in 2×SSC [saline-sodium citrate buffer]) for 60 min and washed twice in 2×SSC for 5 min. Afterwards the slides were denatured at 68°C for 3 min 30 s in 70% formamide in 2×SSC, and immediately dehydrated in a cold ethanol series (70, 80 and 96%; 1 min each). Each slide was treated with a denatured probe mixture containing 20 ng of the probe, 25 µg of salmon sperm DNA in 10µl of 50% deionized formamide, and 10% dextran sulphate in 2×SSC. After overnight hybridization in a dark chamber at 37°C, Cy3-conjugated streptavidin was applied to the slides, followed by a dose of biotinylated antistreptavidin and one more treatment with Cy3-conjugated streptavidin. The slides were counterstained with DAPI (4',6-diamidino-2-phenylindole) contained in Fluoroshield^{TM} (Sigma Aldrich).

Documentation and karyotype analysis

Giemsa stained/FISH chromosomes were documented through an Olympus IX81 microscope equipped with an ORCA-AG monochromatic charge-coupled device camera (Hamamatsu). The FISH images were pseudocolored (blue for DAPI, red for Cy3) and superimposed using ImageJ (v1.45r) (Schneider et al. 2012) or Cell^R software (Olympus Soft Imaging Solutions GmbH). For each specimen with distinct karyotype macrostructure, ten spermatocyte nuclei were selected for a karyotype analysis. The chromosome measurements of relative diploid set length (DSL) were carried out using ImageJ v1.45r (Schneider et al. 2012) with the plugin Levan (Sakamoto and Zacaro 2009). The position of 18S rDNA gene sites was given in % of DSL, expressing the distance between the 18S rDNA gene site and the closest chromosome terminal.

205

206 **Barcoding and phylogenetic analyses**

207 A total of 23 individuals were used in the present study to determine the genetic variation and
208 phylogenetic relationships of the *Gint* species. The sample set included six *Gint* species and
209 their cytotypes (22 individuals in total), and one specimen of *G. calviceps* lacking cytogenetic
210 data. Three *Neobuthus* species were selected for phylogenetic reconstruction as outgroup taxa.
211 The list of *Gint* and *Neobuthus* specimens analyzed in the present study with sample ID, GPS,
212 and GenBank accession codes is given in Table S1.

213 Genomic DNA was isolated from leg muscle tissue using the Tissue Genomic DNA kit
214 (Geneaid) following the manufacturer's protocol. Partial fragments of two mitochondrial genes,
215 a barcode locus cytochrome oxidase subunit I (COI; ca. 660 bp) and the ribosomal 16S rRNA
216 gene (16S; ca. 370 bp), were amplified by PCR with the primer pairs LCO1490 (5'-
217 GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-
218 TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) for COI, and 16Sbr (5'-
219 CGATTTGAACTCAGATCA-3') (Simon et al. 1994) and 16SR (5'-
220 GTGCAAAGGTAGCATAATCA-3') (Gantenbein et al. 1999) for 16S. The PCR protocol,
221 identical for both the gene loci, consisted of initial denaturation step at 95°C for 5 min, followed
222 by 36 cycles of denaturation at 95° for 30 s, annealing at 45°C for 1 min, extension at 72°C for
223 1 min, and terminated by final extension at 72°C for 10 min.

224 Sequences were checked for quality and manually edited using Geneious R11 (Kearse et al.
225 2012). Edited sequences of the two gene loci were aligned in MAFFT v.7 (Katoh and Standley
226 2013), using the G-INS-i strategy recommended for less than 200 sequences with global
227 homology and the 1PAM/K = 2 matrix parameter for aligning DNA sequences of closely
228 related taxa. Gap opening penalty GOP=1.53 and offset value 0.0 were default. Terminal
229 regions of various lengths have been trimmed in both alignments, which shortened their length
230 to 357 bp in 16S and 605 bp in COI. Sequences for protein-coding COI were examined after
231 translation into amino acids with the invertebrate mitochondrial genetic code. No stop codons
232 were detected.

233 To estimate the genetic variation among samples, the MEGA v.7 program (Kumar et al.
234 2016) was used to compute (i) variable, conserved and parsimony informative sites for both
235 gene loci, (ii) inter- and intraspecific pairwise sequence divergence using uncorrected pairwise
236 genetic distances (uncorrected *p*-distances) for COI data. To visualize haplotype diversity and
237 mutation steps between haplotypes, a haplotype network based on COI was constructed using

the TCS network method implemented in PopART v.1.7 (http://popart.otago.ac.nz, Leight and Bryant 2015).

All individuals used in phylogenetic reconstructions represented unique mtDNA haplotypes. Prior to phylogenetic analyses, aligned sequence data from the two gene loci were concatenated, and the best-fit models of nucleotide evolution were calculated for each of the genes and codon position (COI) in PartitionFinder v.1.1 (Lanfear et al. 2012) under the Bayesian information criterion (BIC) using the "greedy" search strategy. The best models were HKY+G for 16S and HKY+I for each codon position in COI. Phylogenetic trees were reconstructed using Bayesian inference (BI) and Maximum Likelihood (ML). The BI analysis was conducted using Markov chain Monte Carlo (MCMC) algorithms in MrBayes v.3.2 (Ronquist and Huelsenbeck 2003). The data set was partitioned by gene and codon position (COI) and the Bayesian analyses were run for 10^7 generations with sampling every 1000 generations to estimate the posterior probability. The consensus tree was calculated after the first 2500 sampled trees were discarded as a burn-in. The ML analysis was conducted in raxmlGUI 1.3 (Silvestro & Michalak 2012) using a non-parametric bootstrap analysis with 1000 replications to estimate branch support. The analysis was run with the GTR+G model applied to all partitions which were the same as in the Bayesian analysis. The final BI and ML trees were visualized in FigTree v. 1.4.3 (Rambaut 2016).

Results

Karyotype analysis

We investigated karyotypes of six *Gint* species with emphasis on meiotic chromosome configurations, 18S rDNA, and telomeric TTAGG repeat distributions. Overall, the species analyzed showed interspecific chromosome variability ranging from 2n = 18 to 2n = 45. Intraspecific chromosome polymorphism was documented in three species, resulting in delimitation of three cytotypes each in *G. dabakalo*, *G. banfasae*, and four cytotypes in *G. amoudensis*.

The multivalent meiotic configuration was present in karyotypes of all species examined, except *G. maidensis* (Fig 2). The number of chromosomes involved in multivalents was either three, four, five or six forming typical trivalent (III), quadrivalent (IV), pentavalent (V) or hexavalent (VI) configurations in post-pachytene I, respectively (Figs 2a–n, 3). The intraspecific variation of multivalent association was found in three species, *G. banfasae*, *G. dabakalo* and *G. amoudensis* (Figs 2a–l, 3a–h).

The number of 18S rDNA loci was predominantly two per 2n, with exceptionally three loci per 2n found in two cytotypes of *G. amoudensis* (Figs 2, 3, S2). The 18S rDNA sites were usually located at subterminal or terminal region of a large chromosome pair, which was often involved in the multivalent (Figs 2b,d,fj,l, 3b,f,j,l). Sometimes the loci were visible on Giemsa stained chromosomes as heterochromatic knobs (Figs 2o, 3k). Surprisingly, *G. gubanensis* showed remarkable size heteromorphism of 18S rDNA sites (Fig. 3j).

The FISH visualization of the telomeric probe revealed no interstitial sites of (TTAGG)n motifs on the chromosomes, not even in species with frequent multivalent associations. All telomeric signals were located in the terminal regions of each chromosome (Fig. 4).

G. banfasae

In this species, we detected intrapopulation variability in diploid number and/or multivalent associations showing the presence of three cytotypes. The position of a single pair of 18S rDNA sites as well as the 18S rDNA bearing chromosome pair involved in the multivalent chain were consistent in all cytotypes.

Cytotype I had 2n = 18 chromosomes which decreased in length from 9.86 % to 1.77 % of DSL (Fig S1). In post-pachytene nuclei, the chromosomes formed seven bivalents and one quadrivalent that was composed of the four largest chromosomes (Fig. 2a). A single pair of 18S rDNA sites was located in the subterminal region (1.13 % of DSL) of the chromosomes Nos. 3 and 4 involved in the quadrivalent (Fig. 2b).

Cytotype II exhibited 2n = 18. Chromosomes decreased in length from 9.79 % to 1.78 % of DSL (Fig. S1). The post-pachytene cells showed six bivalents and one hexavalent (chromosomes Nos. 1, 2, 3, 4, 15 and 18) (Fig. 2c). A pair of 18S rDNA sites was located in the subterminal region (1.08 % of DSL) of the chromosomes Nos. 3 and 4 involved in the hexavalent (Fig. 2d).

Cytotype III had 2n = 19 chromosomes which decreased in length from 9.66 % to 1.78 % of DSL (Fig. 2e, S1). Post-pachytene spermatocytes exhibited five bivalents, one trivalent (chromosomes Nos. 7, 17 and 18) and one hexavalent (chromosomes Nos. 1, 2, 3, 4, 14 and 19). A pair of 18S rDNA sites was situated in the subterminal region (1.12 % of DSL) of the chromosomes Nos. 3 and 4 involved in the hexavalent (Fig. 2f).

G. dabakalo

In this species, three cytotypes were distinguished. The difference in the diploid number, multivalent associations, and the appearance of 18S rDNA-bearing chromosomes were detected at inter- and intrapopulation levels.

Cytotype I had 2n = 23 chromosomes which decreased in length from 7.90 % to 2.55 % of DSL (Fig. S1). The post-pachytene cells showed nine bivalents and one pentavalent that was composed of chromosomes Nos. 1, 4, 5, 10 and 23 (Fig. 2g). The pair of 18S rDNA sites was located in the subterminal region of the largest bivalent (0.63% of DSL) (Fig. 2h).

Cytotype II exhibited 2n = 24. Chromosomes decreased in length from 7.65 % to 2.95 % of DSL (Fig. 2i, S1). The post-pachytene nuclei showed configuration of eight bivalents, one trivalent (chromosomes Nos. 2, 12 and 17) and one pentavalent (chromosomes Nos. 1, 3, 4, 7 and 20). The pair of 18S rDNA sites was situated in the subterminal region (0.85 % of DSL) of chromosomes Nos. 2 and 12 of the trivalent (Fig. 2j).

Cytotype III had 2n = 27. Chromosomes decreased in length from 8.31 % to 1.81 % of DSL (Fig. S1). In the post-pachytene spermatocytes, we observed eight bivalents, two trivalents and one pentavalent (Fig. 2k). The larger trivalent was composed of chromosomes Nos. 2, 9 and 10, the smaller trivalent was composed of chromosomes Nos. 4, 22 and 25, and chromosomes Nos. 1, 3, 5, 8 and 17 formed a pentavalent. The 18S rDNA sites were located in the subterminal region of the chromosomes Nos. 2 and 9 of the larger trivalent (0.80 % of DSL) (Fig. 2l).

G. gaitako

All specimens exhibited 2n = 30 chromosomes which decreased in length from 4.65 % to 1.81 % of DSL (Fig. S1). The post-pachytene cells showed eight bivalents and one quadrivalent (chromosomes Nos. 1, 18, 25 and 30) (Fig. 2m, S1). The pair of 18S rDNA sites was located in the terminal region of the third largest bivalent (chromosomes Nos. 6 and 7) (Fig. 2n).

G. maidensis

The karyotype of *G. maidensis* comprised 2n = 34 chromosomes that decreased in length from 4.22 % to 1.87 % of DSL (Fig. S1). The post-pachytene nuclei exhibited 17 bivalents (Fig. 2o). No multivalent associations were found in this species. One pair of 18S rDNA sites was located in the terminal region of the largest bivalent (Fig. 2p).

G. amoudensis

The most striking intraspecific variability was discovered in *G. amoudensis,* which exhibited four cytotypes differing in the diploid number, multivalent associations and in the number and position of 18S rDNA sites. The cytotypes II, III and IV occurred sympatrically, while the cytotype I was found on a remote locality ca. 150 km far from the other cytotypes.

Cytotype I exhibited 2n = 36. Chromosomes decreased in length from 4.50 % to 1.64 % of DSL (Fig. S1). The post-pachytene nuclei showed 16 bivalents and one quadrivalent (chromosomes Nos. 1, 2, 9 and 15) (Fig. 3a). Three 18S rDNA sites were detected (Fig. 3b), two of them were located in the subterminal region of the chromosomes No. 1 (0.97 % of DSL) and No. 2 (0.45 % of DSL) involved in the quadrivalent and one in the terminal position of chromosome No. 2 in the quadrivalent. This situation was confirmed in metaphase II (Fig. S2).

Cytotype II had 2n = 35 chromosomes which decreased in length from 5.01 % to 1.46 % of DSL (Fig. S1). The post-pachytene spermatocytes exhibited 14 bivalents, one trivalent (chromosomes Nos. 4, 24 and 35) and one quadrivalent (chromosomes Nos. 1, 5, 8 and 23) (Fig. 3c). Three 18S rDNA loci were present (Fig. 3d), one pair was located on the chromosomes No. 1 (subterminal site, 0.95 % of DSL) and No. 5 (terminal site) involved in the quadrivalent, and one single heterozygous loci was situated in the interstitial region of the third largest bivalent (chromosome No. 9; 1.23 % of DSL) (Fig. 3d, S2).

Cytotype III had 2n = 36. Chromosomes decreased in length from 4.71 % to 1.52 % of DSL (Fig. S1). The post-pachytene cells showed 15 bivalents and one hexavalent (chromosomes Nos. 1, 2, 5, 6, 11 and 24) (Fig. 3e). One pair of 18S rDNA sites was located in the subterminal region of the chromosomes Nos. 1 and 2 of the hexavalent (1.21 % and 0.85 % of DSL) (Fig. 3f).

Cytotype IV exhibited 2n = 35 chromosomes which decreased in length from 4.67 % to 1.54 % of DSL (Fig. S1). The post-pachytene nuclei showed configuration of 13 bivalents, one trivalent (chromosomes Nos. 7, 26 and 35) and one hexavalent (chromosomes Nos. 1, 4, 5, 6, 10 and 23) (Fig. 3g, Fig. S1). One pair of 18S rDNA sites was located in the subterminal region of the chromosomes Nos. 1 and 4 of the hexavalent (0.50 %, and 1.39 % of DSL) (Fig. 3h).

G. gubanensis

Karyotype of *G. gubanensis* comprised 2n = 45 chromosomes which decreased in length from 2.97 % to 1.02 % of DSL (Fig. S1). Overall, 21 bivalents and a single trivalent, composed of chromosomes Nos. 7, 33 and 45, were detected in post-pachytene nuclei (Fig. 3i, Fig. S1). One

pair of 18S rDNA loci was found on a terminal part of the heteromorphic chromosome pair (chromosomes Nos. 26 and 44). The overhanging part of chromosome No. 26 (the larger chromosome of the heteromorphic pair) was formed by a single large 18S rDNA site (Fig. 3j).

mtDNA diversity and phylogeny

The sequences for both 16S and COI were obtained from 21 *Gint* individuals. In *G. calviceps*, only 16S gene fragment was successfully sequenced. The final alignment with a total length of 963 bp (16S - 358 bp, COI - 605 bp) consisted of 765 conserved sites, 194 variable sites and 172 parsimony-informative sites.

Based on COI data, *Gint* exhibited low genetic variation at both inter- and intraspecific levels, with the pairwise genetic distances ranging from 0.31 to 1.2% between species, and from 0.0 to 0.30% within species (Table S2). *Gint* included 15 COI haplotypes in total and all cytogenetically examined species, except *G. gubanensis* with only a single specimen analysed, were represented by more than one haplotype (Table S3, Fig. S3). Within the species studied, *G. banfasae* showed the highest within-location haplotype diversity (four haplotypes per locality), however with minor pairwise genetic differences (p-distances: 0.07%). In this species, the relationships between haplotypes were not fully congruent with cytogenetic data, as individuals of both cytotype I and cytotype III did not share the same haplotype. Specimens of cytotype III [S1530] and cytotype II [S1532] formed one haplogroup, whereas specimen of cytotype III [S1533] represented a separate haplotype distant from the haplogroup by one mutational step (Fig. S3). Specimens of cytotype I [S1531 and S1534] forming unique haplotypes were distant from one another by four mutational steps. *G. dabakalo*, comprising three haplotypes, showed the highest intraspecific genetic variation with p-distances: 0.30%. In this species, the genetic distance between individuals of cytotype I and cytotype II from the same locality was higher than between each of them and a specimen of cytotype III from a distinct locality (Table S1, Fig. S3). *G. maidensis*, a species with a stable karyotype 2n = 34, was represented by three haplotypes with p-distances of 0.06%. *G. gaitako* consisted of two haplotypes, where one haplogroup, formed by five specimens, was distant from the other by one mutational step (p-distance: 0.04%). In *G. amoudensis*, individuals of cytotype II, cytotype III and cytytotype IV shared the same haplotype, whereas specimen of cytotype I differed from this haplogroup by 8 point mutations (p-distance: 0.15%).

Both BI and ML mtDNA analyses provided similar tree topologies and nodal supports for clades. Phylogenetic reconstruction depicted *Gint* as a monophyletic group (Fig. 5). Most of the *Gint* species formed well supported clades. The monophyly of the *G. banfasae* specimens

remained unresolved due to low support values. *G. maidensis* formed an early branching clade which was sister to the clade comprising all of the remaining *Gint* species. This clade was further subdivided into two groups: (i) a clade, in which *G. gubanensis* was a sister species to *G. amoudensis*; (ii) a clade consisting of *G. gaitako*, *G. calviceps*, *G. dabakalo*, and *G. bafasae*. In the latter, *G. gaitako* formed a sister lineage to the clade comprising *G. calviceps*, *G. dabakalo*, and *G. bafasae*. The mutual relationships between the species in this group remained unresolved due to low support values.

Discussion

The present study integrates the cytogenetic and molecular approaches in *Gint*, a scorpion genus endemic to the Horn of Africa, elucidating the fundamental mechanisms of karyotype differentiation within this group. Linking the cytogenetic and sequence-based data when addressing the aspects of karyotype evolution in species of interest has been underused in the scorpion order. However, this approach seems to be beneficial for better understanding the observed chromosomal polymorphism or polytypism in scorpion taxa, in which morphological uniformity commonly hampers species delimitation (Štundlová et al. 2019). Buthidae represents the most frequently studied scorpion group from the cytogenetic point of view (Schneider et al. 2019). Concurrently, its representatives are well-known for the presence of chromosomal polymorphism (e.g. Shanahan 1989, Mattos et al. 2013, 2018). Despite this fact, the examination of the genetic structure of polymorphic species and their populations holds great potential that is not yet fully exploited. For this reason, we conducted the current comparative study focused on both the chromosomal and genetic structure of *Gint* species.

Based on present knowledge, buthid genera differ in the degree of karyotype differentiation. Nevertheless, lineages with stable or slightly variable interspecific chromosome numbers seem to be more abundant. The range of chromosome numbers observed in *Gint*, 2n = 18–45 (Kovařík et al. 2013, 2018a; present study), thus ranks among the greatest within the family Buthidae. A similarly diversified karyotype macrostructure has also been detected in other buthid genera inhabiting the Afrotropical region, e.g. *Parabuthus* [2n = 18–36] (Newlands & Martindale 1980, Kovařík et al. 2016b) *Uroplectes* [2n = 20–48] (Newlands & Martindale 1980, Kovařík et al. 2016c) or *Babycurus* [2n = 16–30] (Kovařík et al. 2018b).

A higher degree of karyotype differentiation in *Gint* is apparent not only at the level of interspecific chromosome number variation but also in the incidence of chromosomal rearrangements in the heterozygous state within individual species. Frequent occurrence of chromosomal heterozygosity in males is a characteristic cytogenetic attribute of buthid

scorpions (Mattos et al. 2013, Adilardi et al. 2016, Mattos et al. 2018). For the species investigated herein, the absence of complex multivalent associations was observed only in *G. maidensis*. Of the total number of individuals of all other *Gint* species analyzed, 85 % were heterozygous for one or multiple chromosome rearrangements. Our results show that the frequency and complexity of heterozygous chromosomes differ among *Gint* species and are related to the extent of observed intraspecific chromosomal polymorphism (summarized in Fig. 5). *G. gaitako*, having stable 2n = 30, showed simple interchange heterozygosity since all individuals were carriers of the quadrivalent. The multivalent exhibited a strikingly similar appearance among individuals, which indicates its shared origin in *G. gaitako*. Taking into account that specimens were collected in different years, it is reasonable to assume the heterozygous quadrivalent successfully maintains in the population. However, the question of whether the interchange heterozygosity is fixed in the *G. gaitako* population could be elucidated by extensive sampling. Unlike to *G. gaitako*, karyotypes of other species, *G. banfasae*, *G. dabakalo* and *G. amoudensis*, are characterized by accumulation of distinct types of heterozygous chromosomes and by intraspecific differences in chromosome numbers. Individuals of these species generally showed complex meiotic configurations consisting of three- to six- chromosome elements and appearing in various combinations (summarized in Fig. 5). Moreover, different multivalents were observed in *G. banfasae* and *G. amoudensis* even among closely related individuals with the identical chromosome number. An analogous situation was also observed in other buthid species, such as *Tityus clathratus*, *T. mattogrossensis* and *T. pusillus* (Mattos et al. 2018). These findings point out the importance of analyzing meiosis I nuclei, which may reveal the presence of interchange heterozygosity in buthid scorpions. It should be noted that the chromosomal variability described above probably represents only a piece of the puzzle. With regard to the limited sampling set, we expect that the actual extent of intraspecific chromosomal polymorphism in *G. banfasae*, *G. dabakalo* and *G. amoudensis* might be even greater.

In the genus *Gint*, species boundaries based on morphology are in agreement with observed interspecific karyotype characteristics and are also supported by phylogenetic reconstruction. The chromosomal features may be of taxonomic significance in this scorpion group. However, due to the presence of chromosomal polymorphism, such cytogenetic data should, in any case, be combined with other data sources. Interestingly, conspicuous interspecific differences in chromosome counts contrast with the shallow genetic divergence among the *Gint* species (Table S2). Sequence-based analyses helped us to evaluate the overall chromosomal variability

and to verify whether heterozygous individuals were assigned to given species. In this regard, we did not detect any discrepancies between outcomes of taxonomic, cytogenetic and genetic approaches.

A high incidence of chromosomal heterozygosity in *Gint* points to the ongoing structural changes in genome organization of species of interest. We assume that observed multivalents have originated from distinct types of rearrangements, as depicted in Fig. 6. It follows that major structural mechanisms altering *Gint* species genome makeups seem to be chromosomal fusions, fission and reciprocal translocations. Such types of chromosomal rearrangements were observed in the heterozygous condition across Buthidae (Mattos et al. 2013, Šťáhlavský et al. 2014, Sadílek et al. 2015, Adilardi et al. 2016, Mattos et al. 2018) and seem to be a shared aspect of karyotype differentiation for the whole family.

The widely studied chromosome region-specific markers in scorpions is nucleolus organizer region (NOR), a site of tandemly arrayed major ribosomal RNA genes (45S = 28S, 5.8S a 18S). The majority of data regarding the rDNA distribution is available for buthid species inhabiting the Neotropical region, e.i. *Ischnotelson*, *Jaguajir*, *Physoctonus*, *Rhopalurus* (Ubinski et al. 2018)*, Tityus* (Adilardi et al. 2016, Mattos et al. 2018, Ojanguren-Affilastro et al. 2017), and *Zabius* (Adilardi et al. 2015). Most of the species exhibit one rDNA bearing chromosome pair with rDNA gene loci located in the terminal region. Rare cases deviating from this general pattern have been subsequently identified, showing either a distinct position of rDNA gene sites as a result of chromosome fusion or having multiple rDNA gene loci.

Fluorescence *in situ* hybridization with a (TTAGG)n probe confirmed that chromosomes bear the telomeric sequences exclusively at the ends of their chromosomes. Interestingly, no interstitial telomeric sequences (ITS) were observed in heterozygous chromosomal associations which apparently resulted from the fusion, i.e. trivalent and pentavalent found in *G. dabakalo* cytotypes. This is not an isolated case within Buthidae, as no interstitial telomeric sequence regions were also present in fusion heterozygotes in other species (de Almeida et al. 2017). Absence of visualized interstitial telomeric sequences on fused chromosomes may be a result of partial or total reduction of regions with TTAGG repeats

We assume that observed heterozygous chromosomes in *Gint* resulted from distinct types of interchromosomal rearrangements as follows: (i) reciprocal translocations gave rise to the quadrivalent in *G. gaitako*, *G. banfasae* (Fig. 6b,d), and the hexavalent in *G. amoudensis* (Fig. 6f); (ii) simple fusions/fissions resulted in trivalents in *G. banfasae*, *G. dabakalo* and *G. amoudensis* (Fg. 6a); (iii) step-by-step multiple fusion led to the pentavalent formation in *G.*

dabakalo (Fig. 6c); (iv) a fission of the chromosome involved in the quadrivalent, followed by fusion of emerged chromosome fragment with another chromosome in *G. banfasae* (Fig. 6e). Reciprocal translocations as well as fusions/fission in heterozygous state, as reported herein in *Gint*, are well documented across various buthid genera and assumed to be the primary mechanism of genome reorganization in this scorpion family.

In *G. banfasae*, the cytotype I with 2n = 18 – 7II + IV appears to represent the karyotype from which closely related cytotypes are derived. Cytotype II [2n = 18 – 6II + VI] and cytotype III [2n = 19 – 5II + III + VI] are carriers of the hexavalent, which likely arose from the fusion of the smallest bivalent with the quadrivalent (Fig. 6d). Furthermore, cytotype III having a trivalent showed heterozygosity for chromosome fission. Both chromosome aberrations led to a decrease in bivalent counts in given cytotypes.

Acknowledgements

We are indebted to Hargeisa University and Amoud University, especially their representatives Hassan Sh Abdirahman Elmi and Ahmed Ibrahim Awale for facilitating field collecting in the Republic of Somaliland. We are grateful to Tomáš Mazuch for help with sampling and organizing the collecting trips. Thanks are due to William Bourland for proofreading the manuscript.

Funding information

This study was supported by Grant Agency of the Charles University (GA UK 1324217) and by Ministry of Education, Youth and Sports of the Czech Republic (SVV 260 434/2019).

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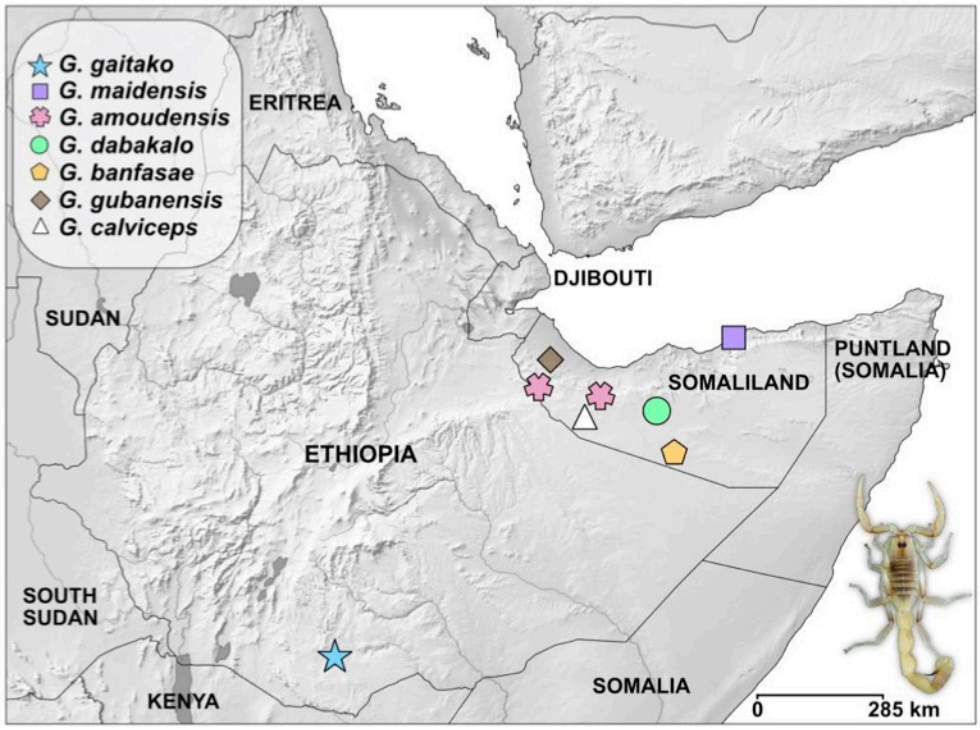
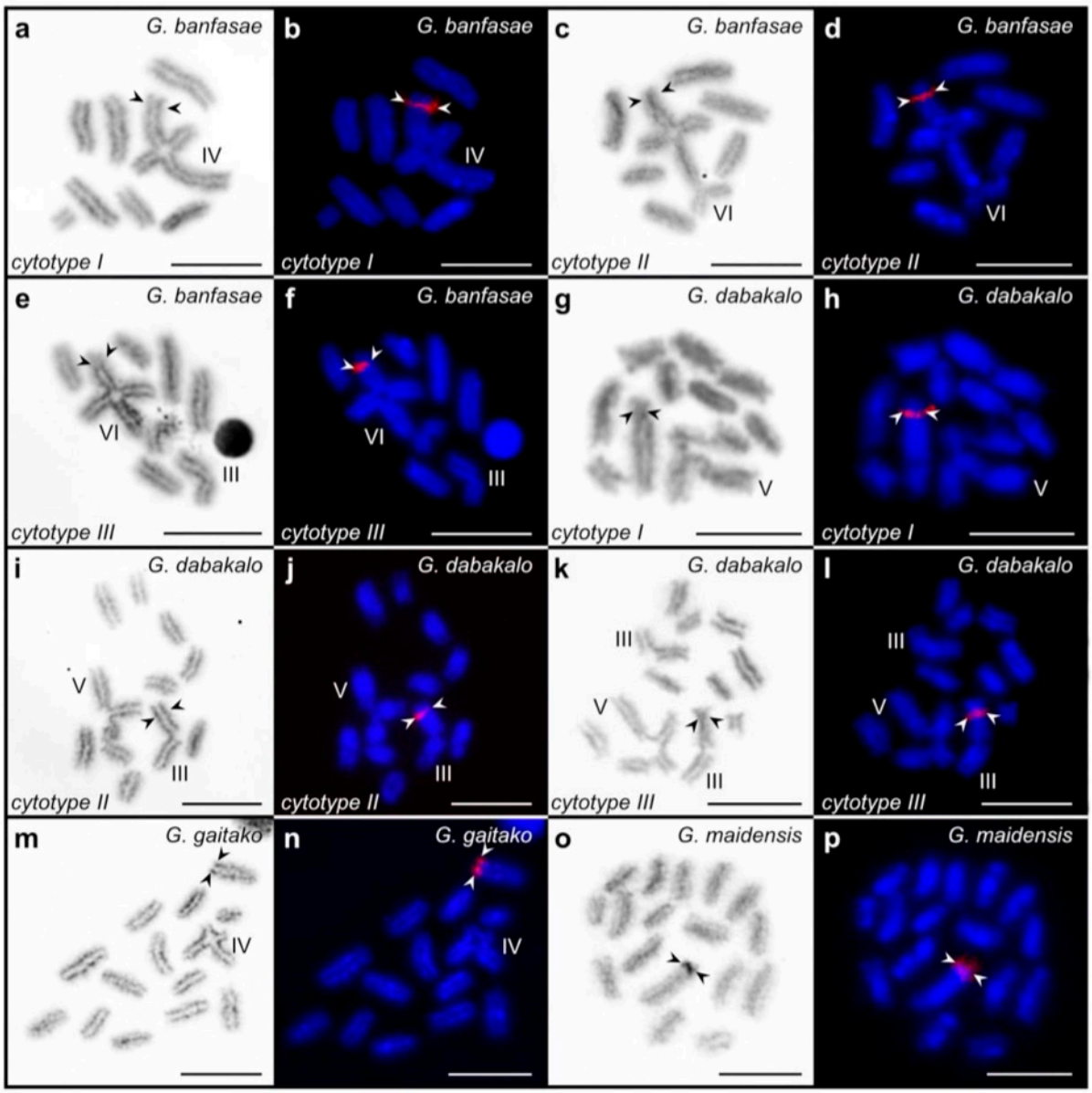


Figure 1. Map showing distribution of the sampled *Gint* spp. Bottom right – *G. amoudensis*.



704

705 **Figure 2.** Post-pachytene cells of *Gint* spp. after Giemsa staining (**a**, **c**, **e**, **g, i, k, m, o**) and
706 FISH with 18S rDNA (red signals) (**b**, **d**, **f**, **h, j, l, n, p**). **a**, **b** *Gint banfasae* cytotype I (2n = 18
707 – 7II + IV). **c**, **d** *Gint banfasae* cytotype II (2n = 18 – 6II + VI). **e**, **f** *Gint banfasae* cytotype III
708 (2n = 19 – 5II + III + VI). **g**, **h** *Gint dabakalo* cytotype I (2n = 23 – 9II + V). **i**, **j** *Gint dabakalo*
709 cytotype II (2n = 24 – 8II + III + V). **k**, **l** *Gint dabakalo* cytotype III (2n = 27 – 8II + 2III + V).
710 **m**, **n** *Gint gaitako* (2n = 30 – 13II + IV). **o**, **p** *Gint maidensis* (2n = 34). Abbreviations: III –
711 trivalent; IV – quadrivalent; V – pentavalent; VI – hexavalent. Arrowheads indicate the position
712 of 18S rDNA. Scale bar = 10 μm

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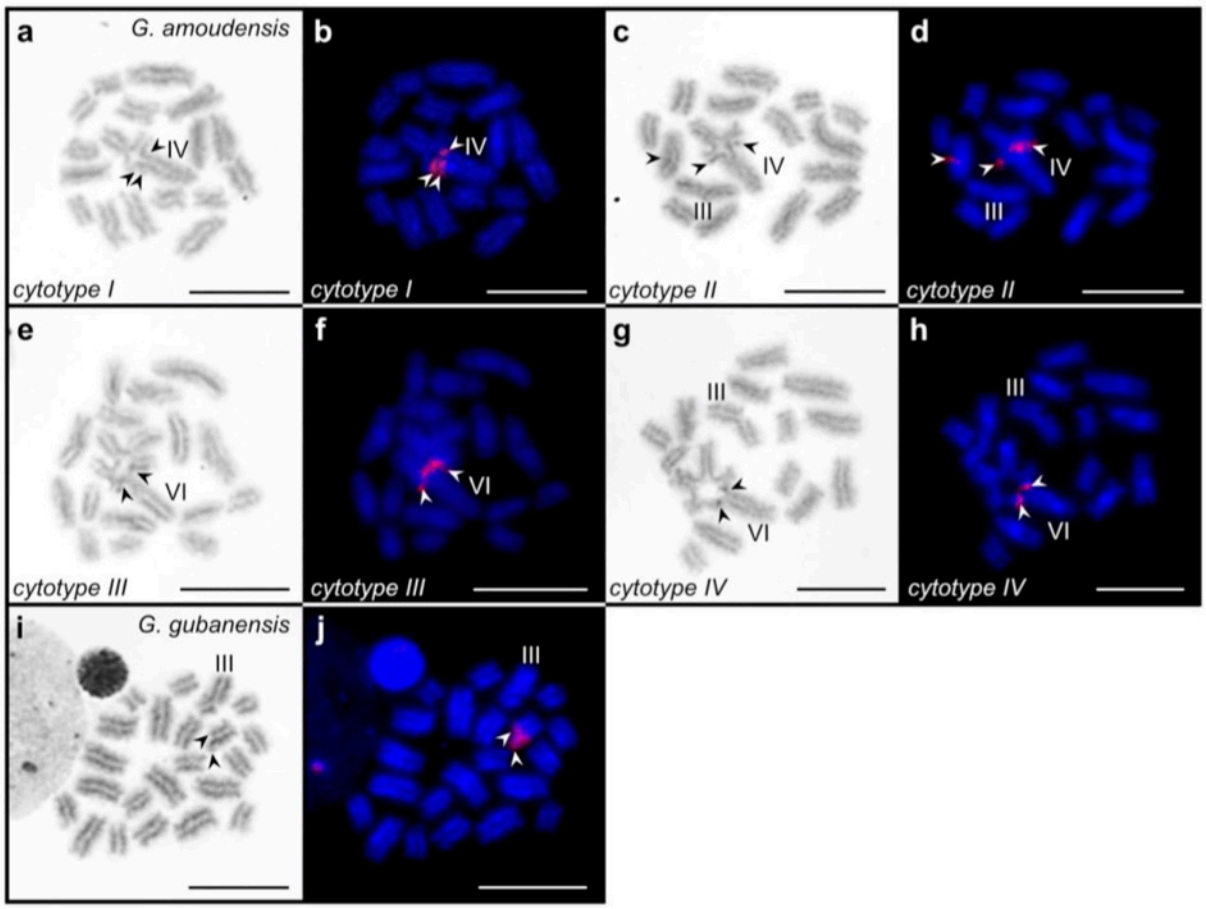
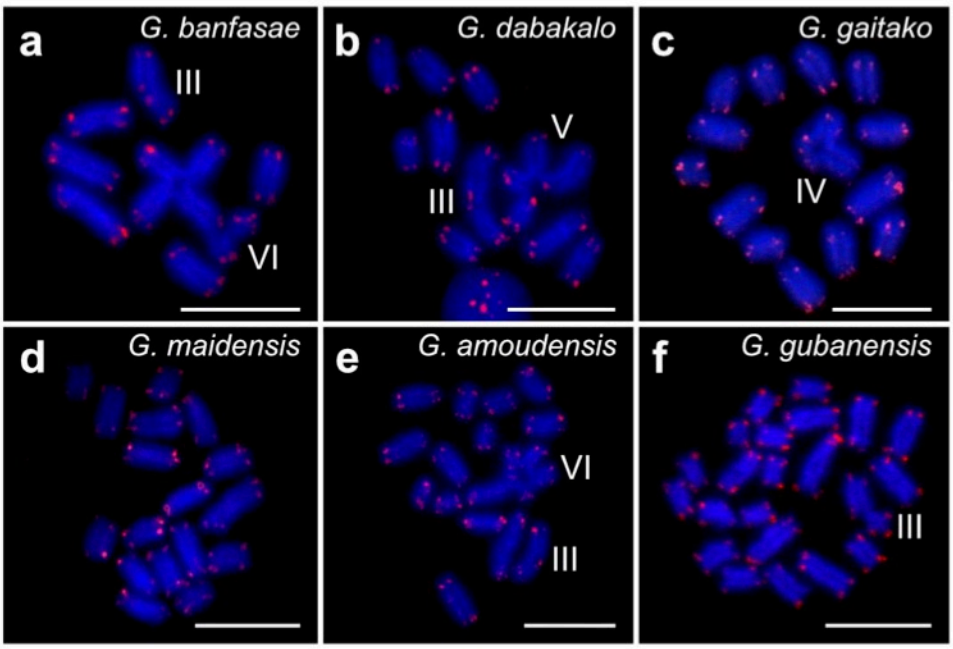
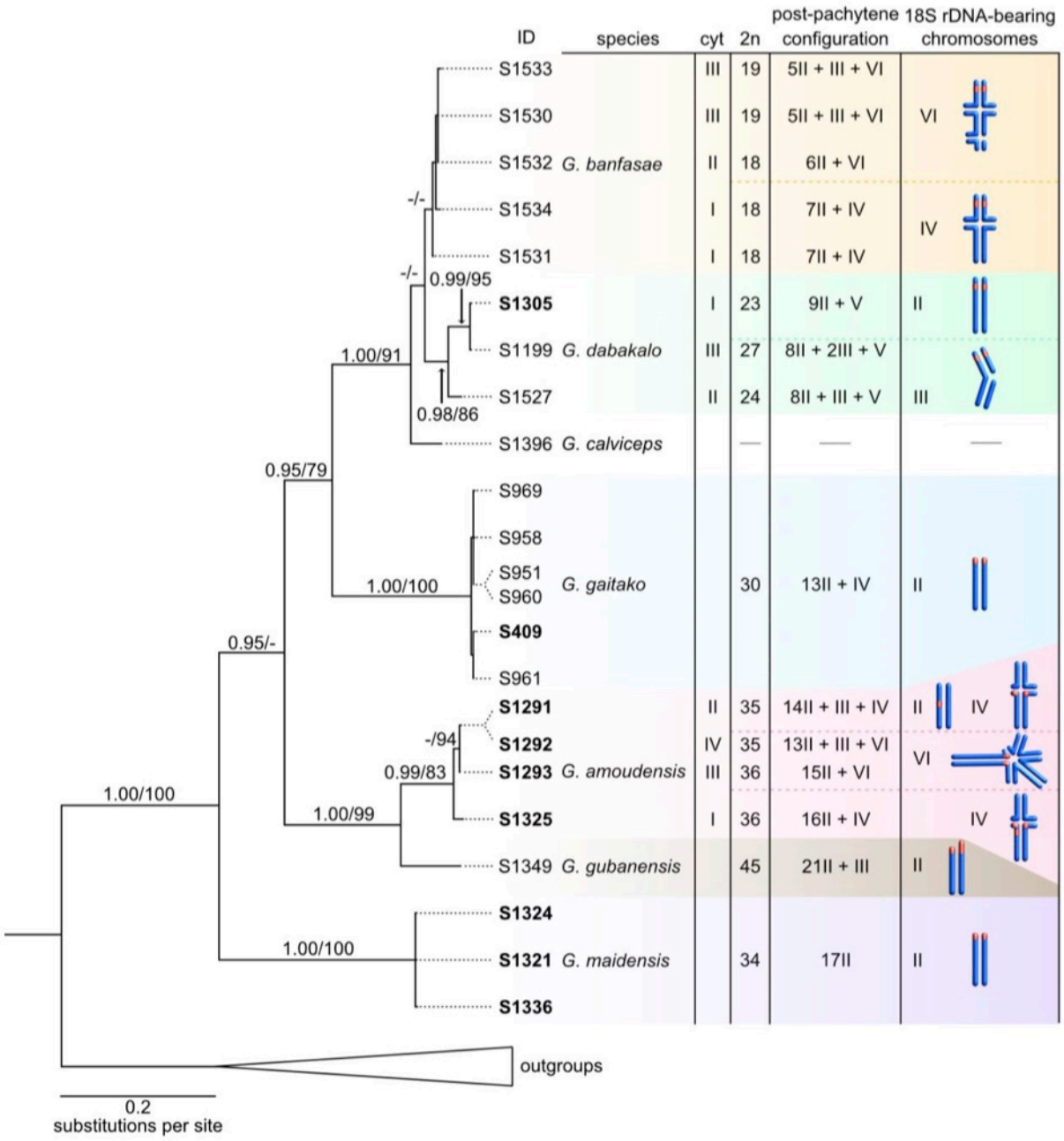


Figure 3. Post-pachytene cells of *Gint amoudensis* and *G. gubanensis* after Giemsa staining (**a**, **c, e, g, i**) and FISH with 18S rDNA (red signals) (**b**, **d**, **f**, **h**, **j**). **a, b** *G. amoudensis* cytotype I (2n = 36 – 16II + IV). **c, d** *G. amoudensis* cytotype II (2n = 35 – 14II + III + IV). **e, f** *G. amoudensis* cytotype III (2n = 36 – 15II + VI). **g, h** *G. amoudensis* cytotype IV (2n = 35 – 13II + III + VI). **i, j** *G. gubanensis* (2n = 45 – 21II + III). Abbreviations: II – bivalent; III – trivalent; IV – quadrivalent; VI – hexavalent. Arrowheads indicate the position of 18S rDNA. Scale bar = 10 μm



723 **Figure 4.** Post-pachytene cells of *Gint* spp. after FISH with (TTAGG)n telomeric probe. **a** *G.*
724 *banfasae* cytotype III [2n = 19 – 5II + III + VI]. **b** *G. dabakalo* cytotype II [2n = 24 – 8II + III
725 + V]. **c** *G. gaitako* [2n = 30 – 13II + IV]. **d** *G. maidensis* [2n = 34 – 17II]. **e** *G. amoudensis*
726 cytotype IV [2n = 35 – 13II + III + VI]. **f** *G. gubanensis* [2n = 45 – 21II + III]. Abbreviations:
727 II – bivalent; III – trivalent; IV – quadrivalent; V – pentavalent; VI – hexavalent. Scale bar =
728 10 μm.

729



730

731 **Figure 5.** Maximum likelihood tree of the *Gint* spp. based on the concatenated data set (16S
732 and COI), complemented with summarized cytogenetic data of the species studied. Numbers
733 above branches correspond to values for highly supported nodes as follows: Bayesian posterior
734 probabilities (PP) > 0.95/maximum likelihood bootstrap > 70%. Specimen IDs depicted in bold
735 indicate individuals based on which the chromosome counts were determined for the
736 corresponding *Gint* species in previous studies (see Kovařík et al. 2013, Kovařík and Mazuch
737 2015, Kovařík et al. 2018a). Abbreviations: cyt – cytotype; 2n – diploid number of
738 chromosomes; II – bivalent; III – trivalent; IV – quadrivalent; V – pentavalent, VI – hexavalent.

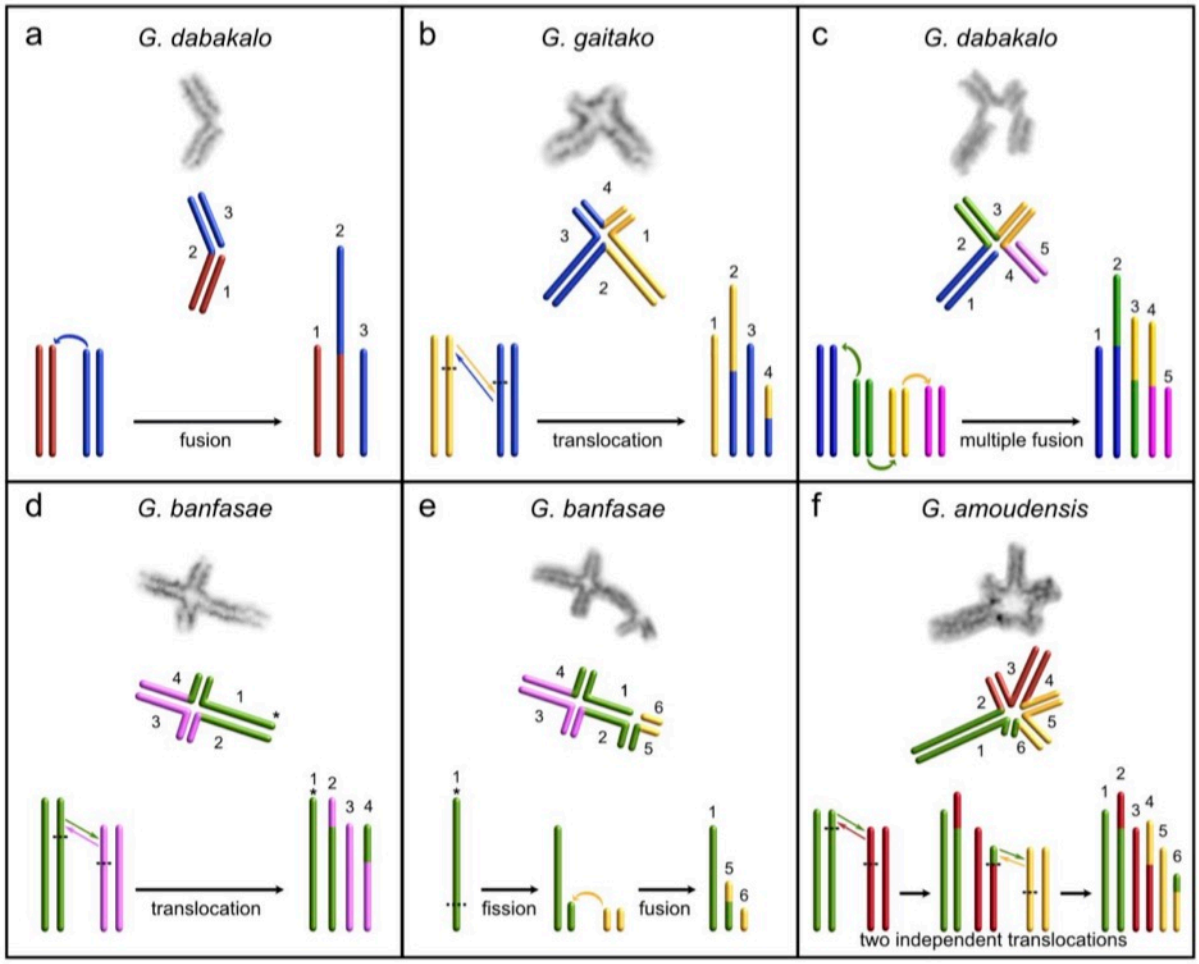


Figure 6. Types of meiotic multivalent associations in *Gint* spp. with schemes of hypothesized interchromosomal rearrangements leading to observed multivalent formation. **a** *G. dabakalo* – a trivalent arising from fusion of two chromosomes. **b** *G. gaitako* – a quadrivalent resulting from reciprocal translocation. **c** *G. dabakalo* – a pentavalent originating from three independent fusion events. **d** *G. banfasae* – a quadrivalent as a result of reciprocal translocation. **e** *G. banfasae* – a hexavalent arising through two independent events: (i) a fission of chromosome 1 involved in quadrivalent (marked by asterisk in **d, e**), (ii) subsequent fusion of emerged chromosome fragment with another chromosome. **f** *G. amoudensis* – a hexavalent originated from two independent reciprocal translocations. Numbers surrounding multivalent denote individual chromosomal elements.

Supplementary files

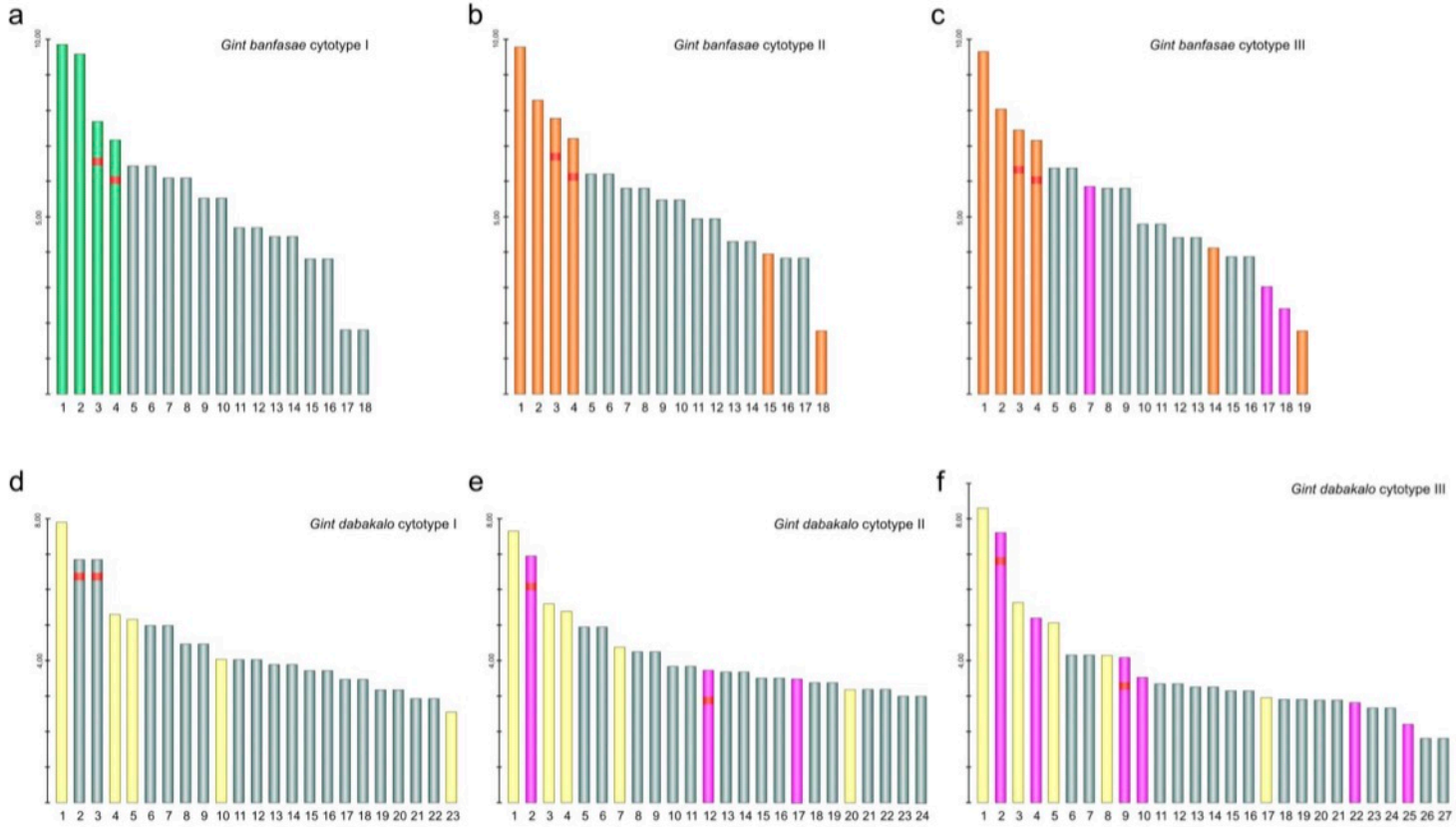


Figure S1. Ideograms of the studied *Gint* spp. showing the relative diploid set length (y axis) and localization of 18S rDNA loci (red signals). **a** *G. banfasae* cytotype I. **b** *G. banfasea* cytotype II. **c** *G. banfasae* cytotype III. **d** *G. dabakalo* cytotype I. **e** *G. dabakalo* cytotype II. **f** *G. dabakalo* cytotype III. **g** *G. gaitako*. **h** *G. maidensis*. **i** *G. amoudensis* cytotype I. **j** *G. amoudensis* cytotype II. **k** *G. amoudensis* cytotype III. **l** *G. amoudensis* cytotype IV. **m** *G. gubanensis*. Grey color – chromosomes forming bivalents; pink color – chromosomes forming trivalents; green color – chromosomes forming quadrivalents; yellow color – chromosomes forming pentavalents; brown color – chromosomes forming hexavalents.

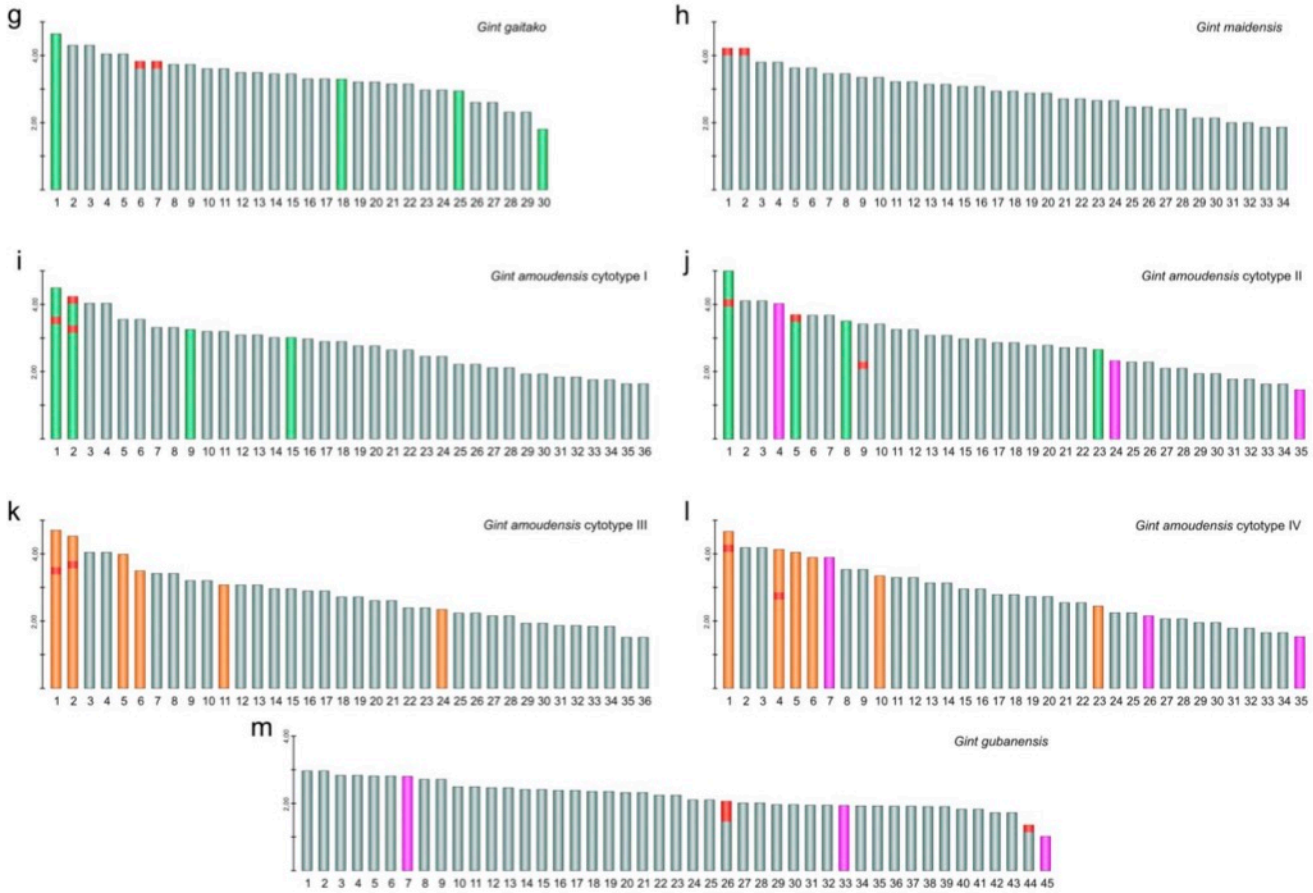


Figure S1. Ideograms of the studied *Gint* spp. showing the relative diploid set length (y axis) and localization of 18S rDNA loci (red signals). **a** *G. banfasae* cytotype I. **b** *G. banfasea* cytotype II. **c** *G. banfasae* cytotype III. **d** *G. dabakalo* cytotype I. **e** *G. dabakalo* cytotype II. **f** *G. dabakalo* cytotype III. **g** *G. gaitako*. **h** *G. maidensis*. **i** *G. amoudensis* cytotype I. **j** *G. amoudensis* cytotype II. **k** *G. amoudensis* cytotype III. **l** *G. amoudensis* cytotype IV. **m** *G. gubanensis*. Grey color – chromosomes forming bivalents; pink color – chromosomes forming trivalents; green color – chromosomes forming quadrivalents; yellow color – chromosomes forming pentavalents; orange color – chromosomes forming hexavalent

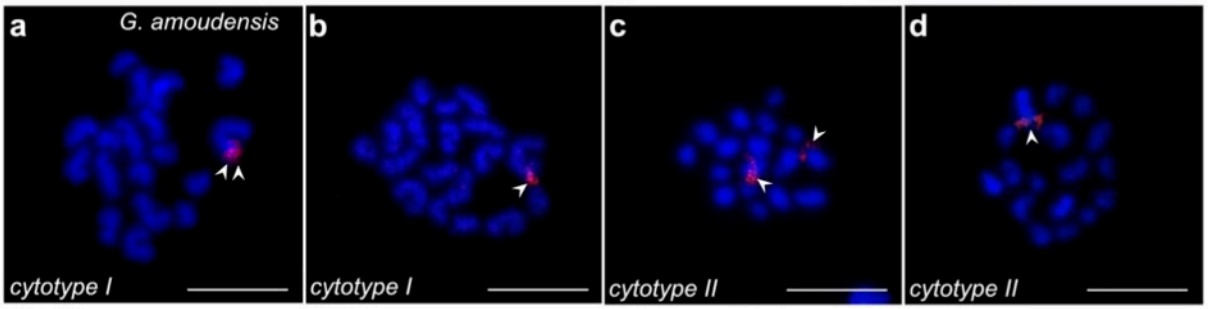
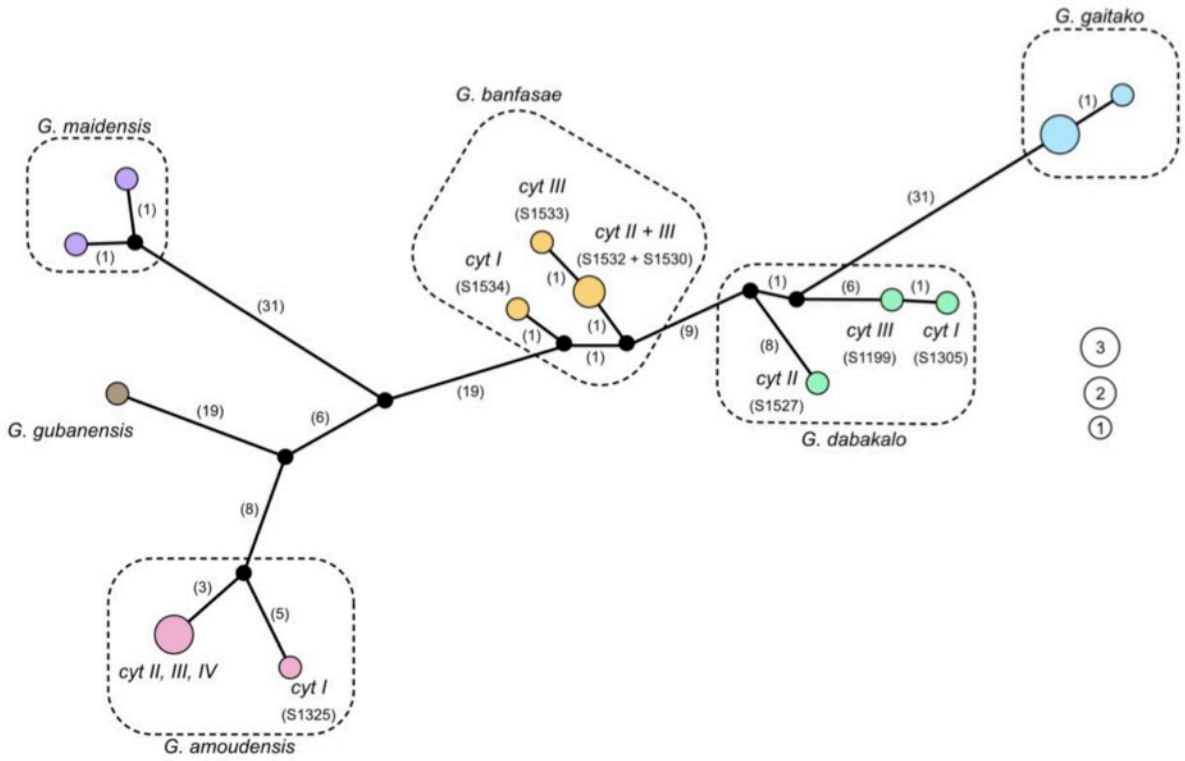


Figure S2. Metaphase II sister cells of *Gint amoudensis* cytotypes after FISH, showing the distribution of 18S rDNA loci (red signals). **a, b** cytotype I. **c, d** cytotype II. Arrowheads indicate the position of 18S rDNA. Scale bar = 10 μm.



766 **Figure S3.** Haplotype network based on COI. Numbers in brackets show mutation steps.
767 Numbers in circles of various size denote number of individuals sharing same haplotype.

768

Table S1. Detailed information and GeneBank accession numbers of specimens used in this study.

ID	species	2n	cyt	country	coordinates	16S	COI
S409	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S951	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S958	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S960	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S961	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S969	*G. gaitako*	30	–	Ethiopia	04°32'33"N 39°03'07"E	XY000000	XY000000
S1199	*G. dabakalo*	27	III	Somaliland	09°31'51"N 45°33'15"E	XY000000	XY000000
S1291	*G. amoudensis*	35	II	Somaliland	09°46'47"N 44°26'43"	XY000000	XY000000
S1292	*G. amoudensis*	35	IV	Somaliland	09°46'47"N 44°26'43"	XY000000	XY000000
S1293	*G. amoudensis*	36	III	Somaliland	09°46'47"N 44°26'43"	XY000000	XY000000
S1305	*G. dabakalo*	23	I	Somaliland	09°33'24"N 45°31'58"E	XY000000	XY000000
S1321	*G. maidensis*	34	–	Somaliland	11°00'03"N 47°06'30"E	XY000000	XY000000
S1324	*G. maidensis*	34	–	Somaliland	11°00'03"N 47°06'30"E	XY000000	XY000000
S1325	*G. amoudensis*	36	I	Somaliland	09°56'49"N 43°13'23"E	XY000000	XY000000
S1336	*G. maidensis*	34	–	Somaliland	11°00'03"N 47°06'30"E	XY000000	XY000000
S1349	*G. gubanensis*	45	–	Somaliland	10°36'01"N 43°26'07"E	XY000000	XY000000
S1396	*G. calviceps*	–	–	Somaliland	09°23'30"N 44°07'10"E	XY000000	–
S1527	*G. dabakalo*	24	II	Somaliland	09°33'24"N 45°31'58"E	XY000000	XY000000
S1530	*G. banfasae*	19	III	Somaliland	08°39'35"N 45°55'49"E	XY000000	XY000000
S1531	*G. banfasae*	18	I	Somaliland	08°39'35"N 45°55'49"E	XY000000	XY000000
S1532	*G. banfasae*	18	II	Somaliland	08°39'35"N 45°55'49"E	XY000000	XY000000
S1533	*G. banfasae*	19	III	Somaliland	08°39'35"N 45°55'49"E	XY000000	XY000000
S1534	*G. banfasae*	18	I	Somaliland	08°39'35"N 45°55'49"E	XY000000	XY000000
S291	*N. awashensis*	–	–	Ethiopia	08°54'N 39°54'E	XY000000	XY000000
S649	*N. kutcheri*	–	–	Ethiopia	05°06'48"N 40°39'18"E	XY000000	XY000000
S878	*N. eritreaensis*	–	–	Eritrea	15°36'58"N 39°22'32"E	XY000000	XY000000

772 **Table S2.** Pairwise genetic distances (uncorrected *p*-distances) among individuals of *Gint* spp. inferred from COI sequences.

	species	ID	cyt	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*G. banfasae*	S1531	I																					
2	*G. banfasae*	S1534	I	0.007																				
3	*G. banfasae*	S1533	III	0.007	0.007																			
4	*G. banfasae*	S1532	II	0.006	0.006	0.002																		
5	*G. banfasae*	S1530	III	0.006	0.006	0.002	0.000																	
6	*G. dabakalo*	S1527	II	0.033	0.037	0.037	0.035	0.035																
7	*G. dabakalo*	S1305	I	0.033	0.037	0.037	0.035	0.035	0.029															
8	*G. dabakalo*	S1199	II	0.031	0.035	0.035	0.033	0.033	0.028	0.002														
9	*G. gaitako*	S969		0.083	0.081	0.083	0.081	0.081	0.075	0.072	0.070													
10	*G. gaitako*	S958		0.081	0.079	0.081	0.079	0.079	0.073	0.070	0.068	0.002												
11	*G. gaitako*	S409		0.081	0.079	0.081	0.079	0.079	0.073	0.070	0.068	0.002	0.000											
12	*G. gaitako*	S951		0.081	0.079	0.081	0.079	0.079	0.073	0.070	0.068	0.002	0.000	0.000										
13	*G. gaitako*	S960		0.081	0.079	0.081	0.079	0.079	0.073	0.070	0.068	0.002	0.000	0.000	0.000									
14	*G. gaitako*	S961		0.081	0.079	0.081	0.079	0.079	0.073	0.070	0.068	0.002	0.000	0.000	0.000	0.000								
15	*G. gubanensis*	S1349		0.083	0.090	0.084	0.086	0.086	0.095	0.088	0.086	0.106	0.105	0.105	0.105	0.105	0.105							
16	*G. amoudensis*	S1291	II	0.066	0.068	0.072	0.070	0.070	0.081	0.081	0.079	0.092	0.090	0.090	0.090	0.090	0.090	0.057						
17	*G. amoudensis*	S1293	III	0.066	0.068	0.072	0.070	0.070	0.081	0.081	0.079	0.092	0.090	0.090	0.090	0.090	0.090	0.057	0.000					
18	*G. amoudensis*	S1292	IV	0.066	0.068	0.072	0.070	0.070	0.081	0.081	0.079	0.092	0.090	0.090	0.090	0.090	0.090	0.057	0.000	0.000				
19	*G. amoudensis*	S1325	I	0.075	0.073	0.075	0.073	0.073	0.086	0.083	0.081	0.084	0.083	0.083	0.083	0.083	0.083	0.061	0.015	0.015	0.015			
20	*G. maidensis*	S1324		0.099	0.097	0.099	0.099	0.099	0.097	0.106	0.105	0.106	0.105	0.105	0.105	0.105	0.105	0.121	0.094	0.094	0.094	0.095		
21	*G. maidensis*	S1321		0.097	0.095	0.097	0.097	0.097	0.095	0.105	0.103	0.105	0.103	0.103	0.103	0.103	0.103	0.119	0.092	0.092	0.092	0.094	0.004	
22	*G. maidensis*	S1336		0.097	0.095	0.097	0.097	0.097	0.095	0.105	0.103	0.101	0.099	0.099	0.099	0.099	0.099	0.121	0.088	0.088	0.088	0.094	0.006	0.006

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ČLÁNEK III

TWO NEW SPECIES OF *EUSCORPIOPS* VACHON, 1980 FROM THAILAND AND MYANMAR (SCORPIONES: EUSCORPIIDAE: SCORPIOPINAE)

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Abstract.— *Euscorpiops artemisae* **sp. nov.** from Myanmar and *Euscorpiops orioni* **sp. nov.** from Thailand are described and compared with other species of the genus *Euscorpiops* Vachon, 1980. A key to the species of *Euscorpiops* is provided. Sexual dimorphism is present, as males of some species have a narrower pedipalp chela than females, while in other species the shape of the chela is the same in both sexes. Males of both new species have the pedipalp chela very narrow, in the male holotype of *E. artemisae* **sp. nov.** the chela length to width ratio is 4.13 and in the male holotype of *E. orioni* **sp. nov.** it is 4.58. In addition to morphological analysis, we describe also the karyotype of male holotype and paratype of *E. orioni* **sp. nov.** Both analyzed specimens have achiasmatic meiosis and the same number of chromosomes (2n=103) with predominance of acrocentric chromosomes gradually decreasing in size. During the first meiotic division we observed one trivalent in both males. This type of multivalent indicates centric fusion or fissions that may cause the differentiation of the karyotypes within the genus *Euscorpiops*.

Key words.— Arachnida, Scorpiones, Euscorpiidae, taxonomy, description, karyotype, Oriental Region, Southeast Asia

INTRODUCTION

The genus *Euscorpiops* represents medium size scorpions of the family Euscorpiidae. This genus was described by Vachon (1980) as a subgenus of the genus *Scorpiops* Peters, 1862, and raised to the status of genus by Lourenço (1998). Vachon (1980) distinguished *Euscorpiops* from *Scorpiops* by the number of external trichobothria on the patella, 17 in *Scorpiops* and 18–21 in *Euscorpiops*. According to Kovařík (2000) the separation of these genera by the number of external trichobothria on the patella is invalid and synonymized

PL ISSN 0003-4541 © Fundacja *Natura optima dux*
doi: 10.3161/00034541ANZ2015.65.1.009

Euscorpiops with *Scorpiops*. Kovařík (2000: 164) furthermore drew attention to the importance of position of the trichobothrium *Eb3* on the external surface of the chela, using as an example *Scorpiops montanus* Karsch, 1879, in which the position differs from other *Scorpiops* species. Soleglad and Sissom (2001) revised the family Euscorpiidae, in which they placed the subfamily Scorpiopinae and confirmed the validity of the genus *Euscorpiops*. Their conclusion was based on the position of trichobothrium *Eb3* rather than on the number of external trichobothria on the patella. Soleglad and Sissom (2001) also transferred *Scorpiops montanus* to *Euscorpiops* and *Euscorpiops lindbergi* to *Scorpiops*.

The genus *Euscorpiops* currently includes 25 species (Rein 2014, present study) inhabiting the Oriental region reaching from Pakistan to Vietnam (e.g. Kovařík 1993, 2000).

In addition to the analysis of external morphology, we also add description of the karyotype of *E. orioni* sp. nov. The cytogenetic data seem to be useful for taxonomy analysis and may help to differentiate morphologically similar species in different groups of arachnids (e.g. Řezáč *et al.* 2008, Zaragoza and Šťáhlavský 2008, Kovařík *et al.* 2009). The information on chromosomes has so far been available for 96 scorpion species (Schneider *et al.* 2014). The order Scorpiones displays a considerable range of diploid numbers, from 5 in *Tityus bahiensis* (Schneider *et al.* 2009a) to 175 in *Urodacus novaehollandiae* (Shanahan 1989). Nevertheless, the potential application of the cytogenetic data to taxonomy of this arachnid order increases the number of cytogenetic analyses, most of which has so far focused on the families Buthidae and Scorpionidae (see Schneider *et al.* 2014); only one species of the subfamily Scorpiopinae, *Euscorpiops neradi*, has been karyotyped. This species has 2n=48 with chromosomes that may be classified into two categories according to their size (Kovařík *et al.* 2013). For the description of standard karyotype is important to include also analysis of meiosis that may reveal unusual pairing. This approach has the ability to disclose specific rearrangements or hybrids (e.g. Gorlov and Tsurusaki 2000, Schneider *et al.* 2009b). Additional information on the karyotypes plays an important role in the understanding of the species diversity of the genus *Euscorpiops*.

Material and methods

Sampling. Scorpions were collected by ultraviolet (UV) detection at night and excavation of burrows during the day. After collection, the obtained material was preserved by standard methods (e.g. Stahnke 1972). Some specimens were taken alive and maintained in the laboratory conditions for later use in cytogenetic studies. Locality data were recorded using portable GPS units (Garmin Oregon 450). Map background was downloaded from MapBox open source mapping platform system and modified in Zoner Callisto, version 5.0. The scorpions were collected under the authority of Doi Inthanon National Park General Management. Holotypes of new species are permanently deposited in the public collection of Faculty of Science, Charles University in Prague (CUP). Paratypes are deposited in the first author collection (FKCP).

Morphological analysis. The specimens were studied under stereomicroscope and measured using an ocular micrometer. Measurements are given in millimeters unless noted otherwise. Nomenclature and measurements follow Stahnke (1970). Designation and description of trichobothria were used according to Vachon (1974). Morphological terminology follows Stahnke (1970) and Kovařík (2009).

Karyotype analysis. For the karyotype analysis we used gonads of the male holotype and one paratype from the type locality of *E. orioni* sp. nov. The chromosome slides were made by the spreading technique described by Traut (1976). The gonads were hypotonised in 0.075 M KCl for 20 minutes and then fixed in methanol : glacial acetic acid (3:1) for at least 20 minutes. Finally small parts of the tissue were dissociated in a drop of 60% acetic acid on a microscope slide and the suspension was evaporated on a warm histological plate (45°C). The chromosome preparations were stained with 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 30 minutes. Additionally, we used C-banding according to the standard method of Sumner (1972). In this case the chromosome preparations were stained with DAPI and the color of the photographs was inverted for higher contrast. The chromosomes were observed in an Olympus AX70 Provis microscope and documented with an Olympus DP72 camera. For the measurements of chromosomes we used eleven well spreaded cells. The photographs were analyzed with the software ImageJ 1.45r (http://rsbweb.nih.gov/ij) with the plugin Levan (Sakamoto and Zacaro 2009). This plugin combines and modifies a criterion for classification described by Levan *et al.* (1964) and Green and Sessions (1991). The relative diploid set length (DSL) was calculated for each chromosome as a percentage of the diploid set.

Taxonomy

Family: **Euscorpiidae** Laurie, 1896

Subfamily: **Scorpiopinae** Kraepelin, 1905

Genus: ***Euscorpiops*** Vachon, 1980

Scorpiops Kraepelin 1899: 179 (in part); Sissom 1990: 114 (in part); Kovařík 2000: 164 (in part); Kovařík 2001: 85 (in part).

Scorpiops (*Euscorpiops*) Vachon 1980: 155 (in part); Tikader and Bastawade 1983: 452 (in part); Bastawade 1997: 104 (in part).

Euscorpiops: Stockwell 1989: 120 (in part); Kovařík 1998: 141 (in part); Lourenço 1998: 246 (in part); Fet 2000: 488 (in part); Soleglad and Sissom 2001: 93; Kovařík 2004: 13 and 17; Kovařík 2005: 1; Qi *et al*. 2005: 14; Kovařík 2009: 32; Kovařík 2012: 1; Kovařík *et al*. 2013: 3.

Type species. *Scorpiops asthenurus* Pocock, 1900.

Diagnosis. Total length 24–70 mm. First to fourth metasomal segments with paired parallel ventral median carinae in adults. Pair of median eyes and three or four pairs of lateral eyes present. Movable fingers of pedipalps with granules in two rows. Ventral edge of cheliceral movable finger with 5–7 denticles. Pedipalp patella with 16–21 external trichobothria. Ventral surface of patella bears 6–18 trichobothria. Ventral trichobothrial serie (*V*) on surface of manus bears 4 trichobothria, of which *V4* is always situated on ventral aspect of chela. Trichobothrium *Eb3* on external surface of manus is between trichobothria *Dt* and *Est*. Annular ring at vesicle/aculeus juncture present on at least the male.

Euscorpiops artemisae Kovařík, Košulič, Šťáhlavský, Plíšková, Dongkhamfu et Wongprom sp. nov.
(Figs 1–7, 43–44, 51–52)

Etymology. The name is given after Artemis, the virgin goddess of the hunt and the Moon from Greek mythology. See also etymology under *E. orioni* sp. nov. below.

Figures 1–7. *Euscorpiops artemisae* sp. nov., male holotype, habitus and trichobothrial pattern: 1 – dorsal view; 2 – chela dorsal; 3 – chela external; 4 – chela ventral; 5 – patella dorsal; 6 – patella external; 7 – patella ventral.

Diagnosis. Total length 43.6 mm. Base color uniformly reddish black to black, legs and telson yellow to reddish brown. Pectinal teeth number 8 in male and 7–8 in female. Pectinal area is yellowish brown. External trichobothria on pedipalp patella number 20 (5 *eb*, 2 *esb*, 2 *em*, 5 *est*, 6 *et*); ventral trichobothria on patella number 14–15. Male has narrow pedipalp segments; chela length to width ratio = 4.13 in male holotype. Pedipalp fingers slightly notched in male. Telson elongate. First metasomal segment wider than long.

Description. The total length of male holotype is 43.6 mm. For habitus of male holotype see Fig. 1; adult female is unknown.

Mesosoma and carapace: The tergites are granulated, dorsally with one median carina, and the seventh sternite is sparsely granulated, with four carinae. The entire carapace is granulated, without carinae. The anterior margin of the carapace is markedly depressed in the middle. Lateral ocular tubercles each possess three eyes, of which two are normal and one is reduced. Pectinal teeth number 8 in the male and 7–8 in female.

Metasoma and telson: The metasoma is finely granulated, with sparse, relatively large granules. The first segment bears 10 carinae, the second to fourth segments bear 8 carinae, and the fifth segment bears 7 carinae, all composed of granules some of which are pointed. The dorsolateral carinae of the third and fourth segments posteriorly terminate in a pronounced tooth. The telson is elongate and is sparsely granulated.

Pedipalps: For position and distribution of trichobothria on the patella of pedipalps see Figs 2–7. External trichobothria on the patella number 20 (5 *eb*, 2 *esb*, 2 *em*, 5 *est*, 6 *et*), and ventral trichobothria on the patella number 14–15 (3 × 14, 1 × 15). The femur is granulated and has four or five granulose carinae, and the patella has five carinae with pronounced internal twin tubercles. The manus dorsally bears fine rounded granules, which in the central part form a longitudinal carina. The external surface of the chela is densely covered by minute granules which in the central part also form a longitudinal carina. The movable fingers bear straight double rows of granules with internal and external granules. The pedipalp fingers dentate margins are slightly notched in the male holotype. The notches on the movable and the fixed fingers alternate perfectly, so the fingers close without any gap.

Legs: The tarsomeres of legs are sparsely hirsute, without bristlecombs but with row of spines on ventral surfaces. The femur and patella are granulated on dorsal surfaces and may bear four to six carinae. The femur bears only solitary setae.

Measurements (*in mm*): Total length of male holotype 43.6; carapace length 7.1, width 7.2; metasoma and telson length 22.4; first metasomal segment length 2.2, width 2.6; second metasomal segment length 2.45, width 2.4; third metasomal segment length 2.55, width 2.2; fourth metasomal segment length 3.2, width 2.15; fifth metasomal segment length 5.7, width 2.13; telson length 6.3; pedipalp femur length 8.5, width 2.85; pedipalp patella length 7.2, width 2.7; chela length 15.7; manus width 3.8; movable finger length 7.4.

Type locality. Myanmar, W Mandalay Division, Pagan (Bagan), Nyaung-U.

Type material. Myanmar, W Mandalay Division, Pagan (Bagan), Nyaung-U, 29–31.v.1997, 1♂, 1♀ juv. (holotype and paratype), J. Rejsek leg.

Distribution. Myanmar (known from the type locality only).

Affinities. They are recounted in the key below.

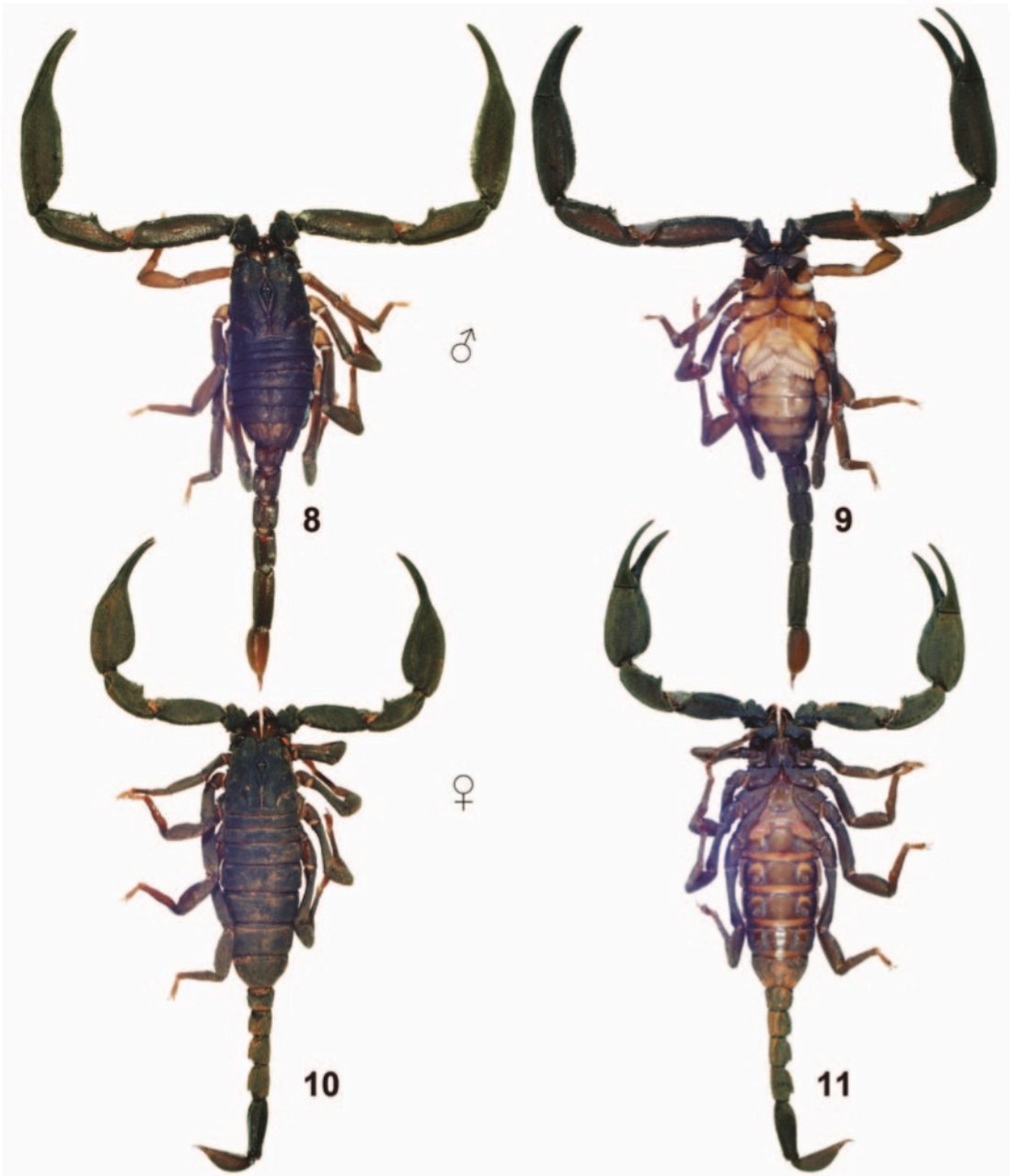
Euscorpiops orioni Kovařík, Košulič, Šťáhlavský, Plíšková, Dongkhamfu and Wongprom sp. nov. (Figs 8–34, 47–55)

Etymology. The scorpion was, according to Greek mythology, created and sent to Earth with the single purpose of killing Orion, the son of Poseidon and a vane hunter who boasted the ability to kill any animal. The scorpion stung Orion, who died. According to another version of the legend, however, he escaped the scorpion and accidentally died by an arrow of Artemis, the virgin goddess of the hunt and the moon, who regretted the accident so much that she installed Orion with his two dogs and a giant scorpion in heavens for all earthlings to see as the constellations of Orion, Lesser and Greater Dogs, and Scorpio. The name is given after Orion, the supernatural hunter of ancient times from Greek mythology.

Diagnosis. Total length 48–66 mm. Base color uniformly reddish black to black, legs and telson yellow to reddish brown. Sternites and pectinal area are yellowish brown. Pectinal teeth number 8–9 in males and 7–8 in females. External trichobothria on pedipalp patella number 19 (5 *eb*, 2 *esb*, 2 *em*, 5 *est*, 5 *et*); ventral trichobothria on patella number 11–13. Male has narrower pedipalp segments than female; chela length to width ratio = 4.58 in male and 3.05–3.44 in female. Pedipalp fingers dentate margin undulate in both sexes. Telson elongate in both sexes, slightly narrower in females. First metasomal segment wider than long in both sexes.

Description. For habitus of male holotype and female paratype see Figs 8–11. Sexual dimorphism is pronounced in the shape of pedipalp segments (see diagnosis and affinities).

Mesosoma and carapace (Fig. 28): The tergites are granulated, with one median carina, and the seventh sternite is sparsely granulated, without carinae. The entire carapace is granulated, without carinae. The anterior margin of the carapace is markedly depressed in the middle. The carapace bears a pairs of median



Figures 8–11. *Euscorpiops orioni* sp. nov.: 8–9 – male holotype, dorsal and ventral views; 10–11 – female paratype, dorsal and ventral views.

eyes and three pairs of lateral eyes, of which two are normal and one reduced. Pectinal teeth number 8–9 in males (8 × 8, 2 × 9) and 7–8 in females (9 × 7, 3 × 8).

Metasoma and telson (Figs 24–27): The metasoma is finely granulated, with sparse, relatively large granules. The first segment bears 10 carinae, the second to fourth segments bear 8 carinae, and the fifth segment bears 7 carinae, all composed of granules some of which are pointed. An additional incomplete lateral carina may be present on the second metasomal segment. The dorsolateral carinae of the third and fourth segments posteriorly terminate in a pronounced tooth. The telson is elongate in both sexes, slightly narrower in females (Fig. 26 versus 27), and is sparsely granulated.

Pedipalps: For position and distribution of trichobothria on the patella of pedipalps see Figs 12–23. External trichobothria on the patella number 19 (5 *eb*, 2 *esb*, 2 *em*, 5 *est*, 5 *et*), and ventral trichobothria on the patella number 11–13 (7 × 11, 11 × 12, 3 × 13). The femur is granulated (more so in males) and has four or

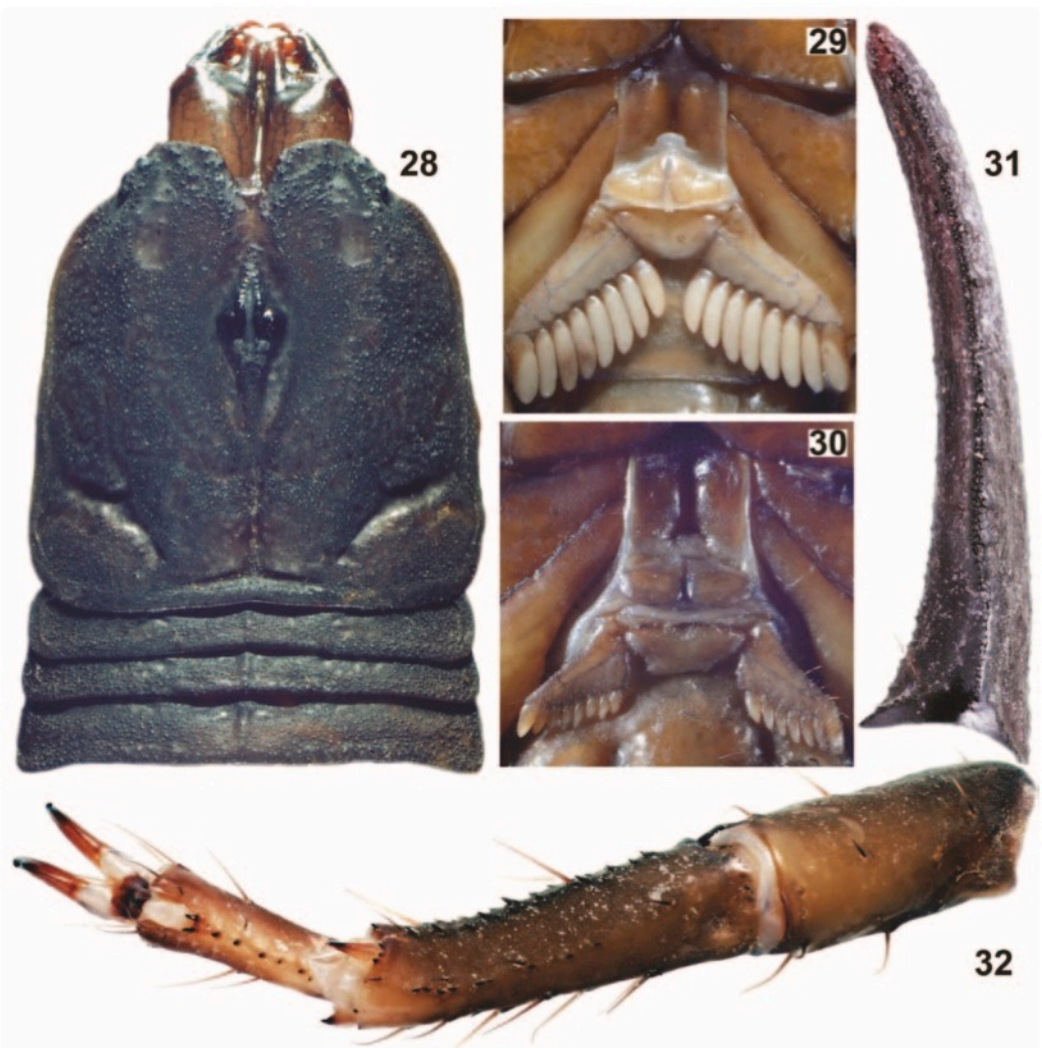
Figures 12–27. *Euscorpiops orioni* sp. nov. 12–17, 24–26 – male holotype, trichobothrial pattern and metasoma with telson ventral (24), dorsal (25) and lateral (26). 18–23, 27 – female paratype, trichobothrial pattern and metasoma with telson lateral. 12 and 18 – chela dorsal; 13 and 19 – chela external; 14 and 20 – chela ventral; 15 and 21 – patella dorsal; 16 and 22 – patella external; 17 and 23 – patella ventral.

five granulose carinae, and the patella has five carinae with pronounced internal twin tubercles. The manus dorsally bears fine rounded granules, which in the central part form a longitudinal carina. The external surface of the chela is densely covered by minute granules which in the central part also form a longitudinal carina. The movable fingers bear straight double rows of granules with internal and external granules (Fig. 31). The pedipalp fingers are undulate in both sexes. The flexures of the movable and the fixed fingers alternate perfectly, so the fingers close without any gap (Fig. 13).

Legs: The tarsomeres of legs are sparsely hirsute, without bristlecombs but with row of spines on ventral surfaces (Fig. 32). The femur and patella are granulated on dorsal surfaces and may bear four to six carinae. The femur bears only solitary setae.

Measurements (*in mm*): Total length of male holotype 49; carapace length 8.6, width 8.1; metasoma and telson length 26.7; first metasomal segment length 2.2, width 3.1; second metasomal segment length 3, width 2.75; third metasomal segment length 3.3, width 2.55; fourth metasomal segment length 4, width 2.5; fifth metasomal segment length 6.7, width 2.3; telson length 7.5; pedipalp femur length 10.8, width 3.2; pedipalp patella length 9.7, width 3.5; chela length 19.7; manus width 4.3; movable finger length 8.9.

Total length of female paratype 57.8; carapace length 8.5, width 8.6; metasoma and telson length 26; first metasomal segment length 2.6, width 3.15; second metasomal segment length 2.7, width 2.7; third metasomal segment length 3.1, width 2.5; fourth metasomal segment length 3.7, width 2.3; fifth metasomal segment length 6.5, width 2.15; telson length 7.4; pedipalp femur

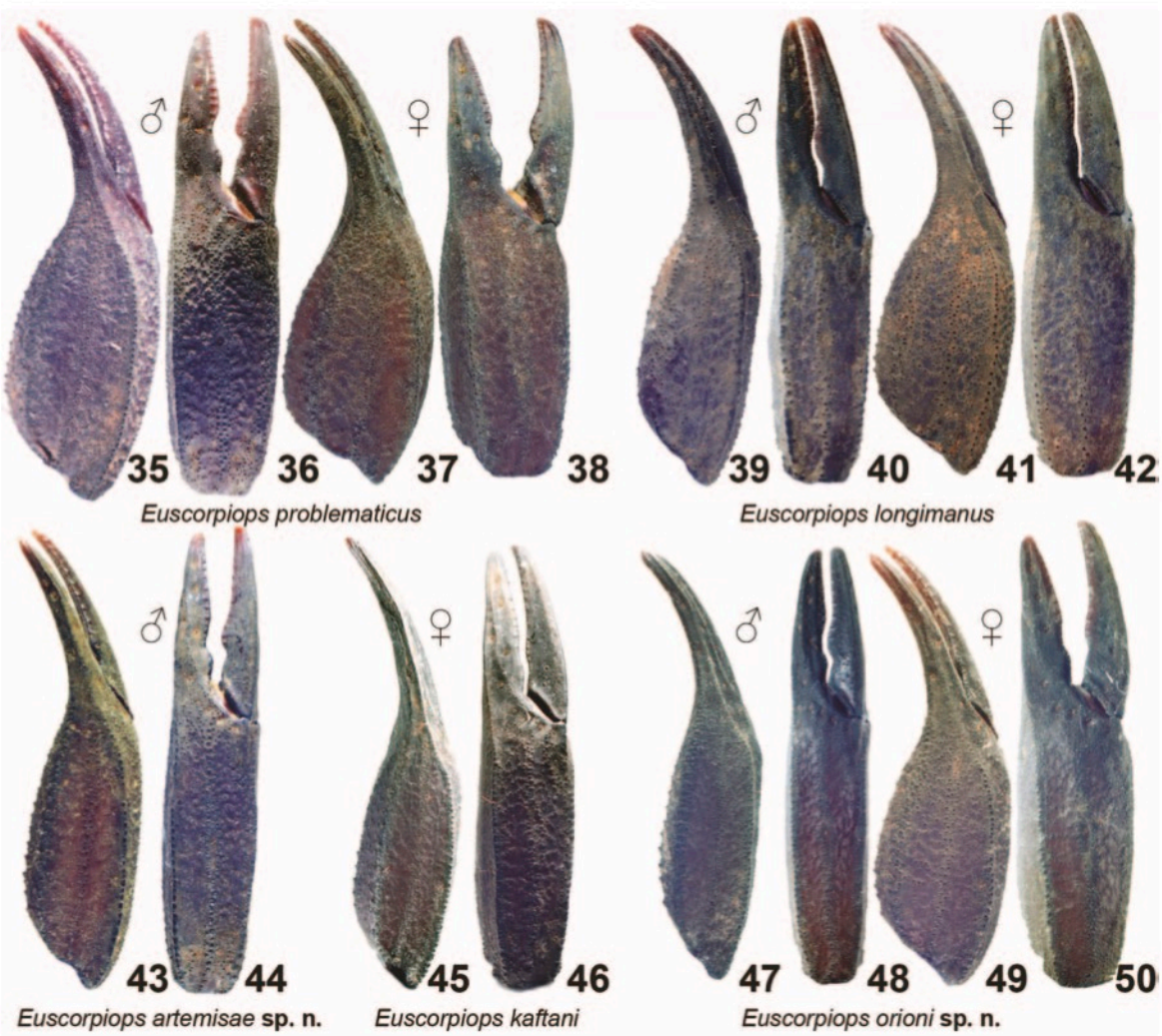


Figures 28–32. *Euscorpiops orioni* sp. nov. 28–29, 31–32: male holotype, carapace with chelicerae and first to third tergites (28), pectinal areas (29), movable finger (31) and fourth leg (32); 30 – female paratype, pectinal areas.

Figures 33–34. *Euscorpiops orioni* sp. nov.: 33 – live specimen, female (paratype); 34 – live specimen, male (paratype).

length 8.9, width 3.1; pedipalp patella length 8.2, width 3.6; chela length 17.2; manus width 5; movable finger length 8.6.

Karyotype: The diploid complement of the male holotype and one paratype is composed of 103 monocentric chromosomes (Fig. 53). The chromosomes gradually decrease in size from 1.8% to 0.36% of DSL. During the first meiotic division we did not observe chiasmata between the homologous chromosomes. This type of male achiasmatic meiosis is typical for the order Scorpiones (e.g. Schneider *et al.* 2009b). Despite the standard bivalents we found one trivalent in both analyzed males (Fig. 54). This fact explains the odd diploid number in the analyzed specimens. We used C-banding because the morphology of the chromosomes is not clearly visible in standard chromosome slides stained directly with Giemsa (see Fig. 54). We found one metacentric bivalent (pair No. 2) and two subtelocentric bivalents (pairs No. 6, 7); the rest of bivalents may be classified as acrocentrics, nevertheless some of them have no visible centromeric region (e.g. pairs No. 41, 46, 50, 51). Some chromosomes possess additional blocks of constitutive heterochromatin at the distal part (pairs No. 16, 25, 26, 36) (Fig. 55). The trivalent consists of one large metacentric chromosome (largest within the karyotype – 1.86 % of DSL) and two smaller acrocentric chromosomes (1.03% and 0.77% of DSL) (Figs 54–55). Size and morphology of chromosomes within the trivalent indicate heterozygous centric fusion or fission. The centric fusion or fission may play an important role in the differentiation of the karyotype within *Euscorpiops*. However the



Figures 35–50. Pedipalp chela of *Euscorpiops* species. 35–38 – *E. problematicus* (Kovařík, 2000); (35–36 – male holotype, 37–38 – female); 39–42 – *E. longimanus* (Pocock, 1893), (39–40 – male, 41–42 – female); 43–44 – *E. artemisae* sp. nov., male holotype; 45–46 – *E. kaftani* (Kovařík, 1993), female; 47–50 – *E. orioni* sp. nov. (47–48 – male holotype, 49–50 – female paratype); 35, 37, 39, 41, 43, 45, 47, 49 – chela dorsal; 36, 38, 40, 42, 44, 46, 48, 50 – chela external.

determination of decreasing (fusion) or increasing (fission) of diploid number in karyotype evolution of *Euscorpiops* is possible after analysis of additional species. The second largest chromosomes (pair No. 2) (1.74% of DSL) are only other metacentric chromosomes in karyotype and their origin may also be explained by centric fusion. We have information only on one other species of *Euscorpiops*, *E. neradi*, which has 2n=48 with the chromosomes of very different size (Kovařík *et al.* 2013).

Variability. Trichobothria on the external surface of the patella do not vary in number (19 in all types), but the series *est* and *et* (terminology according to Vachon 1974) vary markedly in position (Fig. 16 versus 22). In several paratypes the trichobothrium *est*3 is situated very close to *et* trichobothria and could thus be interpreted as an *et* trichobothrium (4 *est* and 6 *et* instead of 5 *est* and 5 *et*).

Type locality. Thailand, Chiang Mai Province, Doi Inthanon, Mae Pan Waterfall track trail, 18°31'N, 98°27'E, 1175 m a.s.l.

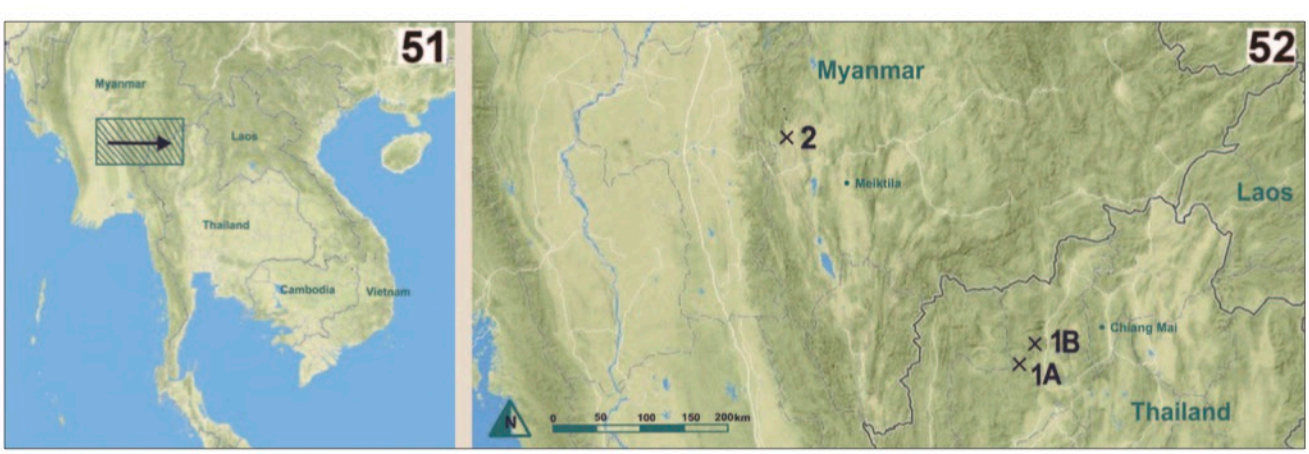
Type material. Thailand, Chiang Mai Province, Doi Inthanon, Mae Pan Waterfall track trail, 18°31'N, 98°27'E, 1175 m a.s.l., 25.XI.2013, 3 ♂♂ (holotype and paratypes), 3 ♀♀, 1♂ juv. (paratypes), O. Košulič leg.; Chiang Mai Province, Doi Inthanon, 18°24'N, 98°32'E, 945 m a.s.l., 26.XI.2013, 3 ♀♀, 1♂ juv. (paratypes), O. Košulič and W. Dongkhamfu leg.

Distribution. Thailand (known from the two localities only – see Fig. 52).

Ecological notes. Specimens of this species have mainly been discovered at night by ultraviolet detection in rocky fissures and deep burrows in the steep soil walls. The area where scorpions were collected was sheltered by evergreen rain forest characterized by very humid conditions (presence of many waterfalls). Adults and juveniles specimens of *E. orioni* were observed in ambush positions resting inverted on overhanging surfaces of rock or soil walls. Some of them occupied more protected places in fissures of cracked rock walls. When disturbed, the scorpions escaped and hide deeper in the rock fissure or soil burrow.

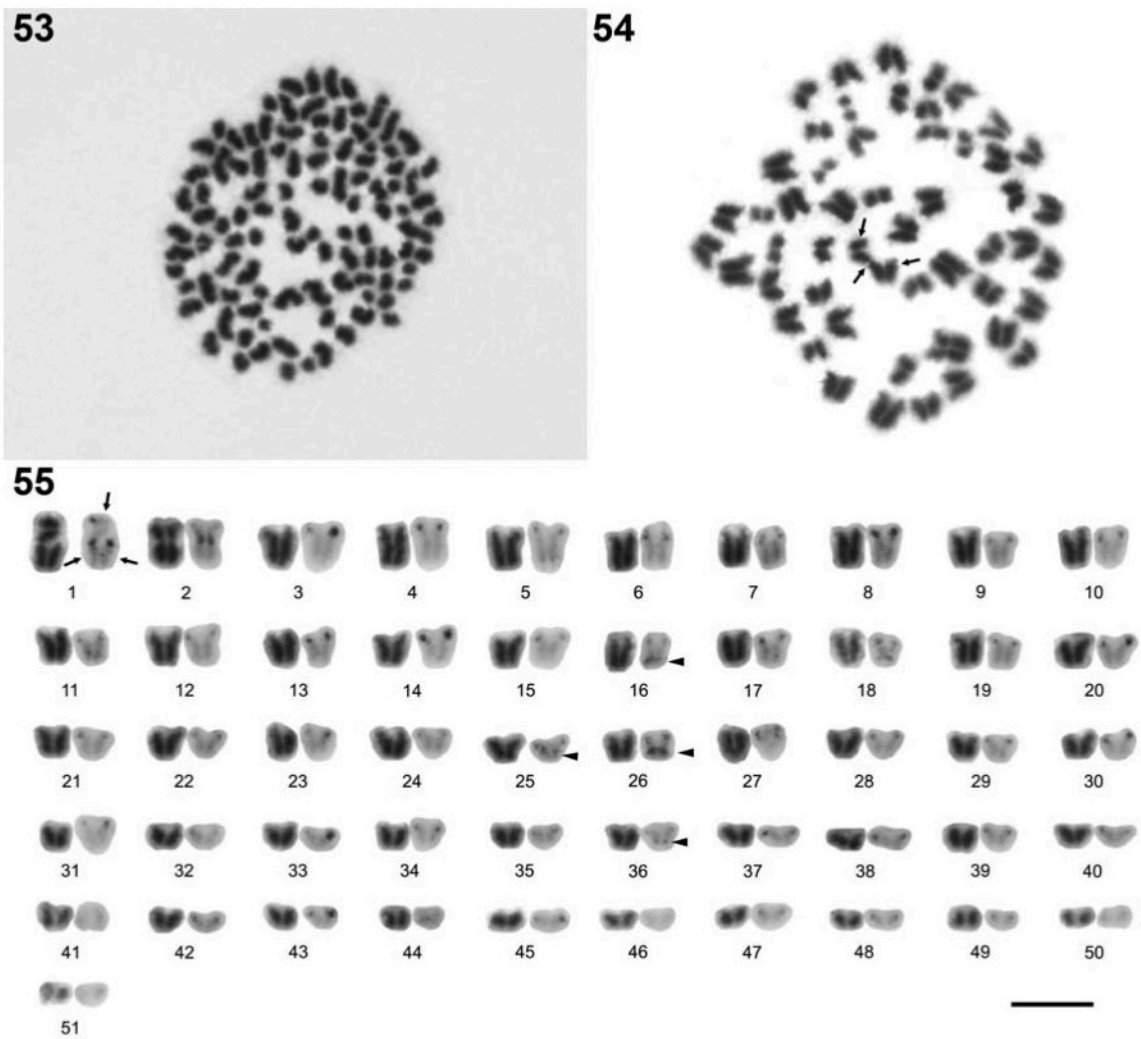
Affinities. The described features distinguish *E. orioni* sp. nov. from all other species of the genus. They are recounted in the key below. *E. orioni* sp. nov. belongs to a complex of species whose adults reach 40–70 mm, have a relatively narrow chela (pedipalp chela length to width ratio is greater than 3) and the number of trichobothria on the pedipalp patella is 18–21 external and 10–15 ventral. This complex contains 13 species which can be divided into two groups on sexual dimorphism expressed in the shape of pedipalp chela. In most of these species both sexes have nearly identical ratio between the chela length and width (Fig. 35 versus 37). These species are *E. binghamii* (Pocock, 1893) (Myanmar, Thailand), *E. kubani* Kovařík, 2004 (northern Laos), *E. problematicus* (Kovařík, 2000) (Thailand), *E. puerensis* Di *et al.*, 2010b (China – Yunnan), *E. thaomischorum* Kovařík, 2012 (Vietnam), *E. validus* Di *et al.*, 2010a (China – Yunnan), *E. yangi* Zhu *et al.*, 2007 (China – Yunnan), and probably also *E. beccaloniae* Kovařík, 2005 from Myanmar (the female of this species is unknown, but the shape of the chela in the male holotype is similar to that in males of the other cited species).

The male is unfortunately unknown in *E. kaftani* (Kovařík, 1993) (Vietnam); the female has the narrowest chela of all 13 species. Its chela length to width ratio is 4.4 (Fig. 45), whereas in females of the other species it is 3.0–3.6 (Figs 37, 41 and 49).



Figures 51–52. Distribution maps of *Euscorpiops artemisae* sp. nov. and *E. orioni* sp. nov. in Southeast Asia: 51 – Larger scale terrain map of Southeast Asia with geopolitical borders and highlighted distribution range of the new species; 52 – Smaller scale terrain map showing locations of the new species. 1A – type locality of *E. orioni* sp. nov., 1B – second location of described paratypes of *E. orioni* sp. nov., 2 – type locality of *E. artemisae* sp. nov.

Only in four species of this group (*E. artemisae* sp. nov., *E. longimanus*, *E. orioni* sp. nov., *E. xui*) the males have markedly narrower pedipalp chela than the females (Figs 39 versus 41). *E. longimanus* (Pocock, 1893) (Bangladesh, India) was erroneously recorded from Myanmar (Henderson 1913: 132; Kovařík 2000: 188), where it apparently has been confused with others species (e.g. *E. artemisae* sp. nov., *E. binghamii*, *E. beccaloniae* or *E. problematicus*), which explains differences of published data on morphometry of the chela. Pocock (1893) regarded the holotype of *E. longimanus* as a female, but his figure (fig. 12, plate XIV, Pocock, 1893) makes it evident that in reality it is a male. After measuring all the specimens we can state that this species has the chela length to width ratio of 3.63–4.23 in males and 3.2–3.45 in females. *Euscorpiops xui* Sun et Zhu, 2010 (China – Yunnan) has the chela length to width ratio 4.14 in males and 3.56 in females. The males of *E. orioni* sp. nov. thus have the narrowest pedipalp chela in the group (length to width ratio 4.58 in males and 3.05–3.44 in females, Figs 47–50). We place here also *E. artemisae* sp. nov., whose adult female is unknown but the male has the chela markedly narrow (Fig. 43). Its chela length to width ratio is 4.13, which is one of the characters distinguishing *E. artemisae* sp. nov. from *E. orioni* sp. nov. Another difference is the presence of 20 external trichobothria on the pedipalp patella (*E. orioni* sp. nov. has 19) and of 14–15 ventral trichobothria on the patella (other cited species have 10–13).



Figures 53–55. Chromosomes of male *Euscorpiops orioni* sp. nov. (2n = 103): 53 – spermatogonial metaphase; 54 – late metaphase I.; 55 – meiotic karyotype based on late metaphase I, left location – chromosomes stained with Giemsa, right location – the same chromosomes after C-banding stained with DAPI (color inversion). Arrows indicate chromosomes intrivalent. Arrowheads indicate additional blocks of constitutive heterochromatin at distal parts of chromosomes. Scale bar = 10 μm.

Key to species of *Euscorpiops* Vachon, 1980

1. External trichobothria on patella number 16–17 . . . . . **2**
–. External trichobothria on patella number 18–21 (Fig. 6) **6**
2. External trichobothria on patella number 16 (5 *eb*, 2 *esb*, 2 *em*, 3 *est*, 4 *et*). Ventral trichobothria on patella number 6. Total length 24–28 mm . . . . . ***E. neradi*** Kovařík *et al.* 2013 Thailand
–. External trichobothria on patella number 17. Ventral trichobothria on patella number 7–18. Total length 42–60 mm **3**
3. Ventral trichobothria on patella number 7–10 . . . **4**
–. Ventral trichobothria on patella number 11–18 . . . . . ***E. montanus*** (Karsch 1879) India, Pakistan . . . . . ***E. shidian*** Zhu *et al.*, 2005 China – Yunnan
4. Ventral trichobothria on patella number 7 . . . . . ***E. bhutanensis*** (Tikader and Bastawade 1983) Bhutan
–. Ventral trichobothria on patella number 8–10 . . . **5**
5. Ventral trichobothria on patella number 8–9 . . . . . ***E. karschi*** Lourenço *et al.* 2005 China – Tibet
–. Ventral trichobothria on patella number 10 . . . . . ***E. vachoni*** Zhu *et al.* 2005 China – Yunnan
6. External trichobothria on patella number 20–21 (5 *eb*, 2 *esb*, 2 *em*, 5–6 *est*, 5–6 *et*) **7**
–. External trichobothria on patella number 18–19 . . . **9**
7. Total length 50–70 mm. Chela length to width ratio in male lower than 4. Ventral trichobothria on patella number 12–13 . . . . ***E. binghamii*** (Pocock 1893) Myanmar, Thailand
–. Total length 38–45 mm. Chela length to width ratio in male higher than 4. Ventral trichobothria on patella number 14–15 . . . . . **8**
8. Chela length to width ratio in male holotype 6.7. Male fingers of pedipalps straight . . . . . ***E. alexandreanneorum*** Lourenço 2013 Laos
–. Chela length to width ratio in male holotype 4.13. Male fingers of pedipalps slightly flexed (Fig. 3) . . . . . ***E. artemisae*** sp. nov. Myanmar
9. *est* trichobothria on patella number 3–4 . . . . . **10**
–. *est* trichobothria on patella number 5 (Fig. 6) . . . **22**
10. Chela length to width ratio lower than 2.8 . . . . . **11**
–. Chela length to width ratio higher than 2.9 . . . . . **12**
11. Ventral trichobothria on patella number 9 . . . . . ***E. sejnai*** Kovařík 2000 Vietnam
–. Ventral trichobothria on patella number 10–11 . . . . . ***E. puerensis*** Di *et al.* 2010 China – Yunnan
12. *eb* trichobothria on patella number 5 (Figs 16 and 22) **14**
–. *eb* trichobothria on patella number 6 . . . . . **13**
13. Total length 54–65 mm. Chela length to width ratio 3.5 (female) to 4.15 (male) . . . . . ***E. xui*** Sun et Zhu 2010 China – Yunnan
–. Total length 39–45.3 mm. Chela length to width ratio 3.1–3.2 in both sexes . . . . . ***E. kubani*** Kovařík 2004 northern Laos
14. Chela length to width ratio of adults higher than 3.9 . . . . . **15**
–. Chela length to width ratio of adults lower than 3.8. . . . . **16**
15. Ventral trichobothria on patella number 12–13 . . . . . ***E. kaftani*** (Kovařík 1993) (Vietnam) (the ratio in female is higher than 4.2)
–. Ventral trichobothria on patella number 7–8 . . . . . ***E. kamengensis*** Bastawade 2006 India – Arunachal Pradesh
16. Male pedipalp fingers flexed **18**
–. Male pedipalp fingers nearly or entirely straight, may be slightly undulate (Fig. 40) **17**
17. Chela length to width ratio 3.63–4.23 in males . . . . . ***E. longimanus*** (Pocock 1893) Bangladesh, India
–. Chela length to width ratio 3.3–3.6 in both sexes . . . . . ***E. karschi*** Lourenço *et al.* 2005 China – Tibet . . . . . ***E. yangi*** Zhu *et al.* 2007 China – Yunnan
18. External surface of chela densely covered by minute granules. Ventral trichobothria on patella number 9–11, very rarely 12 . . . . . **19**
–. External surface of chela smooth, with less than 40 large, rounded granules most of which form a medial carina. Ventral trichobothria on patella number 12. . . . . ***E. beccaloniae*** Kovařík 2005 Myanmar
19. Sexual dimorphism expressed in shape of pedipalp fingers **20**
–. Sexual dimorphism not readily apparent, fingers flexed identically in both sexes . . . . . ***E. thaomischorum*** Kovařík 2012 Vietnam
20. Pedipalp fingers in male flexed, in female nearly straight (very slightly undulate) **21**
–. Pedipalp fingers flexed in both sexes, but strongly in males and only weakly in females . . . . . ***E. validus*** Di *et al.* 2010 China – Yunnan
21. Ventral trichobothria on patella number 8–9. Pectinal teeth number 5–6. . . . . ***E. asthenurus*** (Pocock 1900) Bhutan, India, Myanmar
–. Ventral trichobothria on patella number 10, rarely 9. Pectinal teeth number 7–8 . . . . . ***E. kubani*** Kovařík 2004 northern Laos

22. Ventral trichobothria on patella number 11–13 . . . **23**
–. Ventral trichobothria on patella number 7–9 . . . **25**
23. Chela length to manus width ratio in adult female higher than 4.2 (Fig. 45) . . . . . ***E. kaftani*** (Kovařík 1993) Vietnam
Chela length to manus width ratio in adult female 3.0–3.4 . . . . . **24**
24. Chela length to width ratio 3.0–3.3 in both sexes . . . . . ***E. problematicus*** (Kovařík, 2000) Thailand
–. Male has narrower chela of pedipalp than female, length to width ratio 4.58 in male (Fig. 47) and 3.05–3.44 in female (Fig. 49) . . . . ***E. orioni*** sp. nov.
25. Ventral trichobothria on patella number 9 . . . . . ***E. novaki*** Kovařík 2005 China – Tibet
–. Ventral trichobothria on patella number 7–8 . . . . . ***E. kamengensis*** Bastawade 2006 India – Arunachal Pradesh

Acknowledgements

Authors would like to thank Ivana Hynková who kindly help us with English improving of the paper. This study was financially supported by the European Social Fund and the state budget of the Czech Republic, the project Indicators of Trees Vitality Reg. No. CZ.1.07/2.3.00/20.0265 and the grant received from the Ministry of Education, Youth and Sports of the Czech Republic no. SVV 260 087/2014. The authors declare no conflicts of interest.

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Received: May 20, 2014
Accepted: October 20, 2014

ČLÁNEK IV

ANNALES ZOOLOGICI (Warszawa), 2016, 66(3): 467-476

DESCRIPTION OF A NEW SPECIES OF *HETEROMETRUS* EHRENBERG, 1828 (SCORPIONES: SCORPIONIDAE) FROM THAILAND WITH REMARKS ABOUT THE UTILIZATION OF CYTOGENETIC DATA IN TAXONOMY OF THE GENUS

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Abstract.— A new species of the genus *Heterometrus* is described on the basis of a specimen recently collected in Thailand. *Heterometrus minotaurus* **sp. nov.** is morphologically closest to *H. longimanus*. The newly described species is well distinguished by its smaller overall size (83 mm) and shorter and less narrow metasoma with specific dorsolateral carinae on the fourth metasomal segment consisting of 9 or fewer granules. No females are known and so knowledge of sexual dimorphism in this species is currently lacking. In addition to the morphological characterization of *H. minotaurus* **sp. nov.**, we present here also a description of the male holotype's karyotype. The diploid set of *H. minotaurus* **sp. nov.** consists of 54 chromosomes with a predominance of metacentrics, which gradually decrease in size. The presence of two types of multivalent association observed in postpachytene and metaphase I is commented on. Current knowledge of karyotypes of *Heterometrus* species is briefly summarized and compared with our cytogenetic results. In conclusion, we discuss the possible usefulness of karyotype as another interspecific feature applicable in the taxonomy of this scorpion group.

Key words.— Arachnida, Scorpiones, karyotype, taxonomy, Southeast Asia

INTRODUCTION

Together with three related lineages (*Scorpio* Linnaeus, 1758; *Pandinus sensu lato* Thorell, 1876; and *Opistophtalmus* C. L. Koch, 1837), the genus *Heterometrus* Ehrenberg, 1828 forms the separate subfamily Scorpioninae within the family Scorpionidae. *Heterometrus* has a centre of diversity in Southeast Asia and its members inhabit various biotopes of tropical and subtropical forests of the Indian subcontinent, Southeast Asia, and the Indonesian archipelago (Prendini *et al.* 2003, Tahir and Prendini 2014). Similar to scorpions of the African sister genus *Pandinus*, representatives of *Heterometrus* are among the largest

PL ISSN 0003-4541 © Museum and Institute of Zoology PAS
doi: 10.3161/00034541ANZ2016.66.3.011

extant scorpions in the world (Prendini *et al.* 2003). Our knowledge of the taxonomy of *Heterometrus* is particularly based on several key studies, most of them are listed below. First, in a series of various publications Pocock described a considerable number of species and also established the basic criteria for their identification (see Pocock 1892, 1893, 1894a, 1896, 1897, 1899, 1900). Several decades later, Couzijn (1981) published a monographic revision of the genus *Heterometrus* with a key to all 21 known species. Subsequently, Tikader and Bastawade (1983) focused on all Indian *Heterometrus* species, giving their redescriptions and a determination key and also describing two new species. Recently, Kovařík (2004) published a new revision of the genus *Heterometrus*. On the basis of obtained knowledge, Kovařík (2004) synonymized several species and subspecies as well as subgenera established by Couzijn (1981). Taking into account Kovařík's morphological revision from 2004, there were 31 valid species recognized within the genus *Heterometrus*. Recently, various authors have described four additional species (Lourenço *et al.* 2005, Zhu and Yang 2007, Javed *et al.* 2010, Mirza *et al.* 2012), and thus the genus *Heterometrus* currently includes 35 species (Rein 2015). As Tahir and Prendini (2014) have noted, the total of recently recognized *Heterometrus* species might not reflect the potential real diversity of this genus. Across its entire area of distribution, numerous range-restricted or narrowly endemic species as well as species complexes are expected (Prendini *et al.* 2003).

Many taxonomic studies of various organisms today utilize standard morphological analysis in combination with molecular phylogenetic or cytogenetic approaches (e.g. Fet *et al.* 2014, Granjon and Dobigny 2003, Post *et al.* 2003). Cytogenetic data seem to represent additional suitable characteristics useful for taxonomic analysis and may also help to differentiate morphologically similar species into different groups of arachnids (e.g. Řezáč *et al.* 2008, Zaragoza and Šťáhlavský 2008). Cytogenetic data are currently known for 105 scorpion species (Schneider *et al.* 2015) and the genus *Heterometrus* is among the scorpion groups more frequently studied from a cytogenetic point of view. At present, information on karyotypes is available for 8 *Heterometrus* species (see Schneider *et al.* 2009, Sharma *et al.* 1962, Vítková *et al.* 2005). This group exhibits a wide range of diploid numbers of chromosomes (56–122). Interspecific differences in the number and morphology of chromosomes among related species suggest that a description of karyotype could be another useful specific characteristic applicable in *Heterometrus* taxonomy. For this reason, we describe the karyotype of *H. minotaurus* sp. nov. and discuss the utility of karyotypes as important characteristic features in scorpion taxonomy.

Material and methods

Sampling. The specimen was found at night by UV detection in front of a massive stone at the border between a rubber plantation and a water stream. The area was sheltered by rubber trees and shrubs with high canopy coverage and humidity. No other scorpions were found sympatrically with *H. minotaurus* sp. nov. during a field trip to the studied area.

Morphological analysis. The specimen was studied under stereomicroscope and measured using an ocular micrometre. Measurements are given in millimetres. Nomenclature and measurements follow Stahnke (1970), Kovařík (2009), and Kovařík and Ojanguren Affilastro (2013). The sternum was analysed according to Soleglad and Fet (2003). Trichobothria were denominated and described according to Vachon (1974). Morphological terminology follows Stahnke (1970) and Kovařík (2009).

Karyotype analysis. For the karyotype analysis, we used the gonads of the *H. minotaurus* sp. nov. male holotype. Chromosome slides were made using the spreading technique described by Traut (1976). The gonads were dissected and hypotonized in 0.075 M KCl for 20 min and then fixed in a methanol: glacial acetic acid (3:1) solution for at least 20 min. The small parts of the testes were then dissociated in a drop of 60% acetic acid on a microscope slide, and the suspension was evaporated on a warm histological plate (45°C). The chromosomes were stained with 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 30 min. Additionally, we used the C-banding technique according to the standard protocol of Sumner (1972). In this case, the chromosome slides were stained with DAPI and the photographs' colour was inverted in Adobe Photoshop CS4 11.0 for higher contrast. Chromosomes were observed with an Olympus AX70 Provis microscope and documented with an Olympus DP72 camera. For chromosome measurements, we used ImageJ 1.45r software (http://rsbweb.nih.gov/ij) with the Levan plugin (Sakamoto and Zacaro 2009). Relative diploid set length was calculated for each chromosome as a percentage of the diploid set based on 11 metaphases I. For the cytogenetic analysis, we used meiotic stages that may reveal unusual pairings. This approach has the ability to disclose specific rearrangements or hybrids (e.g. Gorlov and Tsurusaki 2000).

Taxonomy

Family Scorpionidae Latreille, 1802

Subfamily Scorpioninae Latreille, 1802

Genus ***Heterometrus*** Ehrenberg, 1828

Heterometrus minotaurus Plíšková, Kovařík, Košulič et Šťáhlavský sp. nov.
(Figs 1–23)

Type locality. Thailand, Surat Thani province, Phanom district, 8°52'N, 98°36'E, 395 m a.s.l.

Type material. Thailand, Surat Thani province, Phanom district, trail along rubber plantation and water stream, 8°52'N, 98°36'E, 395 m a.s.l., 1 male holotype, 11.VII.2014, O. Košulič leg. Holotype specimen is deposited in the public collection of Faculty of Science, Charles University in Prague (CUP-S651).

Differential diagnosis. The described features distinguish *H. minotaurus* sp. nov. from all other species of the genus. *H. minotaurus* sp. nov. from Thailand seems to be closest to *H. longimanus* (Herbst, 1800) from Indonesia (Java, Kalimantan and Sumatra), Singapore, Malaysia (Kalimantan) and Philippines. In published keys to the *Heterometrus*, the two species are categorized under the *H. longimanus* (Kovařík, 2009: 47–48, couplet 28). Males of the two species can be unequivocally separated by: 1) total length 90–140 mm in *H. longimanus* and 83 mm in *H. minotaurus* sp. nov.; 2) longer and more narrow metasoma in

Figures 1–5. *Heterometrus minotaurus* sp. nov., male holotype: (1) dorsal view; (2) ventral view; (3–5) Metasoma and telson: (3) lateral view; (4) dorsal view; (5) ventral view. Scale bars = 10 mm.

H. longimanus (ratio length to width the first metasomal segment is 0.96–1.04 in *H. longimanus* and 0.78 in *H. minotaurus* sp. nov.); 3) more densely granulated carinae on metasoma in *H. longimanus* (dorsalolateral carinae of the fourth metasomal segment consists of 12 or more granules in *H. longimanus* and 9 or less granules in *H. minotaurus* sp. nov.).

Summary of description. Total length male holotype 83 mm, female unknown. Base colour uniformly black (Figs 1–2), telson reddish brown (Figs 3–5). Chelicerae yellow strongly reticulate, anterior part reddish black (Fig. 6). Pectines with 16/17 teeth in male (Fig. 7). Male with fingers, chela, femur and patella of pedipalp narrow and long, ratio chela length manus width 3.42 in male (Figs 8–14). Chela not lobiform in male, smooth, sparsely tuberculate with pronounced carination. Patella of pedipalp with pronounced internal tubercle. Carapace smooth, sparsely granulated. Metasomal segment I wider than long, ratio 0.78. Telson hirsute, elongate, vesicle approximately as long as aculeus.

Description. Total length male holotype 83 mm. Coloration (Figs 1–2) base uniformly black; telson, tarsomeres of legs and sternopectinal area reddish brown.

Chelicerae (Fig. 6): Chelicerae yellow strongly reticulate, anterior part reddish black. Fingers reddish brown to black. Dentition typical for the genus, teeth sharp. Tegument basally smooth and shiny without granulation.

Pedipalps (Figs 8–14): Trichobothriotaxie type C. Pedipalp femur with three trichobothria, of them only one on internal surface. Patella of pedipalp with 20 trichobothria (Figs 11–13), one internal, two dorsal, three ventral and 14 external from which em3 trichobothrium is accessory (Fig. 12 and figs 80–84 in Vachon, 1974: 918). Chela of pedipalp with standard 26 trichobothria (Figs 8–10). Male with fingers, chela, femur and patella of pedipalp narrow and long, ratio chela length manus width 3.42 in male. Chela not lobiform in male, smooth, sparsely tuberculate with pronounced partly incomplete carination. Patella smooth with seven obsolete carinae developed, with pronounced internal tubercle. Femur smooth, with four granulate carinae developed. Fingers long (ratio chela length movable finger length 1.78 in holotype male, curved and with straight rows of granules and internal and external denticles (Fig. 14).

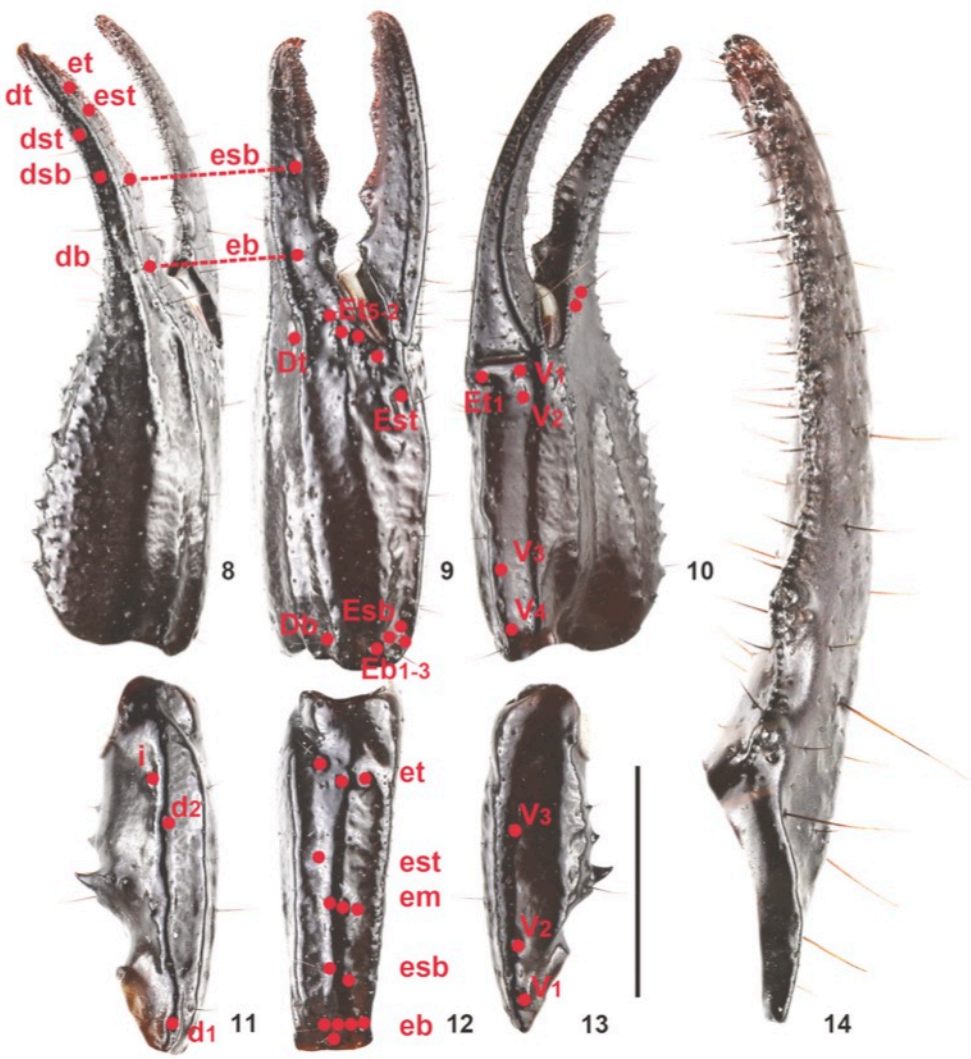
Figures 6–7. *Heterometrus minotaurus* sp. nov., male holotype: (6) chelicerae, carapace and tergites I–III; (7) sternopectinal region and sternites III–VII.

Carapace (Fig. 6): Slightly trapezoidal (narrower anteriorly) and slightly longer than wide; anterior margin strongly concave medially. Tegument smooth sparsely covered by large granules which do not form carinae. Median and posteriolateral furrows wide and deep, other vestigial to absent. Median eyes large and raised; three pairs of lateral eyes same-sized and aligned along each anterolateral corner.

Mesosoma (Figs 1–2, 6–7): Tergites smooth, lack carinae. Sternum (Fig. 7) standard for the genus: type 2 with six sides, posterior emargination and convex lateral lobes, vertical compression absent, apex width slightly narrower than posterior width. Pectines standard-sized for the genus (Fig. 7): short, wide and setose. Tooth count 17 in male. Pectines have 3 marginal lamellae and 4 middle lamellae. Sternites lack carinae, surfaces are smooth and sparsely setose, more setose is sternite III. Posterior margin of sternites IV–VI with smooth median patch.

Legs (Figs 15–18): Retrolateral pedal spurs absent. Lateroapical margins of tarsi produced into rounded lobes. Legs are smooth, without carinae and granules, and unevenly hirsute. Tarsomere I is hirsute. Tarsomere II has spiniform setae and several spines. Spiniform formula of tarsomere II is 4/6: 4/6: 4/6: 4/6.

Metasoma and telson (Figs 3–5): All segments with granulate complete carinae developed. Metasomal segment I wider than long, ratio 0.78. The first metasomal segment has a total of 10 carinae, the second through fourth segments have eight carinae, and the



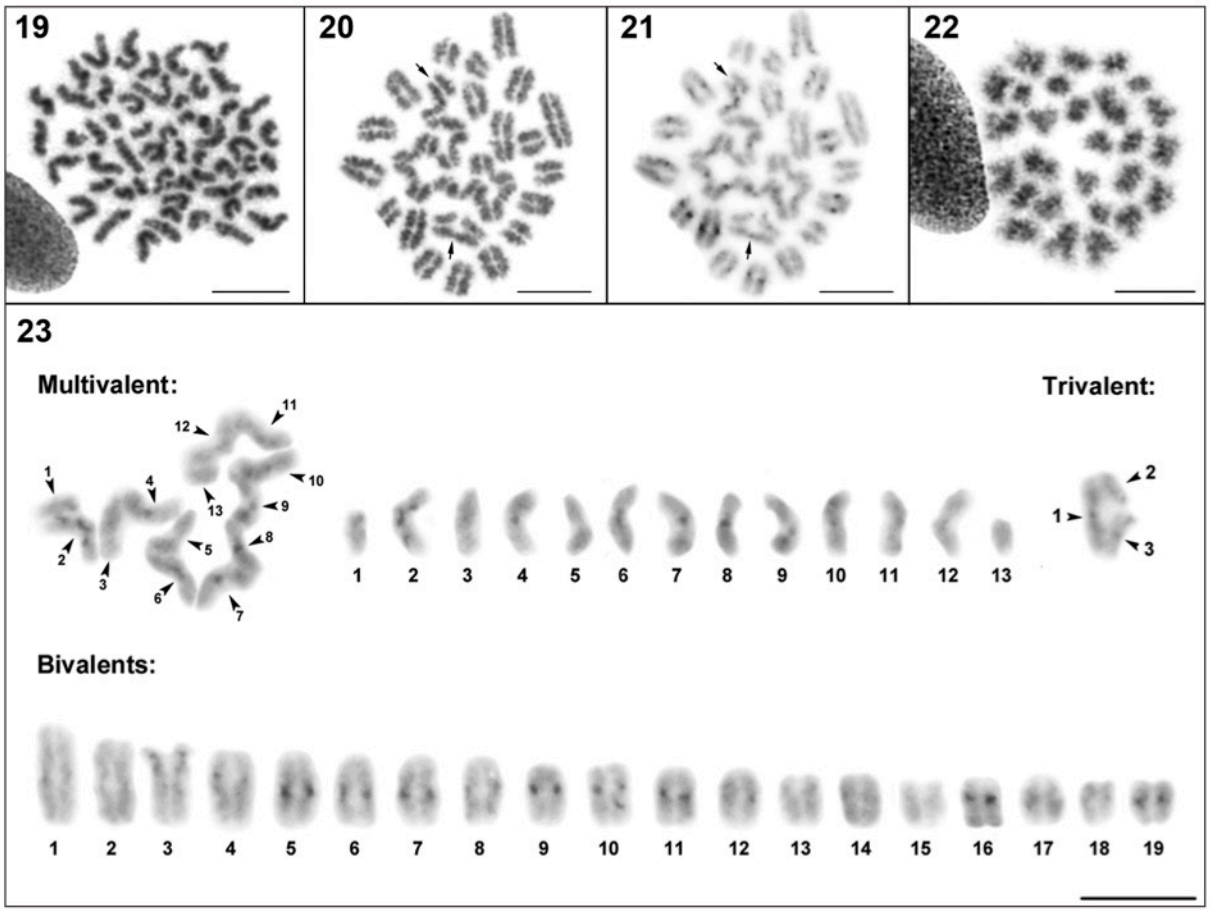
Figures 8–14. *Heterometrus minotaurus* sp. nov., male holotype: 8–10. Pedipalp chela: (8) dorsal view; (9) external view; (10) ventral view; 11–13. Pedipalp patella: (11) dorsal view; (12) external view; (13) ventral view; (14) pedipalp movable finger, dorsal view. The trichoborial pattern is indicated in Figs 8–13. Scale bar = 10 mm.

fifth segment has five to seven carinae. All metasomal segments are smooth, laterally as well as ventrally sparsely granulated by minute granules; dorsal surface entirely smooth without granules. Metasoma is sparsely hirsute by reddish setae. Telson more hirsute by long spiniform setae, smooth. Vesicle elongate ellipse. Aculeus curved, approximately as long as vesicle.

Measurements (in mm): Total length of male holotype 83; carapace length 14.1, width 13.8; metasoma and telson length 48.3; first metasomal segment length 5, width 6.4; second metasomal segment length 5.9, width 5.7; third metasomal segment length 6.5, width 5.35; fourth metasomal segment length 8.2, width 4.85; fifth metasomal segment length 11.4, width 4.5; telson length 11.3; telson width 4.4; telson depth 3.7; pedipalp femur length 15.1, width 5.1; pedipalp patella length 15.45, width 5.3; chela length 28.8; manus width 8.4; movable finger length 16.1.

Karyotype. Karyotype of *H. minotaurus* sp. nov. was established on the basis of meiotic and mitotic cells of male holotype. Specimen exhibits monocentric type of chromosomes that differ one from another by their morphology and size. The chromosome complement consists of 54 chromosomes (Fig. 19), which gradually decrease in size from 2.82% to 1.06% of the diploid set. In all postpachytene and metaphase I nuclei, we observed 19 homomorphic bivalents (Figs 20, 21) and two different types of multivalent association - one trivalent and one multivalent chain composed of 13 distinct chromosomes (Figs 20, 21). Despite the presence of unusual multivalents, all observed metaphases II had the same number of chromosomes (n=27) (Fig. 22). Chiasmata between homologous chromosomes during the first meiotic division were not observed. Cytological observation gave no evidence for existence of diplotene-diakinesis stage during prophase I. No positively or negatively heteropycnotic bodies were present during the meiotic division. We also did not detect presence of morphologically differentiated sex chromosomes in all analysed nuclei. Morphology of some chromosomes was not clearly visible in standard chromosome slides stained directly by Giemsa. For better visualization of centromere, which is formed by constitutive heterochromatin, and thus proper determination the morphology of individual chromosomes, we applied C-banding accompanied by DAPI staining. However, in some cases centromere was not clearly detected even after using C-banding technique. When Giemsa stained chromosomes had noticeable primary constrictions, C-bands corresponded with these areas. The karyotype of *H. minotaurus* sp. nov. is composed mainly by metacentric chromosomes (Fig. 23). Trivalent consists of one large metacentric and two small telocentric chromosomes, multivalent chain is

Figures 15–18. *Heterometrus minotaurus* sp. nov. male holotype, distal segments of legs, retrolateral view: (15) Leg I; (16) Leg II; (17) Leg III; (18) Leg IV. Scale bar = 5 mm.



Figures 19–23. *Heterometrus minotaurus* sp. nov., male holotype, chromosomes: (19) Mitotic metaphase (2n=54), Giemsa staining; (20) Postpachytene, Giemsa staining; (21) Postpachytene, C-banding, DAPI staining (inverted); (22) One sister metaphase II (n=27), Giemsa staining; (23) Karyotype based on postpachytene, DAPI staining (inverted). Arrows indicate multivalents, arrowheads show individual chromosomes in multivalents. Scale bar = 10 μm.

compiled of 10 metacentric, 1 submetacentric and 2 small telocentric chromosomes (multivalent chromosomes No. 1, 13). Nevertheless, position of centromeres in chromosomes associated in multivalent chain was not entirely evidential. In case of standard bivalents, 13 of them are metacentric, 3 submetacentric (pairs No. 4, 13, 18), 2 subtelocentric (pairs No. 15, 17) and 1 telocentric (pair No. 3).

Distribution. Thailand (known from the type locality only).

Etymology. This species is named according to the mystic creature from ancient Greek mythology. Minotaurus was a beast with the head of a bull on the man's body.

Discussion

In this article, we described *Heterometrus minotaurus* sp. nov. from Thailand. As with other scorpionid genera, males of the genus *Heterometrus* are important and essential for species identification and delimitation (Tahir and Prendini 2014). The *H. minotaurus* male holotype is clearly distinguished from the Indonesian *H. longimanus*, the morphologically closest species, by several features mentioned above. Unfortunately, no females of the described species are known, and so we cannot comment on aspects of potential sexual dimorphism. To date, the genus *Heterometrus* includes 36 species. However, Tahir and Prendini (2014) pointed out certain weak spots in some previous taxonomic works, e.g. the recent description of *H. atrascorpius* Mirza *et al.*, 2012 was based on only non-adult specimens. It is therefore possible that a detailed revision of this genus could reveal some taxa of dubious validity. Despite this assumption, the total number of currently recognized *Heterometrus* species might not reflect the real diversity of this endemic Asian genus. A reliable answer to the question of its true diversity requires further

detailed taxonomic studies with wider sampling and ideally additional methodical approaches, e.g. molecular phylogeny or cytogenetics. New methodical approaches and interspecific markers not only could bring us deeper knowledge of species diversity and phylogeography but might also resolve some discrepancies in traditional *Heterometrus* taxonomy, which is based mainly on external morphology. It is highly advantageous to include new markers as a part of prospective descriptions of new species, where the type series is established and analysed. For this reason, we cytogenetically analysed the *H. minotaurus* sp. nov. male holotype, and also preserved muscular tissue in absolute ethanol for prospective DNA analysis. *H. minotaurus* sp. nov. exhibits 54 chromosomes with a typical monocentric nature. This type of chromosome with a localized centromere is typical for scorpions with the exception of the family Buthidae, which represents the only lineage of scorpions displaying chromosomes with a holocentric nature (Schneider *et al.* 2009). Previously published cytogenetic studies have shown that the genus *Heterometrus* exhibits a wide range of diploid numbers of chromosomes from 2n=56 (*H. spinifer* (Ehrenberg, 1828)) to 2n=112 (*H. gravimanus* (Pocock, 1894b)). Accordingly, *H. minotaurus* sp. nov. with 2n=54 represents a species with the lowest known number of diploid sets within its genus. With 8 species cytogenetically analysed so far, the genus *Heterometrus* forms one of the more frequently studied scorpion genera from a cytogenetic point of view (see Schneider *et al.* 2015). Interestingly, all analysed specimens (except for *H. spinifer*) have come from a geographically restricted area, the Indian peninsula, and have displayed great interspecific differences. However, previous information on specific karyotypes cannot be used currently for this group's taxonomy. The authors did not focus on precise determination and delimitation of the specimens analysed and also did not use karyology results for the taxonomy of particular taxa. Moreover, descriptions of karyotypes were published mainly during the 1960s. Since that time, the definition of some *Heterometrus* taxa has changed rapidly, especially during recent decades (e.g. Couzijn 1981, Kovařík 2004). We can illustrate this problem through the example of *H. longimanus*, the species morphologically closest to *H. minotarus* sp. nov. According to Srivastava and Agrawal (1961), the Indian population of this species exhibits 64 chromosomes. As far as we now know, however, *H. longimanus* is endemic to Indonesia and does not occur in India (see Kovařík 2009). Therefore, the obtained cytogenetic data on Indian populations belong to another as yet undefined species. This scenario could be applied similarly to other cytogenetically studied *Heterometrus* species from India.

As indicated above, differences in karyotypes are present among particular *Heterometrus* taxa and these specific markers have the potential to be usable in the taxonomy of this scorpion group. However, it is advisable to analyse more specimens or populations for appropriate and precise results. Moreover, this approach is also necessary to understand potential chromosomal polymorphism in the species analysed, e.g. whether the unusual chromosomal rearrangements detected (see Kovařík *et al.* 2015a, Kovařík *et al.* 2015b) are fixed in particular scorpion populations of concrete species. In the case of *H. minotaurus* sp. nov., unfortunately, we had only one live specimen available for cytogenetic analysis, and a great deal of additional material from the type location would be required to answer this question. This wide-sampling analysis might also help to clarify what role chromosomal rearrangements could play in causing multivalent associations in karyotype differentiation and by extension how they participate in the reproductive isolation of closely related species in the genus *Heterometrus*.

Acknowledgements

OK would like to express thanks to Šárka Mašová and Chaowalit Songsangchote for their companionship during the expedition to southern Thailand. The cytogenetic analysis was supported by grant received from Ministry of Education, Youth and Sports of the Czech Republic no. SVV 260 313 / 2016. The internship of third author in Thailand was partially funded by European Social Fund and the state budget of the Czech Republic under the project Indicators of Trees Vitality Reg. No. CZ.1.07/2.3.00/20.0265.

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Received: December 21, 2015
Accepted: May 10, 2016

ČLÁNEK V

Zoologischer Anzeiger 273 (2018) 173–182

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz

Zoologischer Anzeiger

Application of cytogenetic markers in the taxonomy of flat rock scorpions (Scorpiones: Hormuridae), with the description of *Hadogenes weygoldti* sp. n.☆

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ARTICLE INFO

Article history:
Received 20 October 2017
Received in revised form 9 January 2018
Accepted 9 January 2018
Available online 11 January 2018
Corresponding Editor: Gabriele B Uhl

Keywords:
Karyotype
18S rDNA
Telomere
FISH
Cytotaxonomy
New species

ABSTRACT

In the present study, we performed the first comparative cytogenetic study in *Hadogenes* species using both standard and molecular cytogenetic approaches. Information about the diploid set, number and distribution of 18S rDNA and telomeric sequences was obtained from three South African species, *Hadogenes trichiurus* (Gervais, 1843), *H. zuluanus* Lawrence, 1937 and *H. weygoldti* sp. n.. All species analysed differ considerably in the number of chromosomes (*H. trichiurus* 2n = 48, *H. zuluanus* 2n = 80, *H. weygoldti* sp. n. 2n = 113). In contrast, the number of 18S rDNA clusters and distribution of telomeric sequences represent rather stable cytogenetic characters in *Hadogenes*. Within all karyotypes, we identified one pair of 18S rDNA clusters. The telomeric signals were exclusively on the terminal chromosomal regions. Interestingly, the chromosomal location of 18S rDNA clusters varied from terminal to interstitial in species karyotypes, indicating the presence of hidden structural chromosomal changes. Additionally, the present comparative study is complemented by the description of a new species, *H. weygoldti* sp. n., based on specific karyotype features and morphological characters. Finally, our cytogenetic results are compared with known chromosomal data of other *Hadogenes* species, and the use of cytogenetic approaches in the taxonomy of scorpions is discussed.

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1. Introduction

The scorpions represent ancient arachnids known by their typical appearance, which has not changed much compared to 400 million year old fossils (Dunlop, 2010). This uniform external morphology has often complicated the higher classification and reconstruction of their phylogenetic relationships, which are still the subject of discussion (see Prendini and Wheeler, 2005; Sharma et al., 2015; Soleglad and Fet, 2003a). It is therefore not surprising that the morphological uniformity of certain structures presents difficulties in species delimitation in this order.

The analysis of DNA is a powerful tool for detecting phylogenetically separated lineages, and its use facilitates correct interpretation of morphological variability. This approach helps to detect cryptic genetic diversity, as was shown in widespread taxa, such as the genera *Euscorpius* (Parmakelis et al., 2013) or *Buthus* (Sousa et al., 2012). Knowledge of the genetic structure of analysed species may also help to understand intraspecific variability that may be erroneously interpreted as interspecific differences (see e.g. Kovařík et al., 2017). It should be noted that the delimitation of species based only upon DNA analysis also is rather disputable and may overestimate species diversity, especially in sedentary organisms (e.g. Opatova and Arnedo, 2014; Parmakelis et al., 2013).

Another useful method that can provide insight into cryptic diversity is karyotype analysis. In some morphologically uniform groups karyotypes may vary considerably and have helped to detect cryptic species in different arachnid orders (e.g. Ojanguren-Affilastro et al., 2017; Řezáč et al., 2007; Zaragoza and Šťáhlavský, 2008). The scorpions display great variability in diploid numbers

☆ This article is part of a special issue entitled "In honor of Peter Weygoldt", published in the Journal Zoologischer Anzeiger 273, 2018.

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https://doi.org/10.1016/j.jcz.2018.01.007
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that range from 5 up to 175 (see Schneider et al., 2017). Such distinctive karyotype differences suggest the potential use of cytogenetic markers in solving intricate taxonomic problems. Moreover, the broad variability of cytogenetic markers is magnified by the holocentric or monocentric chromosome organization in this order. These two types of chromosomes basically differ in the distribution pattern of the centromeric areas. While monocentric chromosomes have kinetic activity restricted to a single chromosomal area (i.e. centromere), holocentric chromosomes lack such a localized centromeric region and display kinetic activity diffused along the length of the chromosome (e.g. Melters et al., 2012). The holocentrics are typical for Buthidae, the best cytogenetically explored scorpion family (e.g. Schneider et al., 2009a). This type of chromosome has evolved independently only a few times in arachnids: in some mites (Wrensch et al., 1994) and two families of spiders (Král et al., 2006). Due to their holocentric nature, buthid chromosomes may tolerate frequent fragmentation or fusion, and this is probably a factor underlying high intraspecific variability observed mainly in some *Tityus* species, e.g. *T. bahiensis* (2n = 5–19) (Schneider et al., 2009a) or *T. obscurus* (2n = 11–16) (Almeida et al., 2017). This high intraspecific variability and similar appearance of holocentric chromosomes may complicate direct application of cytogenetic data to taxonomy of this group. On the other hand, the cytogenetic characterization, such as number and position of nucleolar organizing regions (NORs), seems to be stable in buthid scorpions (e.g. Mattos et al., 2014; Sadílek et al., 2015), and differences in this marker can assist in distinguishing species with similar holocentric chromosomes (Ojanguren-Affilastro et al., 2017).

In contrast to the Buthidae, the chromosomes in the other studied families are monocentric (Schneider et al., 2009b), and these scorpions also exhibit distinctive karyotype differences. In the better explored genus *Urodacus* (Urodacidae) from South Australia, multiple chromosomal differences were found not only between seven species examined, but also within their populations. The highest intraspecific variability was found in *U. novaehollandiae* (2n = 66–175) and *U. manicatus* (2n = 29–64), and the karyotypes differing in chromosome numbers were treated as 'cytotypes' (Shanahan, 1989). Shanahan (1989) assumed that populations with different 'cytotypes' may represent several cryptic species. Unfortunately, the cytogenetic data were not used in any subsequent study on morphology or variability of DNA sequences to test this hypothesis.

Within the scorpions, only the genus *Hadogenes*, endemic to the southern part of Africa with 19 recognized species (Rein, 2017), was subject to such a complex analysis that demonstrated that the karyotypes of twelve species differ considerably (2n = 36–174) and this data has taxonomic potential (Newlands, 1980; Newlands and Cantrell, 1985). The utilization of cytogenetic markers in the taxonomy of this genus is limited by the fact that neither Newlands (1980) nor Newlands and Cantrell (1985) specified the locality of the karyotyped samples, and only presented the diploid numbers of chromosomes without providing details about their morphology.

For this reason, we analysed three remaining unkaryotyped species of *Hadogenes* from the southern limit of distribution of the genus of flat rock scorpions to expand our knowledge about the karyotype differences in this genus. Moreover, we also used fluorescence *in situ* hybridization (FISH) to identify the number and position of the 18S rRNA genes and the telomeric sequence, which is the first implementation of this method in the family Hormuridae. Both markers are frequently used for the analysis of karyotype differences and identification of specific chromosomal changes during the karyotype evolution of different taxa (e.g. Grzywacz et al., 2011; Nguyen et al., 2010; Panzera et al., 2012; Rovatsos et al., 2015). However, within scorpions they were only used frequently in buthids with holocentric chromosomes (Adilardi et al., 2014, 2015, 2016; Almeida et al., 2017; Mattos et al., 2014; Ojanguren-Affilastro et al., 2017; Sadílek et al., 2015; Schneider and Cella, 2010).

2. Material and methods

2.1. Sampling

We analysed three species of flat rock scorpion belonging to the family Hormuridae from different localities in South Africa:

Hadogenes trichiurus (Gervais, 1843): Free State: Bankfontein (30.073683°S, 24.883627°E), 1 female, 2 juveniles.

Hadogenes weygoldti sp. n.: Northern Cape: Sutherland (32.289692°S, 20.599503°E), 1 male holotype.

Hadogenes zuluanus Lawrence (1937): KwaZulu-Natal: Ophathe Game Reserve (28.374147°S, 31.389749°E), 1 male, 1 female, 1 juvenile.

2.2. Chromosome preparation and karyotype analysis

Chromosome slides were made from male and female gonads or from mesenteron tissue of juveniles as described by Plíšková et al. (2016). The testes were dissected and exposed to initial hypotonic treatment in 0.075 M KCl for 20 min, then fixed in methanol:acetic acid solution (3:1) for 30 min. Thereafter, tissue fragments were macerated in 60% acetic acid and the resultant cell suspension was spread on slides using a preheated histological plate (45 °C). The chromosome preparations were stained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 30 min. Additionally, we used the C-banding technique according to the standard protocol of Sumner (1972). In this case, the chromosome slides were stained with DAPI (FluoroshieldTM; Sigma-Aldrich) and the images were inverted in Adobe Photoshop CS4 11.0 for higher contrast. Chromosome morphology was documented by an Olympus AX70 Provis microscope using an Olympus DP72 camera and QuickPHOTO CAMERA v2.3 software (Promicra). For *Hadogenes weygoldti* sp. n. and *H. trichiurus*, five mitotic metaphases were selected for karyotype analysis. In *H. zuluanus* we measured five meiotic postpachytenes and we computed the mean values for homologue chromosomes within pairs. Chromosome measurements were performed for the diploid set length (DSL) using ImageJ v1.45r (Schneider et al., 2012) with the plugin Levan (Sakamoto and Zacaro, 2009) (Table A.1). Morphology of chromosomes was classified based on the nomenclature presented by Green and Sessions (1991).

2.3. Probes

Total genomic DNA was extracted from *Euscorpius sicanus* using the Tissue Genomic DNA kit (Geneaid). Obtained gDNA was used as a template for amplification of the 18S rDNA fragment. Unlabelled 18S rDNA probe for FISH was generated by the polymerase chain reaction (PCR) with the use of the 18S-Gal forward (5′-CGAGCGCTTTTATTAGACCA-3′) and 18S-Gal reverse primers (5′-GGTTCACCTACGGAAACCTT-3′), following the conditions described by Fuková et al. (2005). Unlabelled telomeric probe $(TTAGG)_n$ was performed through non-template PCR using the $(TTAGG)_4$ and $(CCATT)_4$ primers, according to protocol described by Sahara et al. (1999). Both probes for FISH were labelled with biotin-14-dUTP by nick translation using a Nick Translation Kit (Abbott Molecular).

2.4. *Fluorescence* in situ *hybridization (FISH)*

For both probes prepared, FISH was carried out according to Fuková et al. (2005), with minor modifications. Briefly, the chromosome slides were initially treated with RNase A (200 μg/ml in 2 x saline-sodium citrate (SSC) for 60 min (37 °C), then denatured

in 70% formamide in 2 x SSC for 3 min 30 s (68 °C). Afterwards, a probe mixture containing 20 ng of probe and 25 ng of salmon sperm DNA in 10 μL of 50% formamide, 10% dextran sulfate in 2 x SSC (per slide) was denatured, applied to slides, and the hybridization process took place overnight (37 °C). The following day, all non-specifically bound probe was removed in a series of stringent washes. The probes were detected with Cy3-conjugated streptavidin and resulting signals were subsequently amplified by biotinylated anti-streptavidin and Cy3-conjugated streptavidin. Chromosomes were counterstained with DAPI (FluoroshieldTM; Sigma-Aldrich) and observed by an Olympus IX81 microscope equipped with an ORCA-AG monochromatic charge-coupled device camera (Hamamatsu). The images were pseudocoloured (red for Cy3 and blue for DAPI) and superimposed with CellR̂ software (Olympus Soft Imaging Solutions GmbH).

2.5. Taxonomy

Nomenclature and measurements follow Stahnke (1971), Kovařík (2009), and Kovařík and Ojanguren-Affilastro (2013), except for trichobothriotaxy (Vachon, 1974) and sternum (Soleglad and Fet, 2003b). Hemispermatophore terminology follows the revised, unified scheme of Monod et al. (2017), except instead of 'stem' we retained the equivalent term 'trunk' for: (i) preservation of nomenclatural stability, as it is widely used in taxonomic and zoological literature, and (ii) consistency with usage of the term 'truncal flexure'. The capsule region was optically cleared by proteolytic digestion (1 mg/ml bovine trypsin, 4 mM $CaCl_2$, 200 mM Tris-NaOH, pH 7.8, 50 °C, 45 min) (Lowe, 2001). Images of serial focal planes were acquired in 15 μm steps and stereoscopic imagery was rendered by Zerenestacker 1.02.

3. Results

3.1. Karyotype analysis

The mitotic metaphases of *Hadogenes weygoldti* sp. n. contain the highest diploid number (2n = 113) compared to the other species analysed in this study (Fig. 1A). Chromosomes in the karyotype gradually decrease in size from 1.96 to 0.36% DSL (Table A.1). The morphology of individual chromosomes could not be determined due to the absence of clearly recognizable centromeres in mitotic chromosomes even after the application of C-banding technique. The pair of 18 rDNA clusters is located in the interstitial region of chromosomes that differ slightly in length, 0.9 and 0.6% DSL (Fig. 1B). The repetitive telomeric sequences $(TTAGG)_n$ revealed two signals at the terminal regions of all the chromosomes without any interstitial location (Fig. 1C).

Hadogenes zuluanus possesses 80 chromosomes in the mitotic metaphase (Fig. A.1G) and 40 bivalents in postpachytene (Fig. 1D). Analysis of meiotic phases (late postpachytene, metaphase II), with chromosomes possessing a well-recognized centromere, enabled us to determine their morphology (Fig. A.1B and C). The karyotype consists of 7 pairs of metacentric (pairs No. 2, 3, 5, 8, 9, 12, 20), two pairs of submetacentric (pairs No. 7, 11), one pair of subtelocentric (pair No.1) and 30 pairs of acrocentric chromosomes (Table A.1). The C-banding confirmed the position of constitutive heterochromatin in centromeric region in the majority of large chromosomes in late postpachytene. Moreover, we identified one additional large block of constitutive heterochromatin on short arm of one pair of chromosomes (Fig. A.1F and H). However, we did not identify conspicious blocks of constitutive heterochromatin in centromeric region especially in small chromosomes during postpachytene (compare Fig. A.1E and F) and in the majority of chromosomes during mitotic meptaphase (compare Fig. A.1G and H). The first pair of chromosomes is considerably longer (2.70% DSL) than the remaining chromosomes, which gradually decrease in length from 1.91 to 0.69% DSL (Table A.1). During meiosis we did not detect any heteromorphic pairs of chromosomes (Fig. A.1A and B) or any atypical chromosome behaviour (Fig. A.1A–D). One pair of 18S rDNA clusters is present at the terminal region of metacentric chromosome pair No. 5 (Fig. 1E). FISH with the repetitive telomeric sequences $(TTAGG)_n$ revealed two signals at the terminal regions in all of the chromosomes without any interstitial locations (Fig. 1F).

The mitotic metaphases of *Hadogenes trichiurus* exhibits the lowest number of chromosomes in this study, 2n = 48 (Fig. 1G). Chromosomes in the karyotype gradually decrease in size from 3.24 to 1.31% DSL (Table A.1). As in the case of *H. weygoldti* sp. n., the analysed C-banded mitotic metaphases did not allow us to establish the morphology of the chromosomes in the karyotype. One pair of the 18S rDNA signals is located at the terminal region of the long chromosome pair (Fig. 1H). The repetitive telomeric sequences $(TTAGG)_n$ are located at the terminal regions in all of the chromosomes without any interstitial signal (Fig. 1I).

3.2. Taxonomy

Hadogenes weygoldti sp. **n**. (Figs. 2–5, A.2–A.5)

Type locality: RSA, Sutherland, 32.289692°S 20.599503°E.

Type material: RSA, Sutherland, 32.289692°S 20.599503°E, 1♂ (holotype) ♀ (paratype), leg. local collector. Types are deposited in the public collection of Faculty of Science, Charles University in Prague.

Etymology: A patronym in honor of Prof. Peter Weygoldt, Germany, for his friendship and lifelong dedication to arachnids.

Diagnosis: Total length 44–48 mm. Color yellowish brown. External trichobothria on pedipalp patella number 35–37; ventral trichobothria on patella number 11–13; femur with three trichobothria, of which only one is dorsal. Ventral edge of cheliceral movable finger without large basal denticle. Sternum longer than wide, type 2 (Soleglad and Fet, 2003b) with posterior emargination and convex lateral lobes. Metasomal segment V with single ventral carina. Male has markedly longer metasoma than female. All metasomal segments longer than wide in both sexes. Telson elongate. Pectinal teeth number 6–7 in female and 11 in male. Legs with one pedal spur; retrolateral spur absent; lateroapical margins of tarsi straight; setation formula of tarsomere II is 3/3.

Description: The adults are 44.3 mm (male) and 48 mm (female) long. The habitus is shown in Fig. 2A–D.

3.2.1. Sexual dimorphism

The mesosoma is matte in the male and glossy in the female; the male has markedly longer metasomal segments (Figs. A.3A–F) and strong pedipalp finger lobe/socket, and the proximal gap is missing in the female (Fig. 3B and K).

3.2.2. Trichobothria (Fig. 3A–G)

Neobothriotaxic major, type C. Pedipalp patella with 35–37 external and 11–13 ventral, one internal, and two dorsal, totally with 49–53 trichobothria; chela with *dt*, *dst*, *dsb*, *db*, *Dt*, *Db*, *et*, *est*, *esb*, *eb*, *Et* 1–5, *Est*, *Eb* 1–3, *Esb*, *it*, *ib*, 18–20 additional external and 19–23 ventral, totally with 59–65 trichobothria; femur with *i*, *d*, *e*, totally with three trichobothria. Total number of trichobothria is 111–121.

3.2.3. Coloration (Fig. 2A–D)

The base color is uniformly yellowish brown. The pedipalp fingers and internal carinae of femur and patella of pedipalp and legs are black.

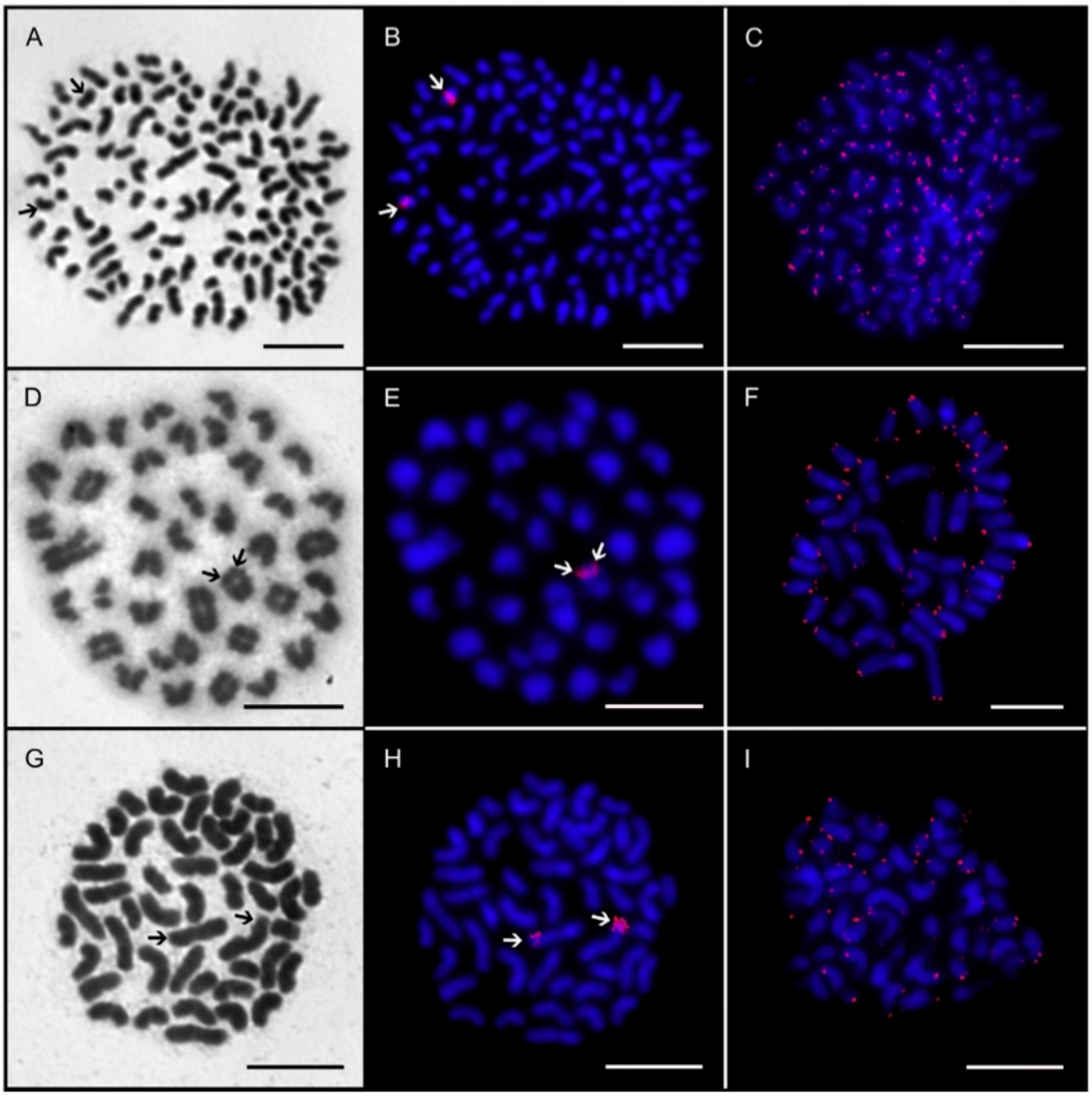

Fig. 1. Chromosomes of *H. weygoldti* sp. n. (2n = 113) (A–C), *H. zuluanus* (2n = 80) (D–F), *H. trichiurus* (2n = 48) (G–I) after Giemsa staining (A, D, G) and after FISH with 18S rDNA (B, E, H) and telomeric sequences $(TTAGG)_n$ (C, F, I). (A–C) Mitotic metaphase (A and B correspond to the same cell). (D and E) The same cell of late postpachytene. (F) Early postachytene. (G–I) Mitotic metaphase (G and H correspond to the same cell). Arrows indicate the position of 18S rDNA. Bar = 10 μm.

3.2.4. Pedipalps (Fig. 3A–L)

The pedipalps are sparsely hirsute and finely granulated. The femur and patella bear four granulate carinae; the ventroexternal and ventrointernal carinae are incomplete; the dorsoexternal and dorsointernal carinae are completely developed. The chela bears two smooth externoventral and externodorsal carinae. The dentate margin of the movable finger is armed with two parallel rows of denticles extending the entire length of the finger.

3.2.5. Metasoma and telson (Fig. A.3A–F)

The metasoma and telson are sparsely hirsute and finely granulated. The metasomal segments I–IV bear a total of 6 often incomplete carinae. The four ventral carinae are reduced on metasomal segments I and more developed on metasomal segments II–IV. The dorsolateral carinae are reduced on metasomal segments I–II and more developed on metasomal segments II–IV; on the third and mainly fourth metasomal segments they terminate in a distinct tooth mainly in the male. The first segment is wider than deep posteriorly. The fifth segment bears five carinae (two dorsal and three ventral), ventral carinae are composed of coarse pronounced granules. The telson is elongated, with the aculeus shorter than the vesicle.

3.2.6. Carapace (Fig. A.2A–B)

The carapace is densely finely granulated, longer than wide, lacks carinae but has a deep sagittal furrow with forked, V-shaped furrow on each side of the posterior part. The anteromedial margin of the carapace is strongly concave. There is a pair of median eyes and three lateral eyes. The carapace and mesosoma are densely, finely granulated.

3.2.7. Mesosoma (Fig. A.2A–C, H)

The mesosoma is densely, finely granulated. Tergites I–II can lack carinae, whereas tergites III–VI bear a carina. Tergite VII bears three incomplete carinael traces. Sternites are smooth with no carinae but bear two symmetric furrows. The sternite V lacks posterior lateral depressions. The pectinal tooth count is 6–7 in female and 11 in male.

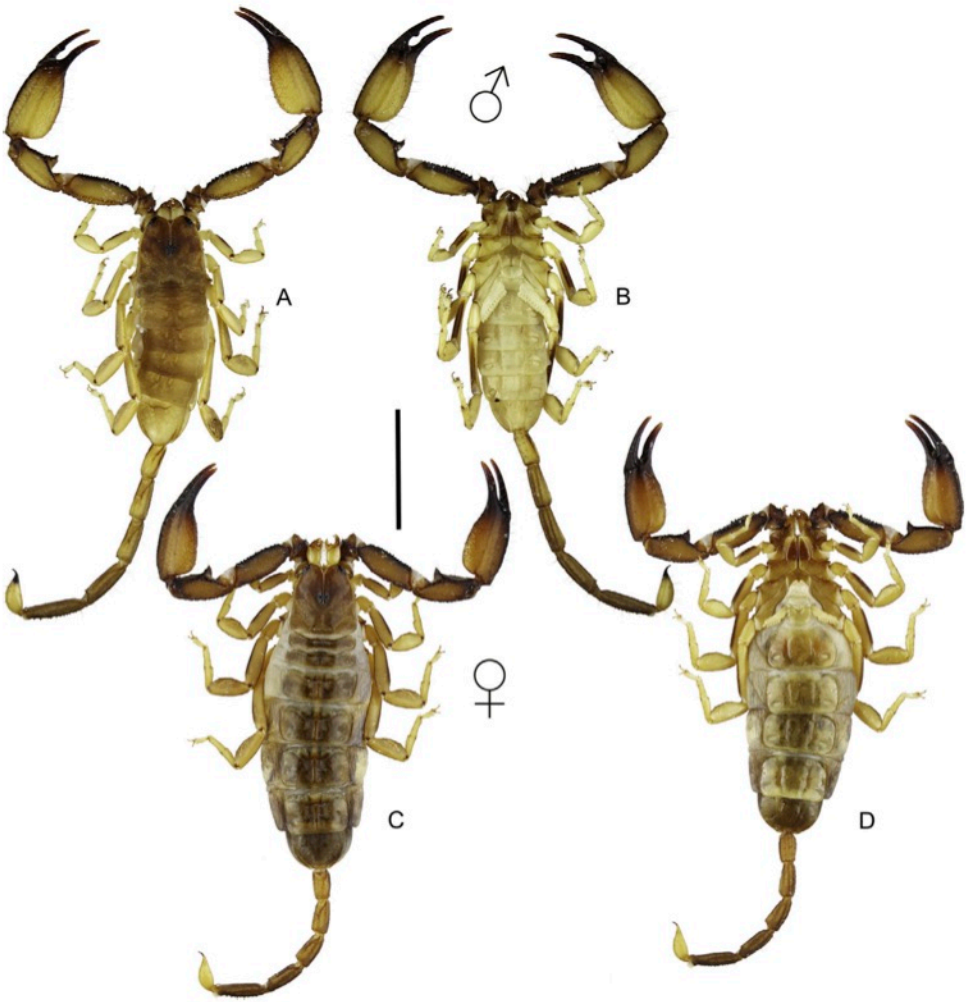

Fig. 2. Habitus of *Hadogenes weygoldti* sp. n. (A) Holotype male, dorsal view. (B) Holotype male, ventral view. (C) Paratype female, dorsal view. (D) Paratype female, ventral view. Bar = 10 mm.

3.2.8. Legs (Fig. A.2D–G)

The legs with one pedal spur, retrolateral spur absent. The lateroapical margins of tarsi straight. The tarsomeres are hirsute with setae and macrosetae. Spiniform formula of tarsomeres II is 3/3.

3.2.9. Hemispermatophore (Fig. 4A–F)

Lamelliform, long and narrow, distal lamina longer than trunk, with conspicuous double hook along basal anterior margin. Section of lamina distal to hook bent anteriorly by angle of about 30° relative to basal section. Apex of lamina rounded, antero-distal margin smooth without a pronounced crest. Hemisolenos tapered, apical margin rounded; clasper prominent, strongly curved with blunt tip. This overall hemispermatophore morphology and structure of the capsule region are similar to what has been reported for other *Hadogenes* spp. (Lamoral, 1979; Monod and Lourenço, 2005; Monod et al., 2017; Newlands and Prendini, 1997; Prendini, 2001, 2005, 2006). However, a distinctive feature is the proximal section of the distal lamina below the hook being exceptionally short, comprising only 14.4% of the total distal lamina length (Fig. 4A–B). In 11 other species of *Hadogenes*, the range was 15.8% – 26.3% (based on published illustrations or photos). In a subset of 11 species, the relative lengths of proximal sections could be either short or long in larger hemispermatophores (taking stalk length as a size metric), but was only short in smaller hemispermatophores (c.f. plot of Fig. 5B with empty upper diagonal area). Length of stalk (i.e. portion of hemispermatophore distal to truncal flexure) was well correlated with carapace length (Fig. 5A; R = 0.901, F = 34.61, P = 0.00037; N = 10, linear regression based on published data), and was thus a good proxy for size of males. The relatively abbreviated proximal distal lamina of *H. weygoldti* sp. n. appears to be related to the fact that it is the smallest member of the genus.

3.2.10. Measurements in mm

Total length of male holotype 44.3; carapace length 6.05, posterior width 5.8; metasoma and telson length 25.23; first metasomal segment length 3.25, width 1.55; second metasomal segment length 3.8, width 1.4; third metasomal segment length 4.0, width 1.2; fourth metasomal segment length 4.75, width 1.2; fifth metasomal segment length 5.23, width 1.17; telson length 4.2; telson width 1.3; telson depth 1.16; pedipalp femur length 6.45, width 2.45; pedipalp patella length 6.1, width 2.65; chela length 11.15; manus width 4.0; manus depth 1.98; movable finger length 5.4.

Total length of female paratype 48; carapace length 6.2, posterior width 6.12; metasoma and telson length 21; first metasomal segment length 2.65, width 1.8; second metasomal segment length 3.03, width 1.2; third metasomal segment length 3.2, width 1.25; fourth metasomal segment length 3.8, width 1.2; fifth metasomal segment length 4.4, width 1.07; telson length 3.9; telson width 1.22; telson depth 1.13; pedipalp femur length 6.1, width 2.25; pedipalp patella length 5.85, width 2.5; chela length 10.9; manus width 3.7; manus depth 1.87; movable finger length 5.4.

Fig. 3. Morphology of *Hadogenes weygoldti* sp. n. (A–C) Male holotype, right pedipalp chela dorsal (A), external (B) and ventrointernal (C). (D–F) Right patella dorsal (D), external (E) and ventral (F). (G–I) Right femur dorsal (G), external (H) and ventral (I). Trichobothrial pattern is indicated (terminology according to Vachon (1974). (J–L) Female paratype, right chela dorsal (J) and external (K), and granulation of movable finger (L). (M and N) Female paratype, chelicera in dorsal (M) and ventral (N) views.

3.2.11. Affinities

H. weygoldti sp. n. is the smallest species of the genus *Hadogenes*. Most of the *Hadogenes* species have total length from 90 to 210 mm. Only *H. tityrus* (total length 50–80 mm) and *H. lawrencei* (total length 47–49.5 mm) have a similar length to *H. weygoldti* sp. n. Both of these species differ from *H. weygoldti* sp. n. morphologically by the shape of the pedipalp segments, which are extremely elongated and narrow, and by sexual dimorphism in the length of the metasoma, which is present in *H. weygoldti* sp. n. and absent in *H. lawrencei* and *H. tityrus* which have the metasoma the same length in both sexes (Fig. A.5A–D). An important difference is in the number of chromosomes: *H. lawrencei* has 2n = 132 and *H. tityrus* 2n = 168 (Newlands, 1980; Newlands and Cantrell, 1985), whereas in *H. weygoldti* sp. n. there is 2n = 113.

3.2.12. Ecological notes (Fig. A.4A–C)

Terrain at the type locality consists of barely vegetated rocky hills with abundant rock slabs and crevices. The surrounding areas are flat and uniformly covered with shrubs. This habitat is characterized by hot summers with sporadic rainfall and cool winters without rain. The type specimens were located in dense populations at the tops of the hills under large and very flat rocks. Their habits differed from other *Hadogenes* species in that they also sheltered under rocks situated on bare soil, not just between rocks and in rock crevices. In this dense population, we some-

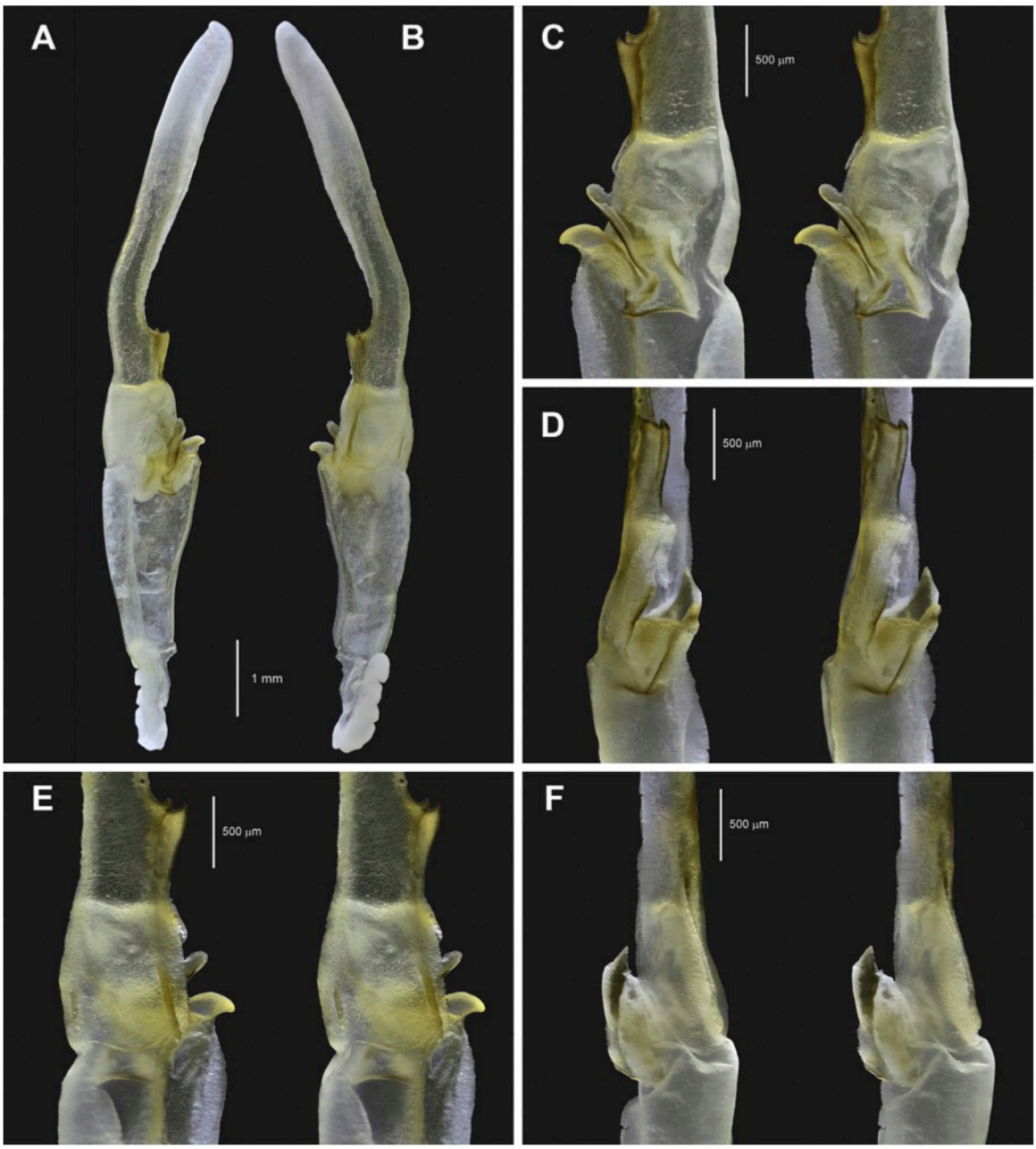

Fig. 4. Hemispermatophore of *Hadogenes weygoldti* sp. n. (A and B) Male holotype, left hemispermatophore, contralateral (interior, concave) (A), and lateral (exterior, convex) (B) views. (C–F) Male holotype, capsule region of right hemispermatophore (enzymatically cleared), contralateral (interior, concave) (C), anterior (sperm duct margin) (D), lateral (exterior, convex) (E), and posterior (F) cross-stereoscopic views. Bar = 1 mm (A and B), 500 µm (C–F).

times observed several juvenile individuals of different instars living together under the same rock, which indicates a very low level of intraspecific aggression. During the summer, females were observed to be heavily gravid or to have recently given birth. The size of litters carried by females ranged between 12 and 15. Individuals at all stages of development were observed. Males were very rare compared to juveniles and females. These were freshly molted to adulthood or waiting directly next to a gravid female, which indicates a mate-guarding behaviour. Other scorpions species observed in this area were *Opistophthalmus austerus* and *Parabuthus capensis* on the flats and *Uroplectes carinatus* at the foothill.

4. Discussion

The scorpions have a wide range of chromosome numbers (2n = 5–175), and the diploid numbers of chromosomes very often differ considerably between species (see Schneider et al., 2017). This suggests that even the basic characteristics of karyotypes seem to be useful for application in scorpion taxonomy, at least in some groups of this morphologically uniform order. However, the direct application of specific cytogenetic markers in delimitation of new scorpion species is still not routinely implemented. Moreover, we have still only limited information about intraspecific variability in this order. The scorpions represent the third best explored arachnid order from the cytogenetic point of view, with 122 karyotyped species, but for taxonomic purposes we do not have enough karyotyped species suitable for comparison. At present, there are only ten genera that have more than three species karyotyped (see Schneider et al., 2017).

Another significant limitation on the application of the older cytogenetic information for taxonomy use is that many of the older studies did not specify sampling localities very precisely,

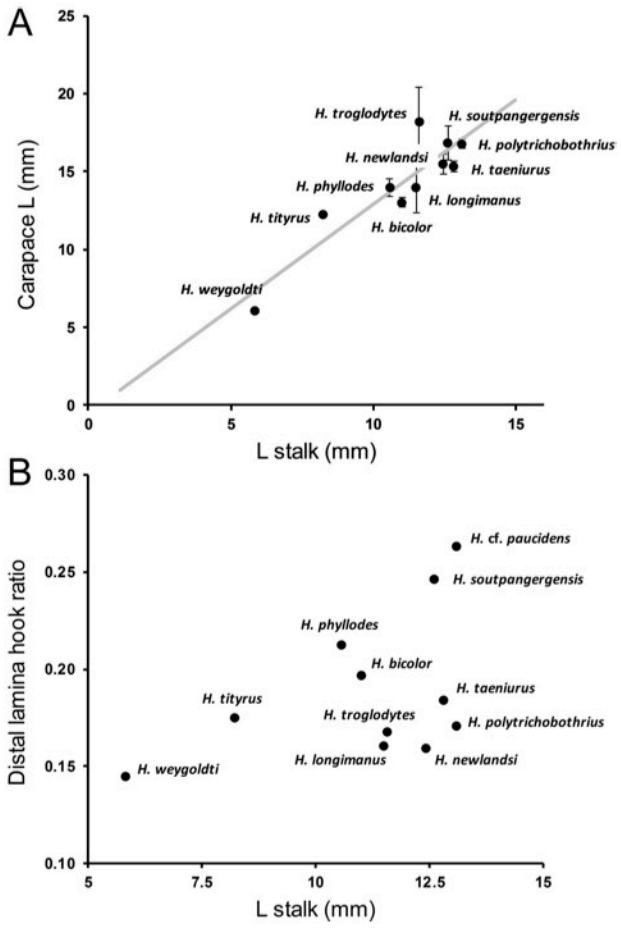

Fig. 5. Comparative biometrics of hemispermatophore distal lamina of *Hadogenes* spp. (A) Scatter plot of carapace length vs. stalk length for hemispermatophores of 10 species of *Hadogenes*. Gray line is a least squares regression. (B) Scatter plot of distal lamina hook ratio (=length of distal lamina from transverse ridge to base of hook/total length of distal lamina) vs. stalk length for hemispermatiophores of 11 species of *Hadogenes*. We computed total length of distal lamina = length of distal lamina from transverse ridge to base of hook + length of distal lamina from base of hook to apex (along an oblique axis, if angled). Measurements from other *Hadogenes* spp. were estimated from published figures (Lamoral, 1979; Monod and Lourenço, 2005; Monod et al., 2017; Prendini, 2001, 2005, 2006). We omitted *H. zumpti* from these plots because Newlands and Prendini (1997) neglected to label the scale bar in their hemispermatophore figure. However, if its hemispermatophore size is correlated with body size as in other species, we predict that it would be consistent with the distribution of points in Fig. 5B. Indeed, it is larger than *H. weygoldti* sp. n., and it has a larger distal lamina hook ratio.

and did not preserve specimens for subsequent taxonomic revision (e.g. Shanahan, 1989; Venkatanarasimhaiah and Rajasekarasetty, 1964a,b). This problem is evident especially in genus *Heterometrus*. In this genus we have information about karyotypes in nine species: *H. bengalensis* (2n = 64) (Sharma et al., 1962), *H. fulviceps* (2n = 86 and 88) (Sharma et al., 1962; Venkatanarasimhaiah and Rajasekarasetty, 1964a), *H. gravimanus* (Venkatanarasimhaiah and Rajasekarasetty, 1964b), *H. longimanus* (2n = 64) (Srivastava and Agrawal, 1961), *H. minotaurus* (2n = 54) (Plíšková et al., 2016), *H. scaber* (2n = 96) (Venkatanarasimhaiah and Rajasekarasetty, 1964a), *H. spinifer* (2n = 56) (Vítková et al., 2005), *H. swammerdami* (2n = 60) (Venkatanarasimhaiah, 1965), *H.* sp. (2n = 62) (Rajasekarasetty et al., 1979). However, the descriptions of karyotypes were published mainly during the 1960s and the definition of some *Heterometrus* taxa has changed significantly since then (e.g. Couzijn, 1981; Kovařík, 2004). Therefore, in this genus matching the karyotypes to the correct species is not possible in some cases, and the utilization of older cytogenetic data in taxonomy is consequently disputable (see Plíšková et al., 2016).

The situation is different in the genus *Hadogenes*. We have available information about the karyotypes of 14 species (Newlands, 1980; Newlands and Cantrell, 1985; present study), which makes it the best explored genus of scorpions with monocentric chromosomes (see Schneider et al., 2017). Newlands (1980) and Newlands and Cantrell (1985) demonstrated considerable differences of the diploid numbers between species (2n = 36–174), and these interspecific traits were also supported by electrophoretic data from venom proteins. They recognized the importance of species-specific chromosome characteristics in the taxonomy of this genus, and incorporated their cytogenetic data into their determination key as one of the valid species characteristics. These results were later revised with the study of additional material, and some species were formally described or redescribed (Newlands and Prendini, 1997; Prendini, 2005, 2006).

Our results support the importance of cytogenetic data in the taxonomy of this genus. We identified another three species-specific karyotypes that differ considerably, and cytogenetic markers helped us to recognize the new species *H. weygoldti* sp. n. The distribution pattern of *Hadogenes* generally displays allopatric or parapatric distribution (Newlands, 1980) (Fig. 6), and the differentiation of the karyotypes may ensure an effective postzygotic barrier (King, 1993). Based on the data obtained here, we are not able to precisely determine the mechanisms of karyotype differentiation in *Hadogenes* species. Limited information about the morphology of individual chromosomes in the karyotypes and the unknown phylogenetic structure of *Hadogenes* species do not allow us to track multiple chromosomal changes. Unfortunately, the C-banding method did not allow us to specify the positions of all centromeric regions in analysed species. Absence of visible centromeres complicated the specification of chromosomal morphology, especially in karyotypes described only after mitotic metaphases. Considering this fact, the analysis of meiotic stages with visible centromere (postpachytene or metaphase II) seems to be more precise for the description of karyotypes. The blocks of constitutive heterochromatin in centromeric regions are probably very small in scorpions. It is one reason why they were not visibile on all chromosomes in other scorpions which also have monocentric chromosomes such as *Urodacus* (Urodacidae) (Shanahan, 1989), *Bothriurus* (Bothriuridae) (Schneider et al., 2009b), and *Heterometrus* (Scorpionidae) (Plíšková et al., 2016). Nevertheless, in view of the fact that the majority of the biarmed chromosomes in *H. zuluanus* represent the long chromosomes, the fusions/fissions of chromosomes seem to be the main mechanism of differentiation of karyotypes. These types of chromosomal rearrangements may be heterozygous and produced multivalents in scorpions with monocentric chromosomes (Plíšková et al., 2016; Schneider et al., 2009b; Shanahan, 1989). The heterozygous fusions/fissions of chromosomes may also explain the odd number of chromosomes in *Hadogenes weygoldti* sp. n. (2n = 113) seen in our study as it was also proposed in *Euscorpiops orioni* (2n = 103) (Euscorpiidae) (Kovařík et al., 2015) or *Urodacus* species (Shanahan, 1989). In contrast to the high variability of diploid numbers, the number of NORs seems to be stable in flat rock scorpions. We identified only one pair of 18S rDNA clusters in all three analysed species. This number is typical for majority of analysed scorpions (e.g. Adilardi et al., 2014, 2015; Mattos et al., 2014; Sadílek et al., 2015) and the one pair of NORs in a terminal position on chromosomes seems to be ancestral for all arachnid orders (Forman et al., 2013). Interestingly, *H. weygoldti* sp. n. has 18S rDNA clusters placed in an interstitial position, which may be a consequence of chromosome rearrangements (peri- or paracentric inversions, tandem fussions), transposable element insertions or ectopic recombination in this species (see Cabrero and Camacho, 2008). We identified telomeric

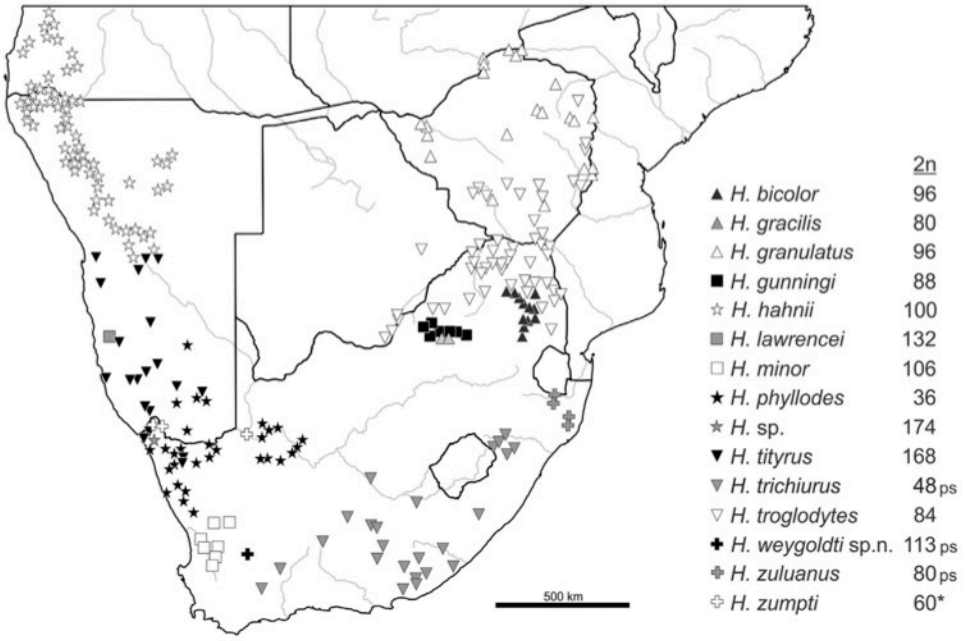

Fig. 6. Geographical distributions of *Hadogenes* species with the known diploid number of chromosomes, based on material examined in present study (ps), Newlands (1980) and Prendini (2005, 2006). The asterisk describes approximate number of chromosomes.

signals exclusively on terminal chromosomal regions in all three analysed species. We did not detect any intestitial telomeric clusters that may reflect the remnants of chromosomal fusion events as was documented in some other organisms (e.g. Pellegrino et al., 2009). The interstitial telomeric sequence is probably eliminated rapidly after chromosomal fusions or is lost during breakage preceding the fusions in scorpions. This is supported by the fact that interstitial signals were not detected even in *Tityus confluens*, a species with intensive chromosomal fusions (Adilardi et al., 2016).

Our results indicate the importance of cytogenetic analysis in scorpions and demonstrate that cytogenetic characterization may represent a valuable tool in the taxonomy of this morphologically uniform order.

Acknowledgments

We would like to thank Pavel Just, and Vera Opatova for their help in laboratory and to Charles R. Haddad for his valuable help in the field in KwaZulu-Natal and Free State and during the preparation of the manuscript. We also thank to anonymous reviewer and Andrés A. Ojanguren-Affilastro for valuable comments and suggestions that improved this paper. The project was supported by Czech Republic Ministry of Education, Youth and Sports grant SVV 260 434/2017 and by grants from the NRF of South Africa (No's 97495 and 105318). Microscopy was performed in the Laboratory of Confocal and Fluorescence Microscopy, co-financed by the European Regional Development Fund and the state budget of the Czech Republic. Project no. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcz.2018.01.007.

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Appendix A

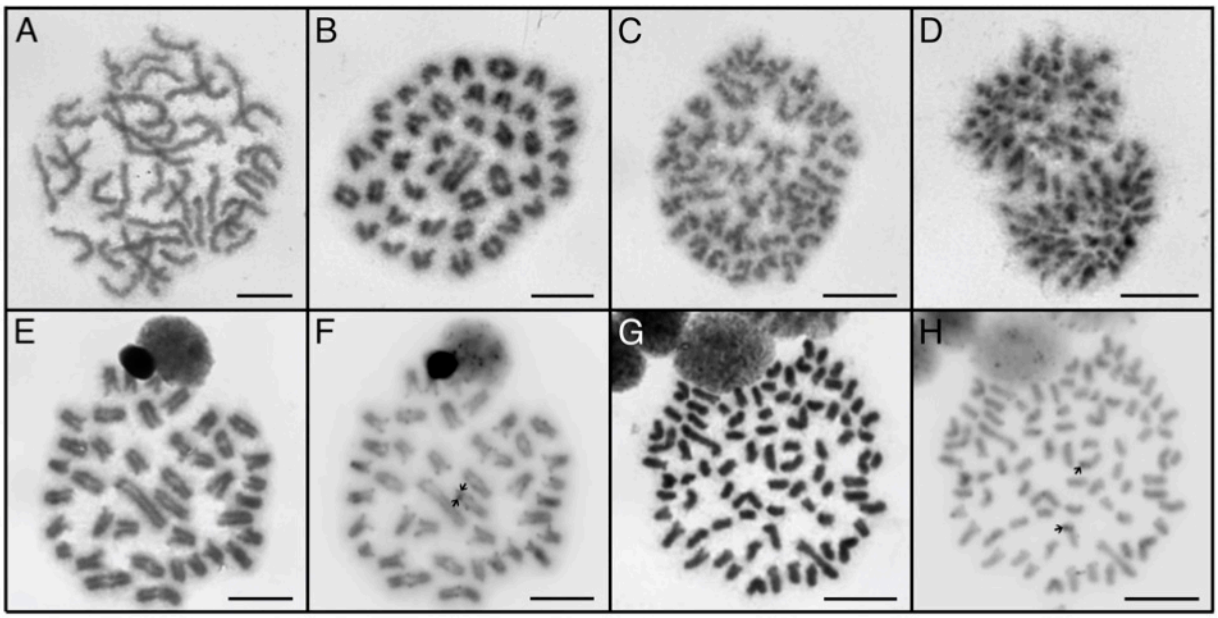

Figure A.1 Meiosis of *Hadogenes zuluanus*. (A) Early postpachytene. (B) Late Postpachytene. (C) One sister metaphase II. (D) Two sister anaphases II. Bar = 10 μm.

Errata:

Figure A.1 Meiotic and mitotic phases of *Hadogenes zuluanus*. (A) Early postpachytene. (B) Late postpachytene. (C) One sister metaphase II. (D) Two sister anaphases II. (E) Late postpachytene. (F) The same cell after C-banding. (G) Mitotic metaphase. (H) The same cell after C-banding. Arrows indicate blocks of constitutive heterochromatin. Bar = 10 μm.

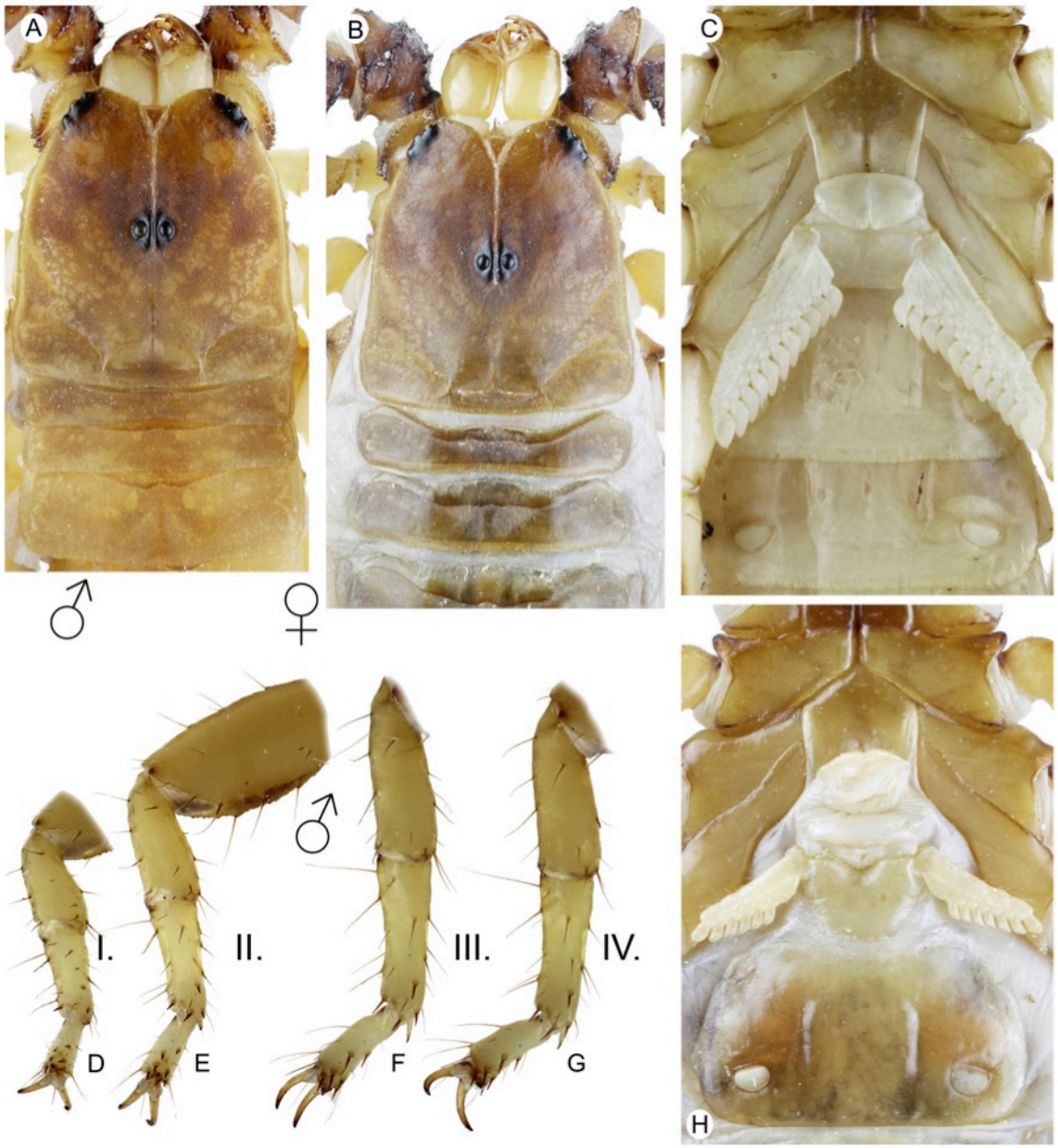

Figure A.2 Morphology of *Hadogenes weygoldti* sp. n. (A) Male holotype, carapace and tergites I–III. (B) Female paratype, carapace and tergites I–III. (C) Male holotype, coxosternal area and sternites III–IV. (D-G) Male holotype, spiniform setation of tarsomeres of right legs I–IV, retrolateral aspect. (H) Female paratype, coxosternal area and sternite III.

Figure A.3 Morphology of *Hadogenes weygoldti* sp. n. (A-C) Male holotype, metasoma and telson, lateral (A), dorsal (B), and ventral (C) views. (D-F) Female paratype, metasoma and telson, lateral (D), dorsal (E), and ventral (F) views. Bar = 10 mm.

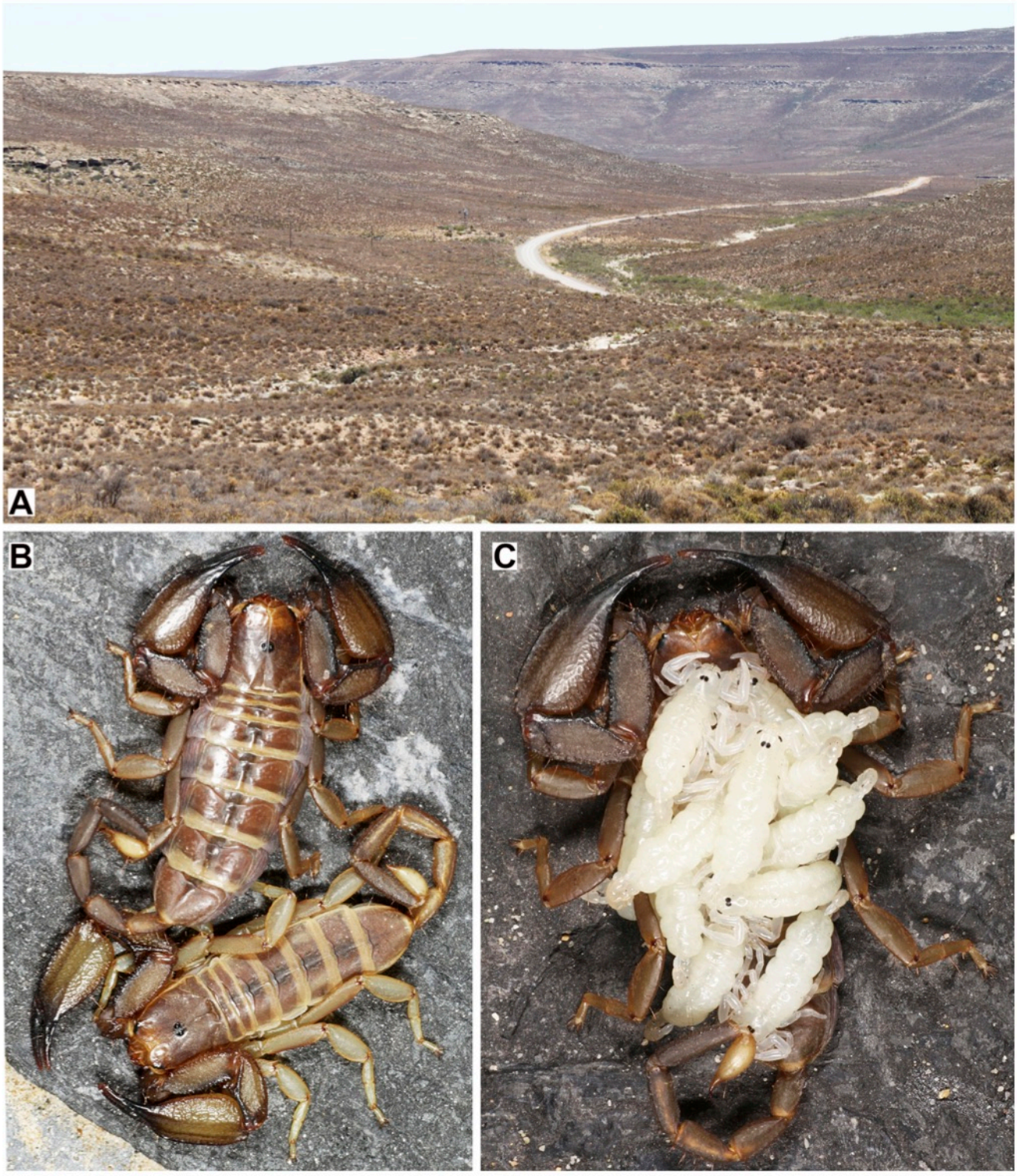

Figure A.4 The type locality of *Hadogenes weygoldti* sp. n. (A), male holotype and female paratype (B), and not type female with newborns (C).

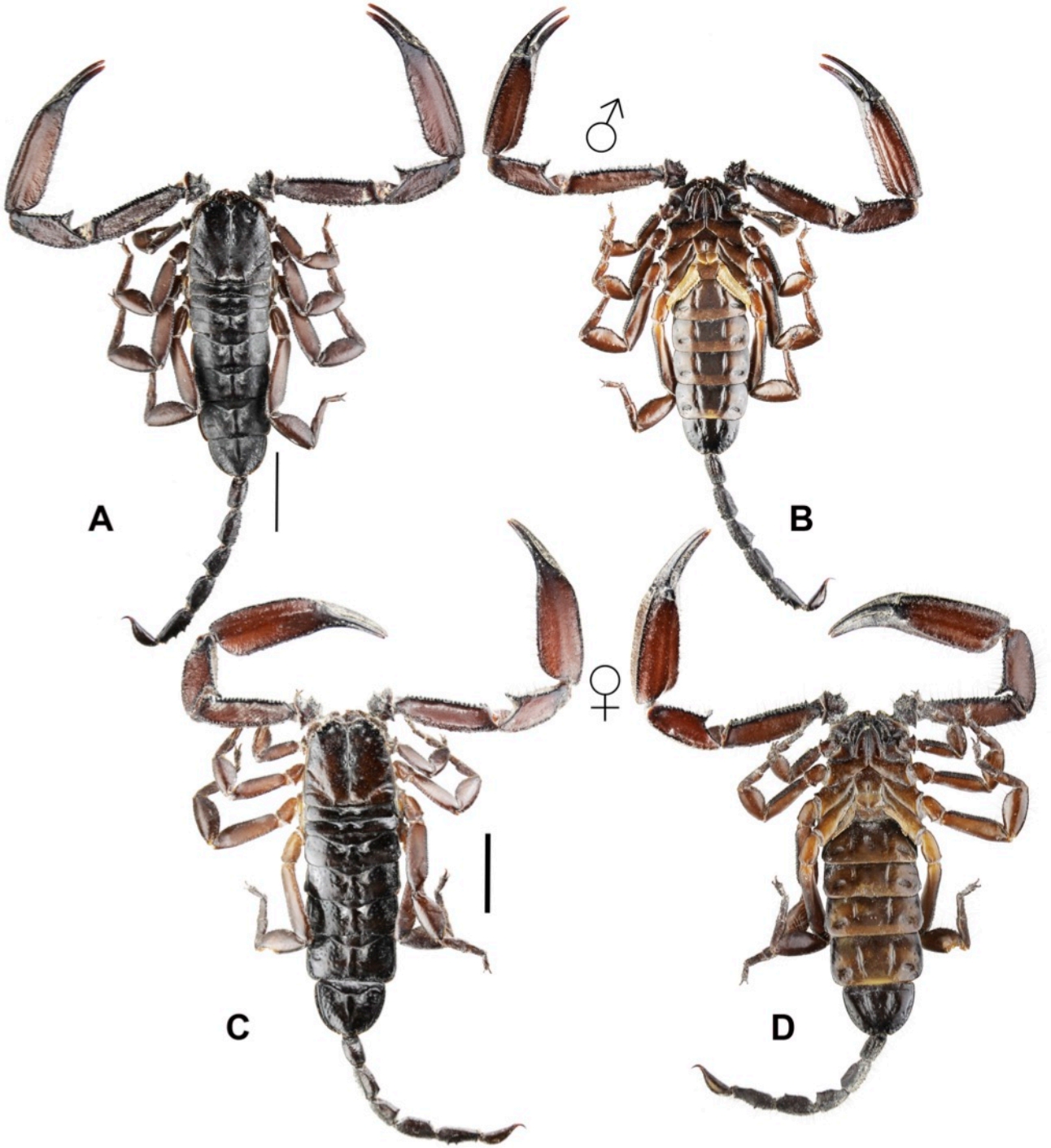

Figure A.5 Habitus of *Hadogenes tityrus* (Simon, 1888) from RSA, Port Nolloth, 29.3041653°S 17.0764537°E. (A) Male, dorsal view. (B) Male, ventral view. (C) Female, dorsal view. (D) Female, ventral view. Bar = 10 mm.

Table A.1 Measurements of the relative chromosome length (% DSL) of analysed *Hadogenes* species from South Africa and in brackets arm ratio of *H. zuluanus*. ± shows standard deviation.

Chrom. No.	*H. trichiurus* (2n=48)	*H. weygoldti* (2n=113)	*H. zuluanus* (2n=80)
1	3.24 ± 0.09	1.96 ± 0.16	2.70 ± 0.16 (5.66 ± 0.99)
2	3.14 ± 0.09	1.74 ± 0.14	
3	3.00 ± 0.06	1.69 ± 0.13	1.91 ± 0.32 (1.26 ± 0.22)
4	2.82 ± 0.04	1.64 ± 0.11	
5	2.78 ± 0.04	1.54 ± 0.09	1.69 ± 0.18 (1.33 ± 0.12)
6	2.73 ± 0.04	1.48 ± 0.03	
7	2.70 ± 0.01	1.45 ± 0.03	1.61 ± 0.12 (>7)
8	2.65 ± 0.02	1.44 ± 0.03	
9	2.63 ± 0.01	1.40 ± 0.05	1.59 ± 0.07 (1.54 ± 0.58)
10	2.49 ± 0.08	1.39 ± 0.05	
11	2.47 ± 0.06	1.37 ± 0.05	1.54 ± 0.10 (>7)
12	2.38 ± 0.03	1.35 ± 0.05	
13	2.36 ± 0.02	1.34 ± 0.05	1.50 ± 0.34 (1.75 ± 1.17)
14	2.31 ± 0.05	1.33 ± 0.05	
15	2.28 ± 0.06	1.31 ± 0.04	1.49 ± 0.36 (1.30 ± 0.25)
16	2.25 ± 0.03	1.30 ± 0.03	
17	2.23 ± 0.03	1.27 ± 0.02	1.47 ± 0.15 (1.37 ± 0.23)
18	2.20 ± 0.02	1.26 ± 0.02	
19	2.19 ± 0.02	1.25 ± 0.02	1.44 ± 0.08 (>7)
20	2.14 ± 0.04	1.24 ± 0.02	
21	2.11 ± 0.03	1.21 ± 0.02	1.39 ± 0.25 (1.71 ± 0.80)
22	2.08 ± 0.01	1.19 ± 0.02	
23	2.06 ± 0.02	1.18 ± 0.02	1.38 ± 0.11 (1.57 ± 0.58)
24	2.05 ± 0.02	1.17 ± 0.02	
25	2.03 ± 0.01	1.16 ± 0.01	1.38 ± 0.07 (>7)
26	1.97 ± 0.03	1.16 ± 0.01	
27	1.95 ± 0.04	1.14 ± 0.01	1.35 ± 0.07 (>7)
28	1.93 ± 0.03	1.13 ± 0.01	
29	1.91 ± 0.01	1.12 ± 0.02	1.32 ± 0.05 (>7)
30	1.90 ± 0.01	1.11 ± 0.02	
31	1.88 ± 0.01	1.11 ± 0.02	1.30 ± 0.05 (>7)
32	1.86 ± 0.02	1.10 ± 0.02	
33	1.84 ± 0.02	1.06 ± 0.02	1.28 ± 0.06 (>7)
34	1.80 ± 0.03	1.06 ± 0.01	
35	1.74 ± 0.04	1.05 ± 0.02	1.24 ± 0.04 (>7)
36	1.74 ± 0.04	1.04 ± 0.02	
37	1.71 ± 0.02	1.03 ± 0.02	1.21 ± 0.03 (>7)
38	1.66 ± 0.01	1.02 ± 0.02	
39	1.62 ± 0.02	1.01 ± 0.02	1.20 ± 0.15 (1.22 ± 0.14)
40	1.57 ± 0.01	1.00 ± 0.03	
41	1.55 ± 0.02	0.99 ± 0.03	1.20 ± 0.04 (>7)
42	1.54 ± 0.01	0.98 ± 0.03	
43	1.52 ± 0.03	0.98 ± 0.03	1.18 ± 0.03 (>7)
44	1.46 ± 0.03	0.96 ± 0.03	
45	1.46 ± 0.03	0.94 ± 0.03	1.16 ± 0.04 (>7)
46	1.38 ± 0.06	0.94 ± 0.03	
47	1.35 ± 0.04	0.93 ± 0.04	1.14 ± 0.04 (>7)
48	1.31 ± 0.02	0.93 ± 0.03	
49		0.92 ± 0.04	1.12 ± 0.05 (>7)
50		0.91 ± 0.03	
51		0.91 ± 0.03	1.11 ± 0.05 (>7)
52		0.89 ± 0.10	
53		0.89 ± 0.03	1.10 ± 0.05 (>7)
54		0.88 ± 0.02	
55		0.87 ± 0.02	1.08 ± 0.07 (>7)
56		0.87 ± 0.01	
57		0.85 ± 0.01	1.05 ± 0.06 (>7)
58		0.84 ± 0.02	
59		0.82 ± 0.01	1.03 ± 0.05 (>7)
60		0.82 ± 0.01	
61		0.82 ± 0.01	1.01 ± 0.04 (>7)
62		0.80 ± 0.03	
63		0.79 ± 0.03	1.00 ± 0.05 (>7)
64		0.78 ± 0.02	

65		**0.78 ± 0.02**	**0.97 ± 0.04 (>7)**
66		0.77 ± 0.02	
67		0.76 ± 0.01	0.95 ± 0.06 (>7)
68		0.76 ± 0.02	
69		0.74 ± 0.03	0.92 ± 0.05 (>7)
70		0.73 ± 0.03	
71		0.72 ± 0.03	0.90 ± 0.05 (>7)
72		0.70 ± 0.04	
73		0.68 ± 0.04	0.87 ± 0.06 (>7)
74		0.67 ± 0.02	
75		0.66 ± 0.03	0.80 ± 0.06 (>7)
76		0.65 ± 0.02	
77		0.65 ± 0.03	0.73 ± 0.04 (>7)
78		0.65 ± 0.10	
79		0.65 ± 0.03	0.69 ± 0.05 (>7)
80		0.64 ± 0.02	
81		0.63 ± 0.02	
82		0.62 ± 0.03	
83		0.61 ± 0.03	
84		0.61 ± 0.02	
85		0.60 ± 0.04	
86		0.59 ± 0.03	
87		0.58 ± 0.03	
88		0.57 ± 0.03	
89		0.56 ± 0.03	
90		0.56 ± 0.03	
91		0.56 ± 0.03	
92		0.54 ± 0.03	
93		0.53 ± 0.03	
94		0.53 ± 0.04	
95		0.53 ± 0.04	
96		0.52 ± 0.03	
97		0.51 ± 0.03	
98		0.50 ± 0.03	
99		0.50 ± 0.03	
100		0.49 ± 0.02	
101		0.49 ± 0.02	
102		0.48 ± 0.02	
103		0.47 ± 0.03	
104		0.47 ± 0.02	
105		0.47 ± 0.02	
106		0.45 ± 0.03	
107		0.45 ± 0.03	
108		0.44 ± 0.02	
109		0.43 ± 0.03	
110		0.43 ± 0.02	
111		0.42 ± 0.03	
112		0.40 ± 0.03	
113		0.36 ± 0.05	

ČLÁNEK VI

Molecular Phylogenetics and Evolution 134 (2019) 152–163

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Cryptic diversity and dynamic chromosome evolution in Alpine scorpions (Euscorpiidae: *Euscorpius*)

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ARTICLE INFO

Keywords:
Cryptic species
Karyotype evolution
Phylogeography
Evolution
Alps
Species delimitation

ABSTRACT

Over time, mountain biota has undergone complex evolutionary histories that have left imprints on its genomic arrangement, geographical distribution and diversity of contemporary lineages. Knowledge on these biogeographical aspects still lags behind for invertebrates inhabiting the Alpine region. In the present study, we examined three scorpion species of the subgenus *Euscorpius* (*Alpiscorpius*) from the European Alps using cytogenetic and molecular phylogenetic approaches to determine the variation and population structure of extant lineages at both chromosome and genetic level, and to provide an insight into the species diversification histories. We detected considerable intraspecific variability in chromosome complements and localization of the 18S rDNA loci in all studied species. Such chromosome differences were noticeable as the existence of three [in *E.* (*A.*) *alpha* and *E.* (*A.*) *germanus*] or four [in *E.* (*A.*) *gamma*] range-restricted karyotypic races. These races differed from one another either by 2n [in *E.* (*A.*) *alpha* 2n = 54, 60, 90; in *E.* (*A.*) *gamma* 2n = 58, 60, 88, 86–92], or by the karyotypic formula [in *E.* (*A.*) *germanus* 2n = 34m + 12sm; 36m + 10sm; 42m + 4sm]. Using mitochondrial (16S rRNA, COI) and nuclear (28S rDNA) genetic markers, we examined genetic variation and reconstructed phylogenetic relationships among the karyotypic races. Both approaches provided evidence for the existence of ten deeply divergent lineages exhibiting the features of local endemics and indicating the presence of cryptic species. Molecular dating analyses suggest that these lineages diversified during the Plio-Pleistocene and this process was presumably accompanied by dynamic structural changes in the genome organization.

1. Introduction

The Alps, the most extensive high mountain system in Europe, have greatly influenced the phylogeography of European biota (Hewitt, 2004). In the past, Alpine species were repeatedly confronted with severe geo-climatic changes and their evolutionary success thus depended on their inherent ecological requirements and migratory abilities (Gugerli et al., 2001; Hewitt, 2004; Louy et al., 2014). These often complex evolutionary histories have inevitably left an indelible imprint on the genetic structure and geographical distribution of species (Hewitt, 2004; Schmitt, 2007). The present-day biodiversity of the Alpine region has been traditionally interpreted in the light of Pleistocene climatic events (Schmitt, 2009; Tribsch and Schönswetter, 2003; Varga and Schmitt, 2008), while the current pattern of local endemism has been considered a relic of refugia, in which particular taxa have persisted over time (Tribsch, 2004; Tribsch and Schönswetter, 2003). Such refuges were especially important for less vagile, stenotopic taxa that could not have migrated across inhospitable mountain barriers, which resulted in their geographical isolation and subsequent diversification (Avise, 2000, 2009; Hausdorf and Hennig, 2003; Schmitt, 2009).

However, diversification of mountain species does not have to necessarily result from recent Quaternary climatic fluctuations but may reflect older events coincident with the orogenic processes during the Miocene and Pliocene (Bryson et al., 2013; Buckley and Simon, 2007; Ceccarelli et al., 2016; Derkarabetian et al., 2011; Riberon et al., 2001).

The evolutionary process of speciation can be accompanied not only by accumulation of point mutations in DNA sequence of the emerging species, but also by changes in their genome organization. Such changes in the genome structure may greatly contribute to development of post-zygotic isolation that serves as an effective barrier to gene flow (King, 1993; White, 1969). White (1978) proposed that the chromosomal changes seem to be involved in speciation particularly in organisms of

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https://doi.org/10.1016/j.ympev.2019.02.002
Received 25 May 2018; Received in revised form 9 January 2019; Accepted 3 February 2019
Available online 08 February 2019
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restricted vagility, whose populations persist in the same area for many generations. The involvement of chromosome rearrangements in speciation, however, arouses controversy and remains the subject of debate (Faria and Navarro, 2010; Rieseberg, 2001; Sankoff, 2003). To better understand the role of chromosomal changes in species diversification, correlation between chromosomal and genetic variability needs to be examined in the context of species' geographical distributions (e.g. Fernandes et al., 2009; Kawakami et al., 2011; Mills and Cook, 2014; Morgan-Richards, 1997).

Scorpions represent a suitable model group to study the potential impact of chromosomal changes on species diversification, as generally they show high variability in diploid numbers of chromosomes (2n = 5–175) (e.g. Newlands and Cantrell, 1985; Shanahan, 1989; Schneider et al., 2018). The correct interpretation of karyotype differences between and within species requires detailed knowledge of phylogenetic relationships of the taxa in question (Kawakami et al., 2011; Morgan-Richards, 1997; Sember et al., 2015). However, this approach has not yet been applied to scorpions.

Members of the subgenus *Euscorpius* (*Alpiscorpius*) (Euscorpiidae) represent range-restricted arachnids, with limited dispersal ability, and narrow ecological preferences (Vignoli and Salomone, 2008). Of the five currently recognized species of the subgenus, three are distributed throughout the European Alps - *E.* (*A.*) *alpha* Caporiacco, 1950, *E.* (*A.*) *gamma* Caporiacco, 1950 and *E.* (*A.*) *germanus* (C. L. Koch, 1837). Species boundaries of the Alpine scorpions have been under debate due to their apparent morphological uniformity (Bonacina, 1980; Caporiacco, 1950; Gantenbein et al., 1999). The use of genetic markers has allowed recognition of three well separated lineages within the Alps and two previously recognized subspecies of *E.* (*A.*) *germanus* were raised to species level as *E.* (*A.*) *alpha* and *E.* (*A.*) *gamma* (Gantenbein et al., 2000; Scherabon et al., 2000). In a pilot genetic study, Gantenbein et al. (2000) proposed that the speciation process of the extant Alpine scorpions predates the Quaternary glaciation events. Based upon the scorpion-specific 16S substitution rate and genetic distances between the species, they estimated the time of the divergence between *E.* (*A.*) *alpha* and *E.* (*A.*) *germanus* to be the Late Pleistocene [2–3 million years ago (Ma)]. However, these results still need to be corroborated by reconstructing a time-calibrated phylogeny of all three species with a fine-scale sampling. Within the whole family of Euscorpiidae, only a single species has been examined cytogenetically so far (Sokolov, 1913). As such, there is no information available on their inter- or intraspecific karyotype variability.

The Alpine species of the subgenus *Euscorpius* (*Alpiscorpius*) thus provide an opportunity for an interdisciplinary study combining cytogenetic and molecular approaches to elucidate the extent of lineage diversification and to establish fundamental mechanisms of species evolution in the context of historical processes in the European Alps. This integrative approach enabled us to reveal potential cryptic lineages and to determine whether scorpions as sedentary organisms exhibit higher accumulation of chromosomal changes and complex genetic structure. A multilocus, time-calibrated phylogeny of all Alpine species from the subgenus *Euscorpius* (*Alpiscorpius*) allowed us to reconstruct the history of their diversification in the topographically complex Alpine ranges and to estimate potential historical events that might have promoted speciation in the group.

2. Material and methods

2.1. Specimens examined

The material studied represents three Alpine species of the subgenus *Euscorpius* (*Alpiscorpius*): *E.* (*A.*) *alpha*, *E.* (*A.*) *germanus*, and *E.* (*A.*) *gamma*. Specimens were systematically collected across the wide area of their distribution (Fig. 1). In total, we obtained 149 individuals from 74 localities (Table S1). Chromosome preparations were made, and leg muscle tissue was taken from freshly dissected specimens. The tissue samples were preserved in 96% ethanol at −20 °C for DNA extraction. All voucher specimens examined were preserved in 80% ethanol for further morphological determination. The species were identified using a key by Vignoli and Salomone (2008), complemented with faunistic and geographical data for each species published by Fet et al. (2001) and Gantenbein et al. (2000). All specimens sampled are deposited in the arachnid collection of the National Museum, Prague, Czech Republic (NMP).

2.2. Chromosome preparation and karyotype analysis

Chromosome slides were prepared from gonads according to Plíšková et al. (2016). Briefly, the procedure involved initial hypotonic treatment of tissue in 0.075 M KCl (20 min), followed by fixation in a methanol-acetic acid solution (3:1; 30 min), maceration in a drop of 60% acetic acid, and spreading of the resulting cell suspension on slides on a heating plate (see Traut, 1976). The chromosome preparations were subsequently stained in 5% Giemsa solution (30 min). Slides selected for the FISH technique remained unstained and were dehydrated with the ethanol series (70, 80 and 96%; 1 min each), air-dried and stored at −20 °C.

Giemsa stained chromosomes were examined and documented through an Olympus AX70 Provis microscope using an Olympus DP72 camera and QuickPHOTO CAMERA v.2.3 software (Promicra). For each karyotypic race, ten spermatocyte nuclei with clearly discernible centromeric regions were selected for a detailed karyotype analysis. The relative diploid set length (DSL) was measured and calculated using ImageJ v1.45r (Schneider et al., 2012) with the plugin Levan (Sakamoto and Zacaro, 2009). This plugin utilizes modified criteria for chromosome classification described by Levan et al. (1964) and Green and Sessions (1991). The morphology of chromosomes was categorized based on nomenclature by Green and Sessions (1991). Metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes were considered bi-armed.

2.3. Fluorescence in situ hybridization with the 18S rDNA probe

Arrayed genes for major ribosomal RNAs (rDNA) were detected by means of FISH with the biotin-labelled probe previously used with success in several arachnid taxa, including scorpions (Forman et al., 2013; Sadílek et al., 2015; Svojanovská et al., 2016). The probe was constructed from a partial sequence of *Dysdera erythrina* (Dysderidae) 18S rDNA according to a protocol described by Forman et al. (2013) and slightly modified by Šíchová et al. (2013). FISH was performed following Fuková et al. (2005). Briefly, chromosome preparations were treated with RNase A and denatured at 68 °C for 3 min 30 s in 70% formamide in 2× SSC. A probe mixture containing about 20 ng of the 18S rDNA probe and 25 μg of salmon sperm DNA in 10 μL of 50% formamide, 10% dextran sulfate in 2× SSC was denatured, applied to slides, and hybridized overnight at 37 °C. The slides were then stripped of the excess and non-specifically bound probe in a series of stringent washes and detected with Cy3-conjugated streptavidin. The resulting signals were further amplified by biotinylated antistreptavidin and Cy3-conjugated streptavidin. Chromosomes were stained by DAPI and slides were mounted in DABCO-based antifade.

Results of FISH were documented by an Olympus IX81 microscope equipped with a monochromatic CCD camera (ORCA-AG, Hamamatsu). The images were pseudocolored (red for Cy3 and blue for DAPI) and superimposed with Cell^R software (Olympus Soft Imaging Solutions). The relative position of the 18S rDNA signal and DSL of its chromosome carrier were determined as described above in Section 2.2.

2.4. DNA extraction, PCR amplification, and sequence analysis

Three genetic markers were selected to reconstruct the phylogeny of Alpine *Euscorpius* (*Alpiscorpius*) species: two fragments of mitochondrial

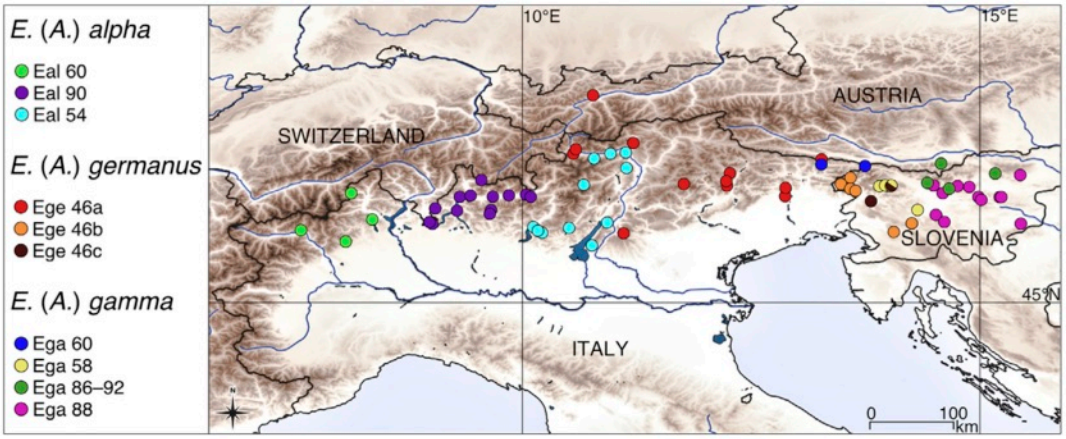

Fig. 1. Map of the geographical distribution of the *Euscorpius* (*Alpiscorpius*) species and their karyotypic races in the Alps [*E.* (*A.*) *alpha*: Eal 60 – karyotypic race I (2n = 60), Eal 90 – karyotypic race II (2n = 90), Eal 54 – karyotypic race III (2n = 54); *E.* (*A.*) *germanus*: Ege 46a – karyotypic race I (2n = 36m + 10sm), Ege 46b – karyotypic race II (2n = 34m + 12sm), Ege 46c – karyotypic race III (2n = 42m + 4sm); *E.* (*A.*) *gamma*: Ega 60 – karyotypic race I (2n = 60), Ega 58 – karyotypic race II (2n = 58), Ega 86–92 – karyotypic race III (2n = 86–92), Ega 88 – karyotypic race IV (2n = 88)].

DNA (mtDNA), namely the ribosomal 16S rRNA (16S; ca. 370 bp) and protein-coding cytochrome *c* oxidase subunit I (COI; ca. 614 bp), and a fragment of the nuclear 28S rDNA gene (28S; ca. 1100 bp).

Genomic DNA was extracted from leg muscle tissue using the Genomic DNA Extraction Kit (Geneaid), following the manufacturer's protocol. The markers were amplified by polymerase chain reaction (PCR) using the primers and PCR conditions listed in Table S2.

Chromatograms were examined and sequences manually edited using Geneious v.6 (Kearse et al., 2012). Homology between obtained sequences and the target genes was checked by NCBI's blastn search (https://blast.ncbi.nlm.nih.gov). In the nuclear 28S gene, heterozygous positions were identified and coded following the IUPAC ambiguity codes. Sequences of the mitochondrial protein-coding gene COI were checked for the presence of stop codons, which would suggest that pseudogenes were amplified.

In total, 90 specimens from 67 localities were included in the genetic analyses (Table S1). Six species representing members of other related subgenera [*E.* (*Polytrichobothrius*) *italicus*, *E.* (*Euscorpius*) *avcii*, *E.* (*E.*) *carpathicus*, *E.* (*E.*) *celanus*, *E.* (*E.*) *erymanthius*, *E.* (*Tetratrichobothrius*) *flavicaudis*] were used as outgroups (Table S1). We aligned the three genes using the online version of MAFFT v.7 (Katoh and Standley, 2013) with the Q-INS-i method applied for the 16S and 28S alignments and the 'auto' settings applied for the COI alignment. Gblocks with low stringency options (Castresana, 2000; Talavera and Castresana, 2007) was applied to the 16S and 28S alignments to trim poorly aligned regions with gaps and terminal regions of various length. This shortened the 16S and 28S alignments to 367 bp and 1100 bp, respectively. The length of the COI alignment was 614 bp.

2.5. Phylogenetic analyses, divergence time estimation and allele network analysis

We reconstructed the phylogeny of the Alpine *Euscorpius* (*Alpiscorpius*) species by means of Bayesian inference in BEAST v.1.8 (Drummond et al., 2012) with two different data sets. Data set 1 consisted of all samples (90 ingroups + 6 outgroups) and all three genes concatenated. Each gene was considered as an independent partition and their best-fit models of nucleotide evolution were identified in jModelTest v.2.1 (Darriba et al., 2012) by the BIC selection criterion. The best models were GTR + I for 16S, GTR + I + G for COI, and HKY + I for 28S. COI was further partitioned by codon positions. Base frequencies were set to empirical and a relaxed uncorrelated lognormal clock prior was selected for all partitions. Other prior settings were as follows: Yule process tree prior with a uniform birth rate (lower: 0, upper: 1000), uniform prior for GTR base substitutions (0, 100), uniform prior for alpha (0, 10), uniform relative rate parameters for codon positions (0, 10), uncorrelated lognormal relaxed clock with mean = 0 and st. dev. = 1 for COI, and mean = 0.1 and st. dev. = 1 for 28S (both relative to the 16S rate). We ran all BEAST analyses three times independently to make sure stationarity and convergence have been reached. The analyses were run for 10^8 generations with parameters logged every 10^5 generations. Posterior trace plots, stationarity, convergence and effective sample sizes (ESS) were inspected in Tracer v.1.5 (Rambaut and Drummond, 2007). We used LogCombiner v.1.7 to combine the three log files and to discard 10% of the posterior trees from each run as burn-in, and TreeAnnotator v.1.7 (both programs are part of the BEAST package) to generate the maximum clade credibility tree (MCC). We also conducted a maximum likelihood (ML) analysis in RAxML-HPC2 v.8.2.9 (Stamatakis, 2014) using a non-parametric bootstrap analysis with 1000 pseudoreplications to assess nodal support. We used the same partitioning scheme as for the BEAST analysis with the GTR + CAT model applied to all partitions. Samples with identical haplotypes were not included in the ML analysis.

Once we recovered the phylogenetic structure within the Alpine *Euscorpius* (*Alpiscorpius*) species using data set 1, we performed a *BEAST analysis (Heled and Drummond, 2010) to infer a time-calibrated phylogeny of the karyotypic races in a coalescent-based species tree framework. This data set (data set 2) contained only samples that had all three genes sequenced, with the exceptions of two samples missing COI and one missing 28S. The samples included in this analysis (59 in total) are listed in Table S1. The outgroup species were not included in this analysis. Alleles of the nuclear 28S gene were inferred using PHASE v.2.1 (Stephens et al., 2001) with the probability threshold set to 0.7 that produced an alignment with no heterozygous positions. We used SeqPHASE (Flot, 2010) for converting the input and output files. Because mtDNA genes share genealogical history, we concatenated the COI and 16S alignments into one partition for this analysis. Evolutionary models were identified as above and were as follows: GTR + I + G for the mtDNA and HKY + I for the 28S. Input units ('species') need to be defined prior to the species tree analysis. Based on the tree structure from analysis of data set 1 in combination with results of the cytogenetic comparisons (see above) we defined 10 'species' as the input units.

We calibrated the species tree using scorpion-specific mutation rates for the two mtDNA genes. Mean mutation rates of 0.005 and 0.007 with a standard deviation of 0.003 and lognormal distributions were selected for 16S and COI, respectively (Gantenbein et al., 2005; Gantenbein and Largiadèr, 2002). Although these evolutionary rates were originally

Table 1
List of the karyotype characteristics in *Euscorpius* (*Alpiscorpius*) in the Alps (DSL – diploid set length, N – number of karyotyped individuals, m – metacentric, sm – submetacentric, st – subtelocentric, t – telocentric, triv – trivalent).

Species	Code	N	2n	Karyotypic formula	rDNA location (DSL)
E. (A.) alpha					
karyotypic race I	Eal 60	5	60	34m + 14sm + 2st + 10t	subterminal m (2.17)
karyotypic race II	Eal 90	24	90	10m + 10sm + 2st + 68t	subterminal t (1.19)
karyotypic race III	Eal 54	24	54	38m + 12sm + 2st + 2t	subterminal sm (2.04)
E. (A.) germanus					
karyotypic race I	Ege 46a	30	46	36m + 10sm	subterminal m (1.77)
karyotypic race II	Ege 46b	13	46	34m + 12sm	subterminal m (1.76)
karyotypic race III	Ege 46c	6	46	42m + 4sm	terminal m (1.74)
E. (A.) gamma					
karyotypic race I	Ega 60	2	60	48m + 12sm	subterminal m (2.17)
karyotypic race II	Ega 58	10	58	42m + 10sm + 2st + 4t	subterminal m (2.31)
karyotypic race III	Ega 86–92	2	86	28m + 2sm + 2st + 54t	subterminal m (2.23)
		2	87	25m + 2sm + 4st + 56t	subterminal triv (2.49)
		1	90	20m + 6sm + 2st + 62t	subterminal m (2.14)
		2	92	20m + 2sm + 4st + 66t	subterminal m (2.23)
karyotypic race IV	Ega 88	18	88	20m + 2sm + 6st + 60t	subterminal t (1.06)
		2	87	21m + 2sm + 6st + 58t	subterminal t
		1	89	19m + 2sm + 6st + 62t	subterminal t

calculated for buthid scorpions (Buthidae), they are widely used for calibrating phylogenies of different scorpion families (Bryson et al., 2013; Bryson et al., 2014; Graham et al., 2013a,b) including Euscorpiidae (Parmakelis et al., 2013) and have also been corroborated by an alternative calibration approach (Ceccarelli et al., 2016). The clock rate for 28S was estimated relative to the mtDNA rates with a lognormal prior distribution and a relative mean rate of 0.1. The other BEAST parameters were set as described above for data set 1. All analyses were run on the CIPRES Science Gateway (Miller et al., 2010).

To infer genealogical relationships between the karyotypic races on the nDNA level alone we used the phased alignment (see above) to reconstruct an allele network for the 28S gene. The network was constructed using the statistical parsimony algorithm (Templeton et al., 1992) as implemented in TCS v.1.21 (Clement et al., 2000) with 95% connection limit. The resulting network was visualized using tcsBU (dos Santos et al., 2016).

2.6. Species discovery approaches

To explore whether the Alpine *Euscorpius* (*Alpiscorpius*) species contain potential cryptic evolutionary lineages not detected by the karyotype analysis we performed three delimitation approaches that do not rely on a priori information regarding sample membership. First, we used the distance-based method implemented in the Automatic Barcode Gap Discovery (ABGD) online software (Puillandre et al., 2012) for the 16S and COI alignments. ABGD identifies the maximum number of evolutionary units the dataset can be partitioned into with the requirement that the genetic distance between any two sequences from different groups is larger than a given threshold (i.e. barcode gap). We used the simple distance option with the relative gap width parameter set to 1.0 and number of bins to 100. The remaining parameters were set to default (P_{min} = 0.001, P_{max} = 0.100, Steps = 10).

Secondly, we estimated boundaries between evolutionary lineages by the general mixed Yule coalescent (GMYC) method (Pons et al., 2006). Contrary to ABGD that compares distances in individual genes, GMYC uses single locus data (e.g. all mtDNA genes) to identify the shift from interspecific (speciation) to intraspecific (coalescence) process of diversification. GMYC needs an ultrametric guide tree that we generated in BEAST based on a concatenated mtDNA dataset as described above in Section 2.5 for data set 1. We ran the analysis in R using the *splits* package (Ezard et al., 2009) under the single-threshold method. Outgroup species were removed from the tree before the analysis.

Finally, we employed the multi-rate Poisson tree process (mPTP) of Kapli et al. (2017) that models intra- and interspecies processes by directly using the number of substitutions represented by branch lengths (Zhang et al., 2013). mPTP is the latest implementation of the PTP methods. Compared to its predecessors, it accounts for divergent intraspecific variation and thus improves the estimate of the number of evolutionary lineages in clades that have different rates of speciation-coalescence across their phylogeny. As in the GMYC analysis, we used a mtDNA-based BEAST tree with the outgroup taxa removed. To run mPTP we used the mPTP web server (http://mptp.h-its.org).

3. Results

3.1. Karyotype variability and genomic location of 18S rDNA

Chromosome characteristics were determined in 142 (138 males, 4 females) of the 149 collected specimens by the analysis of meiotic or mitotic metaphases (Table S1). In general, the species analysed shared common cytogenetic features such as the monocentric type of chromosomes, the presence of homomorphic bivalents (i.e. homologous chromosomes), and achiasmatic male meiosis with the absence of diplotene-diakinesis stages. No morphologically differentiated sex chromosomes and specific heteropycnosis in nuclei were detected.

Detailed karyotype analysis revealed the presence of intraspecific karyotype variation related to the diploid chromosome number (2n) [*E. (A.) alpha*, *E. (A.) gamma*] or karyotypic formula [*E. (A.) germanus*]. Although FISH with the 18S rDNA probe showed a stable number of rDNA clusters (two per 2n), their chromosomal localization differed among the intraspecific karyotypes. Observed intraspecific chromosome differences showed a strong geographical pattern in all species analysed. Based on distinctive karyotype features, geographically delimited populations were recognized as well-defined karyotypic races (Fig. 1, Table S3).

An abbreviation consisting of the species name and chromosome number is assigned to each karyotypic race. For each species, karyotypic races are described according to their geographical distribution from west to east. Cytogenetic results are summarized in Table 1.

3.1.1. Euscorpius (Alpiscorpius) alpha

We detected three highly distinct karyotypic races with an allopatric distribution covering the Northwestern Alps in Italy and Switzerland and the westernmost part of the Italian Southeastern Alps (Fig. 1, Table S3).

Karyotypic race I (Eal 60) exhibits 2n = 60 chromosomes. The

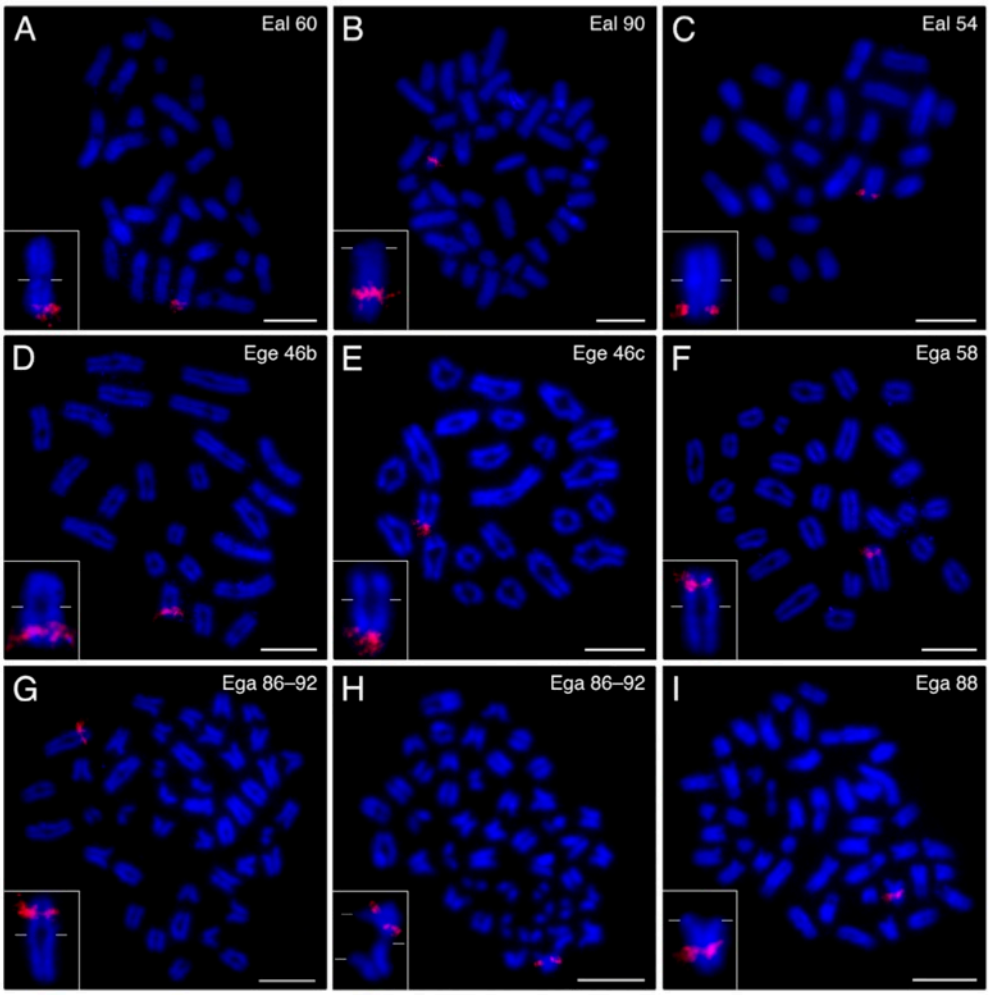

Fig. 2. The localization of the 18S rDNA (red signals) in metaphase I of *Euscorpius* (*Alpiscorpius*) *alpha* (A–C), *E.* (*A.*) *germanus* (D, E), and *E.* (*A.*) *gamma* (F–I) (DAPI counterstaining – blue). (A) Eal 60 – karyotypic race I (2n = 60), (B) Eal 90 – karyotypic race II (2n = 90), (C) Eal 54 – karyotypic race III (2n = 54), (D) Ege 46b – karyotypic race II (2n = 34m + 12sm), (E) Ege 46c – karyotypic race III (2n = 42m + 4sm), (F) Ega 58 – karyotypic race II (2n = 58), (G) Ega 86-92 – karyotypic race III (2n = 86–92), a specimen with 2n = 86, (H) Ega 86-92 – karyotypic race III (2n = 86–92), a specimen with rDNA-bearing trivalent, 2n = 87, (I) Ega 88 – karyotypic race IV (2n = 88). Narrow white lines in the insets indicate position of centromeres. Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

karyotype is composed of 34 metacentric, 14 submetacentric, 2 subtelocentric, and 10 telocentric chromosomes (Fig. S1A). The rDNA clusters are located in the subterminal region of the long arms of the metacentric chromosome pair 4 (Fig. 2A, Fig. S1A).

Karyotypic race II (Eal 90) has 2n = 90 chromosomes. The karyotype is composed of 10 metacentric, 10 submetacentric, 2 subtelocentric, and 68 telocentric chromosomes (Fig. S1B). The rDNA clusters are localized in the subterminal region of the long arms of the telocentric chromosome pair 21 (Fig. 2B, Fig. S1B).

Karyotypic race III (Eal 54) possesses 2n = 54 chromosomes. The karyotype is composed of 38 metacentric, 12 submetacentric, 2 subtelocentric, and 2 telocentric chromosomes (Fig. S1C). The rDNA clusters are located in the subterminal region of the long arms of the submetacentric chromosome pair 23 (Fig. 2C, Fig. S1C).

3.1.2. Euscorpius (Alpiscorpius) germanus

This species occurs in the Eastern Alps in Italy, southern Austria, and western Slovenia where its distribution continues into the high karst plateau of the Dinaric Alps (Fig. 1, Table S3). All specimens from the whole area of distribution exhibit a stable chromosome complement 2n = 46. The karyotypic formula and chromosomal location of the rDNA clusters defined three karyotypic races within *E.* (*A.*) *germanus*.

Karyotypic race I (Ege 46a) has a karyotype composed of 36 metacentric and 10 submetacentric chromosomes (Fig. S1D). The rDNA clusters are situated in the subterminal region of the long arms of the metacentric chromosome pair 11 (Fig. S1D).

Karyotypic race II (Ege 46b) has a karyotype composed of 34 metacentric and 12 submetacentric chromosomes (Fig. S1E). The rDNA clusters are situated in the subterminal region of the long arms of the metacentric chromosome pair 11 (Fig. 2D, Fig. S1E).

Karyotypic race III (Ege 46c) has a karyotype composed of 42 metacentric and 4 submetacentric chromosomes (Fig. S1F). The rDNA clusters are located in the terminal region of the long arms of the metacentric chromosome pair 14 (Fig. 2E, Fig. S1F).

3.1.3. Euscorpius (Alpiscorpius) gamma

We detected four distinct karyotypic races with specific geographical distribution in the Southeastern Alps in Italy, Slovenia and Austria, and the Slovenian Dinaric Alps (Fig. 1, Table S3).

Karyotypic race I (Ega 60) exhibits 2n = 60 chromosomes. The karyotype is composed of 48 metacentric and 12 submetacentric chromosomes (Fig. S2A). In both specimens examined, 28 bivalents and one quadrivalent chain were present in metaphase I. The rDNA clusters are located in the subterminal region of the short arms of the metacentric chromosome pair 6 (Fig. S2A).

Karyotypic race II (Ega 58) has 2n = 58 chromosomes. The karyotype is composed of 42 metacentric, 10 submetacentric, 2 subtelocentric and 4 telocentric chromosomes (Fig. S2B). The rDNA clusters are situated in the subterminal region of the short arms of the metacentric chromosome pair 6 (Fig. 2F, Fig. S2B).

Karyotypic race III (Ega 86–92) possesses a conspicuous variation in diploid chromosome number ranging from 86 to 92, both between and/or within populations (Fig. S2C and D; Table S1). Observed polymorphism, presumably caused by the presence of chromosome fusion/fission events, resulted in four different karyotypes: 2n = 86 (homozygous state, 43 bivalents; Fig. S2C), 2n = 87 (heterozygous state, 42

bivalents and one trivalent; Fig. S3A), 2n = 90 (heterozygous state, 42 bivalents and two trivalents; Fig. S3B) and 2n = 92 (homozygous state, 46 bivalents; Fig. S2D). The rDNA clusters are situated in the subterminal region of the short arms of the largest metacentric chromosome pair 1; in the case of karyotype 2n = 87 it was detected on the heterozygous trivalent (Fig. 2G and H, Fig. S2C and D).

Karyotypic race IV (Ega 88) has 2n = 88 chromosomes. The karyotype is composed of 20 metacentric, 2 submetacentric, 6 subtelocentric and 60 telocentric chromosomes (Fig. S2E). Polymorphism for chromosomal fusion/fission is present in three specimens from different localities (Table S1). Their karyotypes are: 2n = 87 (heterozygous for fusion, 42 bivalents and one trivalent; Fig. S3C) and 2n = 89 (heterozygous for fission, 43 bivalents and one trivalent; Fig. S3D). The rDNA clusters are situated in the subterminal region of the long arms of the telocentric chromosome pair 27 (Fig. 2I, Fig. S2E).

3.2. Phylogenetic analyses, divergence time estimation and allele network analysis

The Bayesian and ML phylogenetic analyses produced mostly similar topologies. The only difference was in *E.* (*A.*) *alpha*, in which the BEAST analysis reconstructed a cluster of all Eal 54 samples (but without support), while in the ML analysis one of the Eal 54 clades was sister to the clade of Eal 60 and Eal 90.

The results of the phylogenetic analysis of data set 1 showed a clear separation of all karyotypic races and their grouping in three distinct clades (Fig. 3). The first clade contained four karyotypic races of *E.* (*A.*) *gamma* (Ega 60, Ega 58, Ega 88, and Ega 86–92), of which Ega 60 was the earliest to diverge and with Ega 58 being sister to the remaining two, Ega 88 and Ega 86–92. The second clade represented the species of a stable chromosome number, *E.* (*A.*) *germanus*, with three genetically distinct and relatively deeply separated lineages that corresponded to the described karyotypic races, Ege 46a from Italy and Austria, and Ege 46b and Ege 46c from Slovenia. Monophyly of all groups and their mutual relationships within *E.* (*A.*) *gamma* and *E.* (*A.*) *germanus* were highly supported (posterior probability (pp) ≥ 0.95). The third clade was formed by three karyotypic races of *E.* (*A.*) *alpha* (Eal 60, Eal 90, Eal 54). Monophyly of the whole *E.* (*A.*) *alpha* clade as well as of the Eal 60 and Eal 90 groups was well supported (pp = 1.0 for all nodes); however, mutual relationships between the groups were not resolved due to low support (pp = 0.91 for a sister relationship of Eal 60 and Eal 90, pp = 0.84 for Eal 54). *E.* (*A.*) *germanus* and *E.* (*A.*) *alpha* were supported as sister species (pp = 1).

Topology of the species tree reconstructed based on data set 2 (Fig. 4A) matched that for data set 1 except that in *E.* (*A.*) *gamma* Ega 60 and Ega 58 were sisters in the species tree, but without support (pp = 0.58). The results of divergence dating indicate that the initial differentiation between the three species took place 11.0 million years ago (Ma), [highest posterior density (HPD) confidence interval: 8.3–13.6], which separated *E.* (*A.*) *gamma* from the other two species and was followed by a split between *E.* (*A.*) *germanus* and *E.* (*A.*) *alpha* 8.6 Ma (HPD: 6.3–10.9). The split within *E.* (*A.*) *gamma* separating the four karyotypic races took place 4.6 Ma (HPD: 3.2–6.0) and the split between Ega 88 and Ega 86–92 took place 1.5 Ma (HPD: 0.8–2.3). The diversification between the karyotypic races of *E.* (*A.*) *germanus* was dated to 4.2 Ma (HPD: 2.9–5.6) and the split between the races Ege 46b and Ege 46c took place 2 Ma (HPD: 1.1–3.0). The basal split within *E.* (*A.*) *alpha* was the youngest of the three species as it was dated to 2.8 Ma (HPD: 1.9–3.8).

The allele network reconstructed for the nuclear 28S gene showed different levels of allele sharing between the karyotypic races of the three species (Fig. 4B). While the three karyotypic races of *E.* (*A.*) *germanus* (Ege 46a, Ege 46b, Ege 46c) did not share any alleles and there was limited allele sharing in *E.* (*A.*) *gamma* only between Ega 88 and Ega 86–92, all three karyotypic races of *E.* (*A.*) *alpha* shared a common central allele, from which only Eal 54 and Eal 60 had other derived alleles.

3.3. Species discovery approaches

The number of evolutionary lineages identified by the different delimitation approaches varied depending on the method (Fig. 3). The three most significant partitions of the ABGD method found nine groups for 16S, while 14 groups were identified for COI. The GMYC analysis recovered 14 lineages according to the likelihood function and the lineage-through-time plot ($logL_{null} = 757.1$, $logL_{GMYC} = 761.0$, LRT = 7.9, $p < 0.05$). The significant result of the LRT indicates that the null model with a single population could be rejected. mPTP partitioned the mtDNA tree into 16 lineages. For full results of the individual delimitation analyses see Figs. S4 and S5. The results of the ABGD analysis of the 16S alignment were of the genetic delimitation approaches most similar to our karyotypic race assignments with the only difference being the races Ega 88 and Ega 86–92 that were recognized as one lineage in the ABGD. The partitioning identified by the mPTP method was generally congruent with the ABGD of COI and GMYC, only the races Ege 46a and Ege 46b of *E.* (*A.*) *germanus* were each partitioned into three lineages by this method.

4. Discussion

4.1. Tracking the extent of cryptic diversity in Alpine Euscorpius (Alpiscorpius) scorpions

Phenotypic conservatism is typical for many scorpion taxa. It causes difficulties in the delimitation of species boundaries, which thus raises the question of the actual diversity of scorpions (Ojanguren-Affilastro et al., 2016; Parmakelis et al., 2013). The onset of phylogenetic studies has provided insights into hidden variation and diversity in a variety of scorpion groups (e.g. Husemann et al., 2012; Luna-Ramirez et al., 2017), including *Euscorpius* (*Alpiscorpius*) from the European Alps (Gantenbein et al., 2000; Scherabon et al., 2000). Although these pilot studies have pointed out the possibility of a cryptic diversity in the Alpine lineage of *Euscorpius* (*Alpiscorpius*), the precise delimitation of evolutionary lineages has remained unresolved since a significant part of the Alps has not been sampled at that time. We herein fill in this gap and provide a robust sampling of the Alpine *Euscorpius* (*Alpiscorpius*) populations to assess the extent of species diversification in the high mountain system and to investigate in depth the mechanisms of its karyotype evolution in phylogenetic context. This study is a pioneer of its kind in scorpions as it combines an extensive sampling that covers the Alpine part of the subgenus range and an integrative multidisciplinary approach of multilocus time-calibrated phylogenetic analyses with up-to-date cytogenetic techniques.

As our results show, the Alpine *Euscorpius* (*Alpiscorpius*) species are exceptionally diverse in their karyotypes with chromosome numbers varying from 2n = 46 to 2n = 92, which contrasts with their highly conserved external morphology. Each species represents a complex of karyotypically distinct populations (termed karyotypic races) with discrete geographical distributions, which are either completely isolated or occur in close proximity (e.g. Ega 88 and Ega 86–92). The three *Euscorpius* (*Alpiscorpius*) species studied here comprise 10 karyotypic races that can be readily distinguished either by chromosome counts (2n = 46–92) or by the karyotypic formula and chromosomal location of the rDNA [e.g. the three karyotypic races of *E.* (*A.*) *germanus*, all with 2n = 46]. The results of the phylogenetic analyses corroborated this karyological evidence for cryptic diversification. The results of genetic delimitation support the presence of 9–16 evolutionary lineages (depending on the approach), most of them congruent with the chromosomal evidence (Fig. 3). Seven of the ten karyotypic races were recovered with all delimitation approaches, but some of them were composed of more distinct genetic sublineages [e.g. Ege 46a and Ege 46b of *E.* (*A.*) *germanus*, Eal 54 of *E.* (*A.*) *alpha*]. However, because these

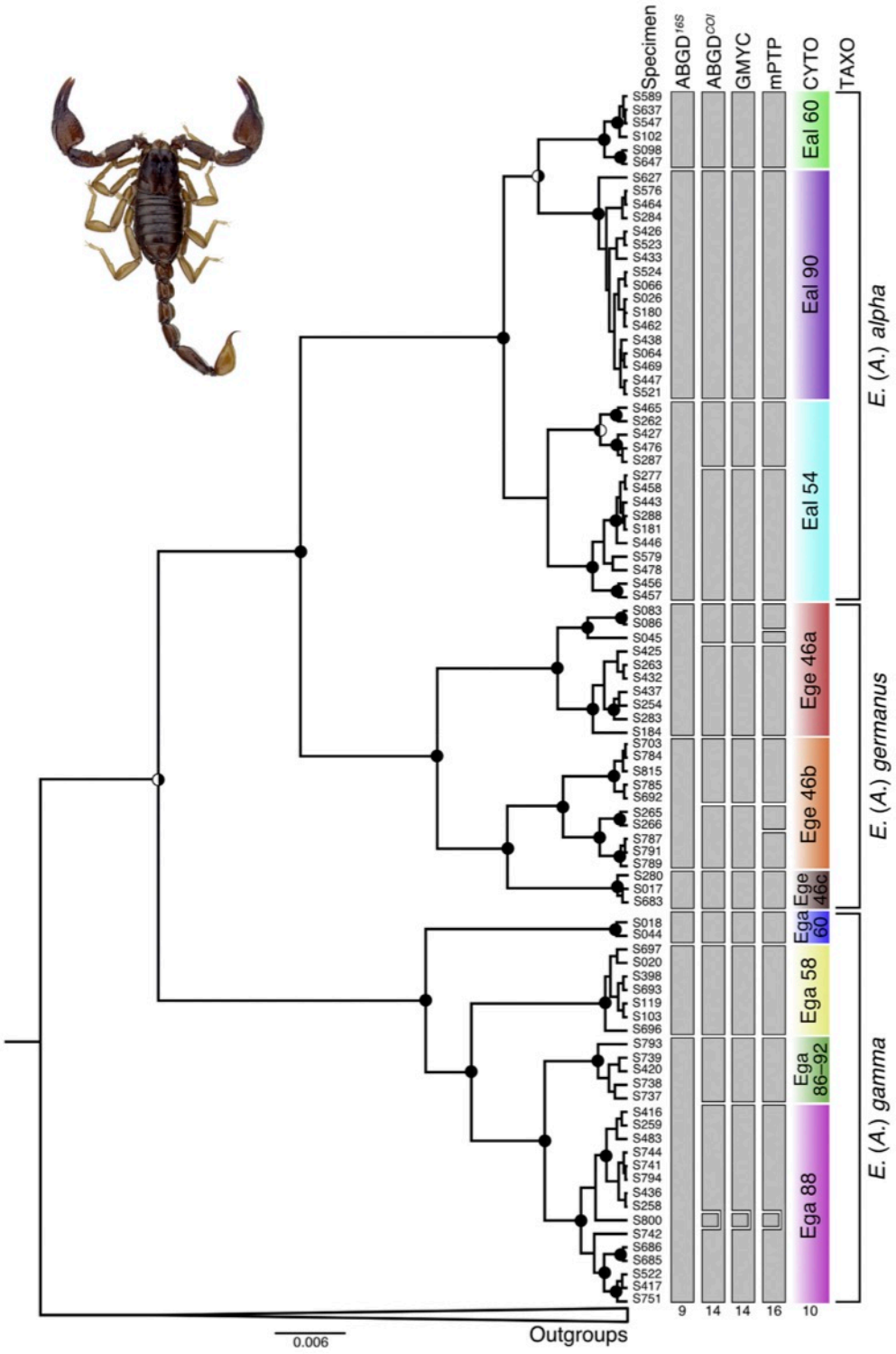

Fig. 3. MCC tree of the three *Euscorpius* (*Alpiscorpius*) species studied based on data set 1. Dots on nodes denote support as follows: left semi-circles are Bayesian posterior probabilities (PP) and right ones are maximum likelihood bootstraps, black = PP > 0.95, ML bootstrap support > 70%, white = topology not supported. Results of the four delimitation approaches are shown next to the sample names as grey columns. Specimen ID codes match those listed in Table S1. Codes of the karyotypic races correspond to Fig. 1.

sublineages do not possess unique karyological characteristics that would allow them to be defined as distinct karyotypic races, we consider them to represent profound genetic structuring within the karyotypic races, a phenomenon typically observed in geographically disparate populations. Moreover, the performance of single locus-based species discovery approaches, such as those employed here, is known to be sensitive to many factors. This includes, for instance, the number of species included, varying population sizes, level of gene flow, and number of singletons in the input trees (Ahrens et al., 2016; Fujisawa and Barraclough, 2013; Puillandre et al., 2012). We believe that the number of genetic lineages was overestimated in our case and we therefore treat the karyotypic races as reliable evolutionary units.

Even though our results indicate the presence of several independent taxa, their taxonomic recognition will require a thorough

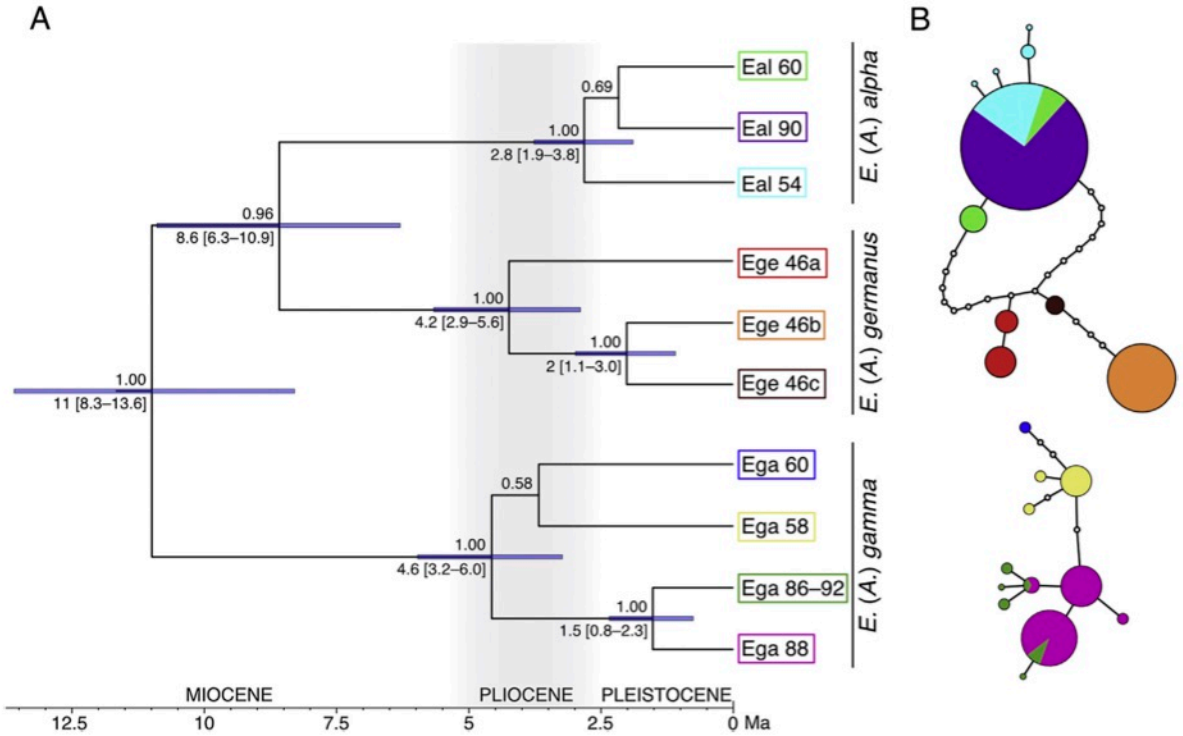

Fig. 4. (A) MCC tree resulting from the species-tree analysis showing divergence time estimates of the karyotypic races of the Alpine *Euscorpius* (*Alpiscorpius*). Upper numbers by nodes are Bayesian posterior probabilities (PP), numbers below branches show the mean and 95% HPD intervals of the estimated node ages; the HPD is also shown as blue bars. Age estimates are shown only for supported nodes. (B) Nuclear allele network of the phased sequences of 28S rDNA. Circle sizes are proportional to the number of alleles and white circles represent mutational steps. Colours correspond to the lineages in the tree. Codes of the karyotypic races correspond to Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

taxonomic revision of the Alpine *Euscorpius* (*Alpiscorpius*) species including analyses of morphological traits and examination of the type material. The present dataset includes the material of *E.* (*A.*) *alpha* from the type locality (Varenna, Italy) belonging to the lineage Eal 90, which may thus correspond to *E.* (*A.*) *alpha sensu stricto*. However, given the considerable complexity of the taxonomy of this group, we refrain from any speculations and believe that our results will provide a solid basis for a future taxonomic revision.

High levels of cryptic diversification and local endemism, as discovered here for Alpine *Euscorpius* (*Alpiscorpius*) species, have previously been documented for other Alpine biotas (Haubrich and Schmitt, 2007; Kotrbová et al., 2016; Riberon et al., 2001; Smyčka et al., 2017; Tribsch and Schönswetter, 2003). The Alps present a topographically complex mountain range with deep-cut valleys that witnessed regional tectonic activity until the Late Pliocene. Since then, the effects of climatic fluctuations began to dominate (Kuhlemann et al., 2002; Schmid et al., 2004). Such topographic and environmental heterogeneity of mountain ranges both in space and time were likely the key factors that have shaped the level of differentiation in invertebrates (Garrick, 2011), most notably those that are sensitive to spatial isolation due to their low dispersal capabilities and narrow ecological requirements, such as scorpions (Bryson et al., 2013; Ceccarelli et al., 2016; Husemann et al., 2012; present study).

4.2. High degree of karyotype variability in Alpine Euscorpius (Alpiscorpius) and its origin

Our cytogenetic examination shows that Alpine *Euscorpius* (*Alpiscorpius*) exhibits a wide variation in the karyotypic macrostructure (e.g. number of chromosomes, karyotypic formula). However, apart from this, differences between karyotypes may also be concealed in the distribution of specific chromosomal markers, such as the extensively investigated major rRNA genes (28S, 5.8S, and 18S) (e.g. Gornung, 2013; Nguyen et al., 2010). Mapping of rDNA by means of FISH has been previously performed in scorpions, although only on a limited number of Buthidae and Hormuridae species (e.g. Almeida et al., 2017; Adilardi et al., 2015, 2016; Mattos et al., 2014; Šťáhlavský et al., 2018). Our comparative FISH mapping with 18S rDNA thus provides the first data on the rDNA distribution for the family Euscorpiidae. The karyotypes of Alpine *Euscorpius* (*Alpiscorpius*) comprise a single rDNA-bearing chromosome pair, which is typical for most scorpion taxa examined so far (Adilardi et al., 2014; Sadílek et al., 2015; Schneider and Cella, 2010; Šťáhlavský et al., 2018). In contrast to the stable number of the rDNA loci, the morphology of the rDNA-bearing chromosome (bi-armed vs. one-armed) as well as the position of rDNA clusters on the monocentric chromosome (short arms vs. long arms) show striking differences between the karyotypes (Fig. S6). Such specificity of the rDNA-bearing chromosome pair contributed to the delimitation of karyotypically variable Alpine *Euscorpius* (*Alpiscorpius*) populations into the karyotypic races (e.g. highly polymorphic Ega 86–92 classified on the basis of a specific rDNA-bearing chromosome pair).

Karyotype differences detected at various structural levels (macrostructure vs. rDNA location) point to dynamic evolutionary alterations in the genomic organization of the Alpine *Euscorpius* (*Alpiscorpius*) populations. Geographical pattern and deep genetic structure of the karyotypic races suggest that chromosomal mutations accumulated independently in isolated demes across the Alps, and thus most likely did not induce the diversification process, but rather accompanied it. Populations of *E.* (*A.*) *alpha* and *E.* (*A.*) *gamma* have undergone remarkable karyotype diversification through successive centric fusion/fission events. These large-scale rearrangements directly lead to alterations in the chromosome number, the arm ratio, and also changes in the morphology of the rDNA-bearing chromosome without affecting the rDNA chromosomal location (Hirai et al., 1994, 1996). Individuals heterozygous for fusion/fission indicate that such interchromosomal rearrangements still occur within certain populations and their

maintenance may be facilitated by their ability to segregate during meiosis (Baker and Bickham, 1986; Shanahan, 1989). In contrast to *E.* (*A.*) *alpha* and *E.* (*A.*) *gamma*, the highly similar karyotypes of *E.* (*A.*) *germanus* populations differentiated at an intrachromosomal level through both types of inversions. The variation in karyotypic formulas (morphology of chromosomes) is likely the result of pericentric inversions, whereas distinct rDNA chromosomal localization seems to be a consequence of paracentric inversions, relocating rDNA sites from subterminal to terminal position.

The accumulation of chromosomal changes within Alpine *Euscorpius* (*Alpiscorpius*) populations may be facilitated by ecological (stenotopy, low vagility) and historical demographic (shifts in population size) factors (White, 1969, 1978). Karyotype differentiation via fixation of new chromosome rearrangements may be driven by random genetic drift within small isolated populations (Lande, 1985; White, 1978; Wright, 1940). The effect of other evolutionary forces, such as female meiotic drive or positive selection of individuals homozygous for a new chromosomal variant, may be also involved in the process of fixation (Hedrick, 1981; de Villena and Sapienza, 2001).

Structural changes in genome organization have the potential to form an effective barrier to introgression upon secondary contact (Baker and Bickham, 1986; King, 1993; White, 1969). It is reasonable to assume that the extensive level of karyotype differences between some closely related Alpine *Euscorpius* (*Alpiscorpius*) populations (e.g. Ega 60 and Ega 90) may hinder their potential crossbreeding as the resulting hybrids would be most likely less viable (Baker and Bickham, 1986; Shanahan, 1989). However, whether strong post-zygotic barriers exist between all intraspecific populations, especially between those with minor differences in their karyotypes, and how the chromosomal changes contribute to reproductive isolation, remains to be tested.

4.3. Historical diversification of Euscorpius (Alpiscorpius) across the Alps

Our time-calibrated phylogenetic reconstruction shows that the basal divergence of the Alpine *Euscorpius* (*Alpiscorpius*) took place in the Late Miocene (ca. 8–14 Ma). This finding is congruent with the pre-Pleistocene diversification of the Alpine scorpions hypothesized by Gantenbein et al. (2000). However, as our results show, this event may be significantly older than the Late Pliocene (2–3 Ma), an age formerly estimated for the split of *E.* (*A.*) *alpha* and *E.* (*A.*) *germanus* (Gantenbein et al., 2000).

The tempo of diversification as well as the estimated ages of the intraspecific genetic lineages differ for each of the Alpine *Euscorpius* (*Alpiscorpius*) species. Such contrasting evolutionary histories of the Alpine scorpions may be linked to the heterogeneity of the different mountain ranges that the species inhabit, their topographic and climatic gradients, and unique geomorphological histories (Dewey et al., 1973; Hewitt, 2004; Huntley and Webb, 1989).

In *E.* (*A.*) *alpha*, the westernmost of the species, three genetic lineages occur in allopatry in the Northwestern Alps and the westernmost part of the Southeastern Alps in Italy (see Fig. 1 and Table S3 for more details). They all emerged in a relatively short succession during the Pliocene-Pleistocene transition. Their rapid diversification started 2.8 Ma (HPD: 1.9–3.8), which temporally corresponds to the culmination of climatic cooling of the Northern Hemisphere ca. 2.75 Ma that resulted in extensive Alpine glaciations (Ruddiman, 2010). The glacial activity thus seems to have left a strong imprint on the present-day phylogeography of *E.* (*A.*) *alpha* by fragmentation of its habitat. The geographical distribution of the intraspecific genetic lineages correlates with the regions permanently unglaciated throughout the Plio-Pleistocene period (see Mey et al., 2016; Tribsch and Schönswetter, 2003). These geographically restricted areas have been regarded as important refuges for the glacial survival of various Alpine plant taxa (Schönswetter et al., 2005), and they likely played an equally crucial role in the diversification of *E.* (*A.*) *alpha* populations. The glacier Lake Maggiore and the stream valley Val Camonica in northern Italy form the borders between the *E.* (*A.*) *alpha* lineages and thus act as an effective barrier to gene flow and hinder their potential secondary contact. Considering the long-term geographical isolation and highly diversified karyotypes (Eal 54, Eal 90, Eal 60), the partial sharing of the 28S alleles detected between the three lineages most probably reflects incomplete lineage sorting as a remnant of the rapid diversification.

In *E.* (*A.*) *germanus*, the three lineages show clear genetic separation with no nuclear allele sharing in spite of having the same chromosome number (2n = 46). Their distribution stretches across the Eastern Alps in Austria, Italy and Slovenia from where it continues to the northern Dinaric Alps. The lineage Ege 46a occupies the western part of the range reaching the Slovenian border, while the ranges of the two other lineages (Ege 46b, Ege 46c) are restricted to western Slovenia where they overlap (see Fig. 1 and Table S3 for more details). Their basal split represents an old event dated to the Early Pliocene, ca. 4.2 Ma (HPD: 2.9–5.6). This initial divergence of the Austrian-Italian (Ege 46a) and Slovenian clades (Ege 46b, Ege 46c) seems to have taken place in allopatry and could have been triggered by the after-effect of the Messinian climate changes (5.3–5.9 Ma) which caused the decrease of the Alpine riverbeds and the formation of deep valleys (Pfiffner, 2014; Willett et al., 2006). Subsequent separation of two Slovenian lineages occurred in the Late Pleistocene, ca. 2 Ma (HPD: 1.1–3.0). During this period, the area inhabited by these lineages underwent a dramatic cooling, accompanied by local glaciations (Bavec and Verbič, 2011), which could cause their isolation and consequent divergence.

Contrary to the two species above, *E.* (*A.*) *gamma* inhabits Alpine regions of a low and less rugged relief. The four intraspecific lineages occur in allopatry or partial parapatry throughout the Southeastern Alps in Slovenia with peripheral distribution in the Slovenian Dinaric Alps (see Fig. 1 and Table S3 for more details). The basal split took place in the Early Pliocene, ca. 4.6 Ma (HPD: 3.2–6.0). This may correspond with the hypothesized pre-Pleistocene development of the Sava river system (Bavec and Verbič, 2011), which currently forms the natural boundary between the western (Ega 60, Ega 58) and eastern clades (Ega 88, Ega 86–92). Of all the Alpine *Euscorpius* (*Alpiscorpius*) clades, the eastern genetic lineages Ega 88 and Ega 86–92 separated most recently in the Early Pleistocene, ca. 1.5 Ma (HDP: 0.8–2.3). Their distributions partially overlap in northern Slovenia and they show a low degree of chromosomal heterozygosity and a partial allele sharing in the 28S nuclear marker. The allele sharing may either result from an incomplete lineage sorting of ancestral polymorphism as we believe to be the case in *E.* (*A.*) *alpha* (see above) or from ongoing gene flow between these populations. The latter is supported by the geographical proximity of the populations sharing the 28S allele and the fact that samples further away from the contact zone have alleles that are unique for their respective karyotypic race. Nevertheless, to properly understand the mechanisms underlying the generation and maintenance of these distinct karyotypic races more precise delimitation of the contact zone and denser sampling along it are needed.

5. Conclusion

Our integrative study demonstrates the potential strength of combining the cytogenetic and multilocus molecular data for uncovering hidden biodiversity. Our results show that, contrary to its phenotypical conservatism, the Alpine *Euscorpius* (*Alpiscorpius*) represents an exceptionally diverse scorpion group both at the chromosomal and genetic level. The combination of different data sources has helped to delimit ten deeply divergent lineages that meet the attributes of local endemics throughout the European Alps. Generation of high endemic diversity through allopatry may be promoted by the low vagility of scorpions, topographic heterogeneity of the montane system, and geoclimatic events during the Pliocene-Pleistocene. We assume that macrostructural chromosomal changes most likely do not pose a pivotal driving force in diversification, but rather represent a consequence of this process. Taken together, we suggest that the evolutionary lineages

within the Alpine *Euscorpius* (*Alpiscorpius*) most likely represent cryptic species and that this subgenus requires a taxonomic revision with a comprehensive morphological analysis.

Acknowledgements

We would like to thank J. Štundl, M. Řezáč, M. Peprný, H. Peprný, P. Just, M. Šlachta, L. Juřičková, P. Dolejš, and J. Dolanský who helped us collect specimens for this research, and to F. Kovařík who contributed to species determination. We thank J. Štundl for generating the map of species distributions. We are grateful to V. Opatova for helpful comments on earlier versions of the manuscript. We would like to thank J. Moravec, who initially assisted with the research. We appreciate all the valuable comments provided by the reviewer A. A. Ojanguren-Affilastro that helped improve the manuscript. This work was financially supported by the Charles University, GA UK No. 1350214, the Ministry of Education, Youth and Sports of the Czech Republic (grant no. SVV 260 434/2019) and the Ministry of Culture of the Czech Republic (DKRVO 2018/14, National Museum, 00023272). P. Nguyen was supported by grant 17-17211S of the Czech Science Foundation. Microscopy was performed in the Laboratory of Confocal and Fluorescence Microscopy, co-financed by the European Regional Development Fund and the state budget of the Czech Republic. Project no. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.02.002.

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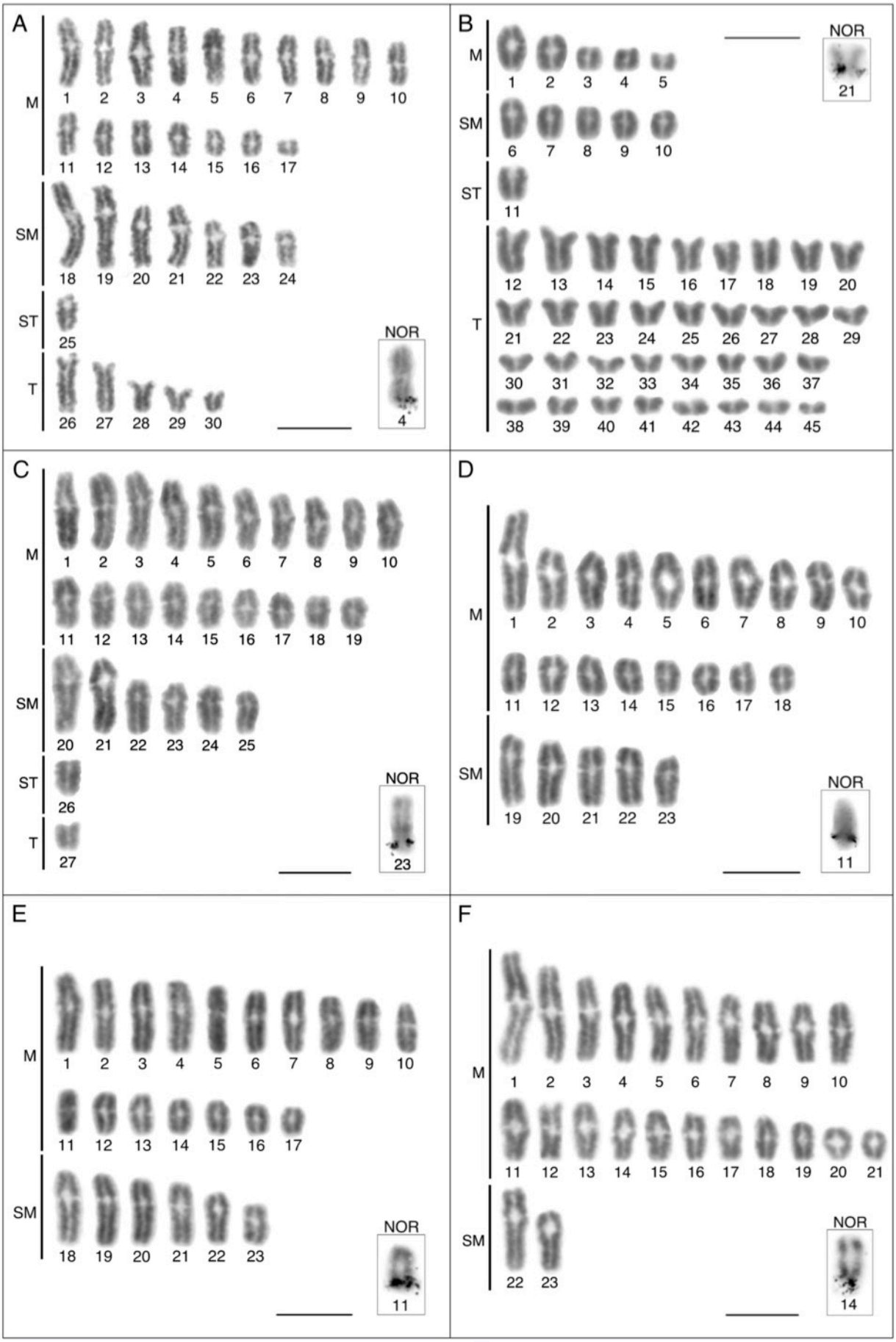

Figure S1. Karyograms of the Alpine *Euscorpius* (*Alpiscorpius*). *E.* (*A.*) *alpha* (A–C), *E.* (*A.*) *germanus* (D–F). (A) karyotypic race I (Eal 60), 3.18–0.57% DSL, (B) karyotypic race II (Eal 90), 1.90–0.44% DSL, (C) karyotypic race III (Eal 54), 3.06–0.92% DSL, (D) karyotypic race I (Ege 46a), 3.4–1.08% DSL, (E) karyotypic race II (Ege 46b), 3.14–1.10% DSL, (F) karyotypic race III (Ege 46c), 3.88–1.08% DSL. Bar=10μm.

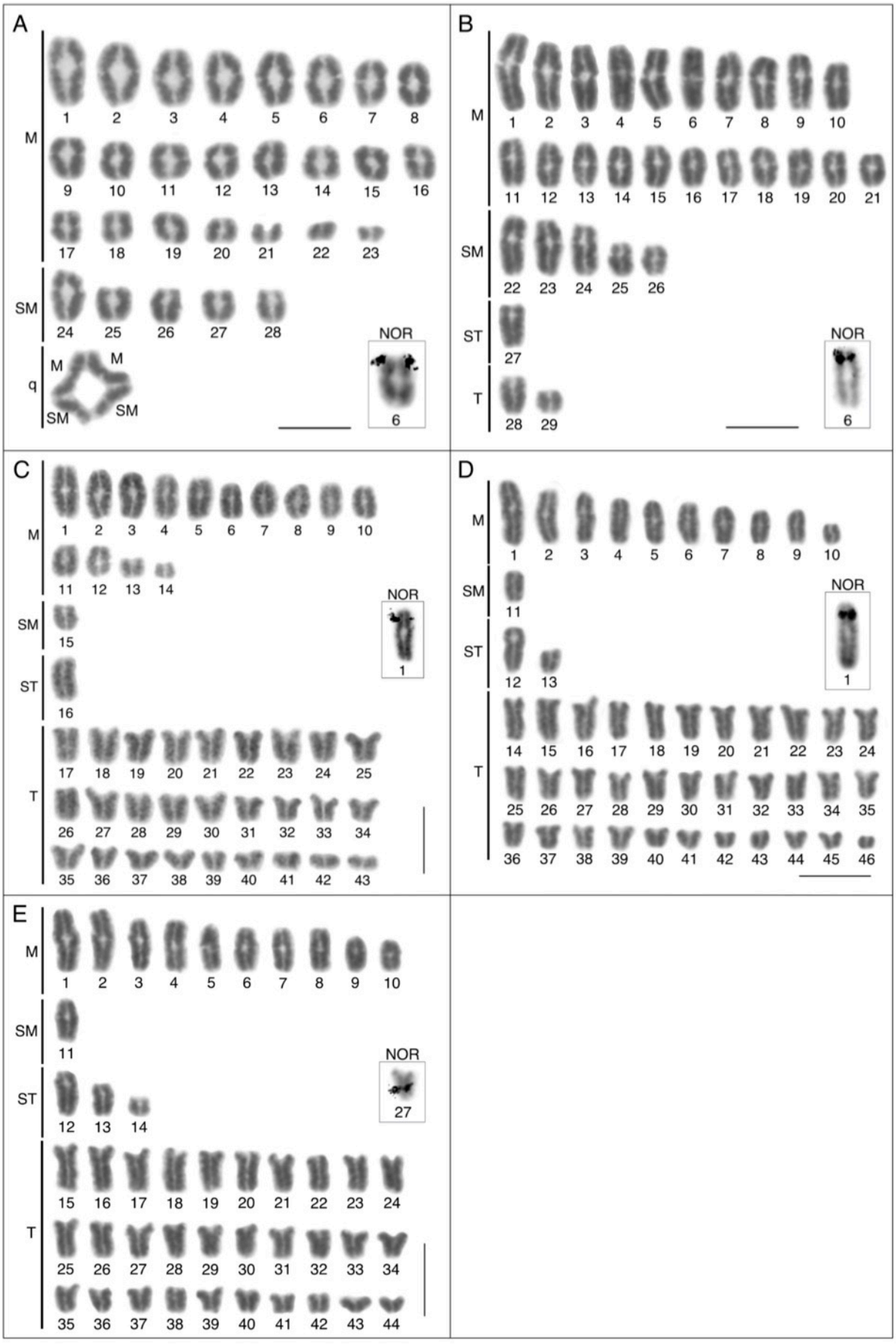

Figure S2. Karyograms of the Alpine *Euscorpius* (*Alpiscorpius*) *gamma*. (A) karyotypic race I (Ega 60), 2.72–0.54% DSL, (B) karyotypic race II (Ega 58), 2.69–0.80% DSL, (C) karyotypic race III (Ega 86–92, 2n = 86), 2.23–0.50% DSL, (D) karyotypic race III (Ega 86–92, 2n = 92), 2.23–0.39% DSL, (E) karyotypic race IV (Ega 88), 2.31–0.46% DSL. Bar = 10μm.

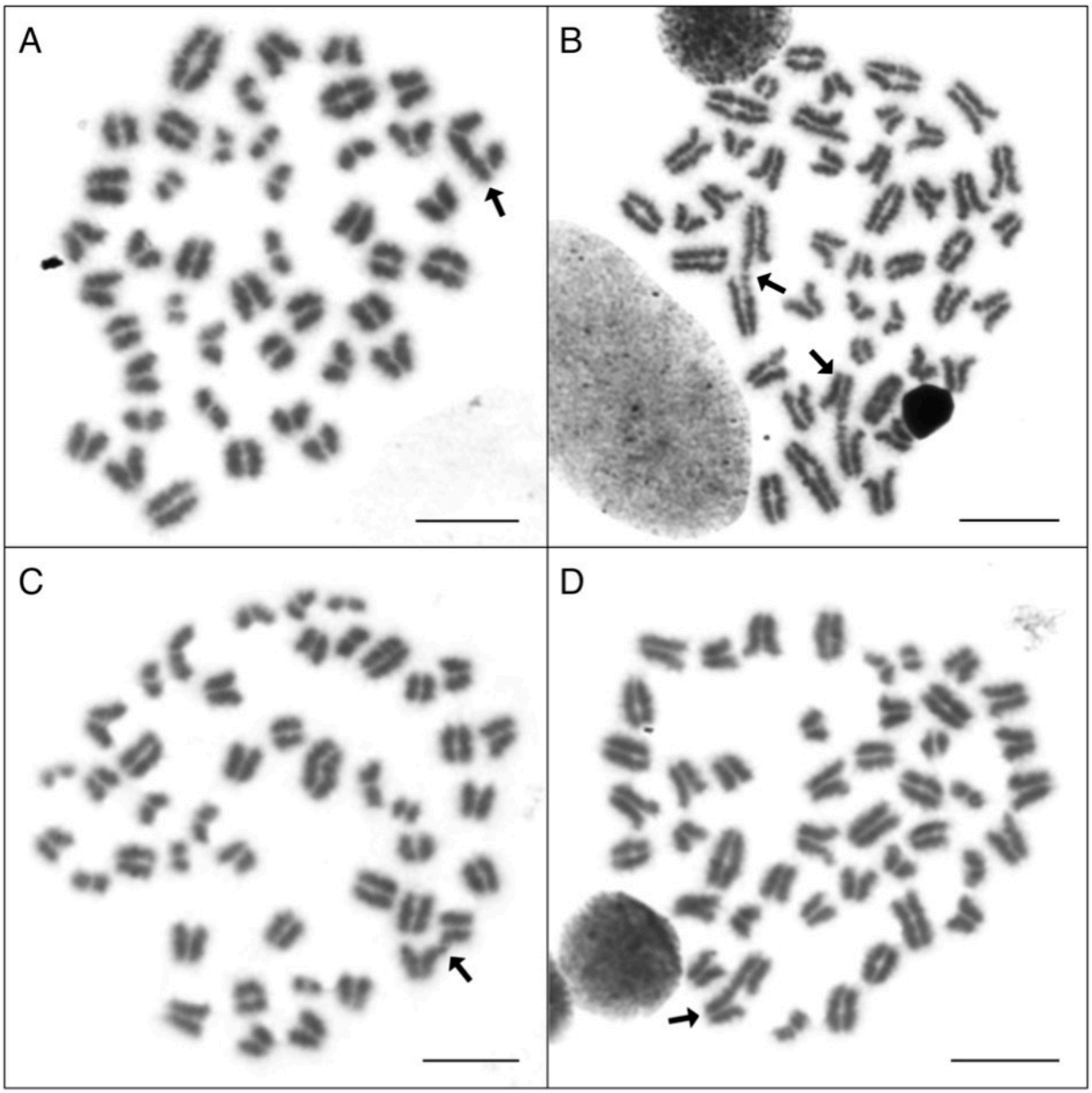

Figure S3. The heterozygous centric fusions/□ssions in *Euscorpius* (*Alpiscorpius*) *gamma*. Karyotypic race Ega 86–92 (A–B), karyotypic race Ega 88 (C–D). (A) heterozygosity for fusion/□ssion in individual with 2n = 87 (42 bivalents + trivalent), (B) heterozygosity for fusion/□ssion in individual with 2n = 90 (42 bivalents + 2 trivalents), (C) heterozygosity for fusion in individual with 2n = 87 (42 bivalents + trivalent), (D) heterozygosity for □ssion in individual with 2n = 89 (43 bivalents + trivalent). Arrows indicate trivalent. Bar = 10μm.

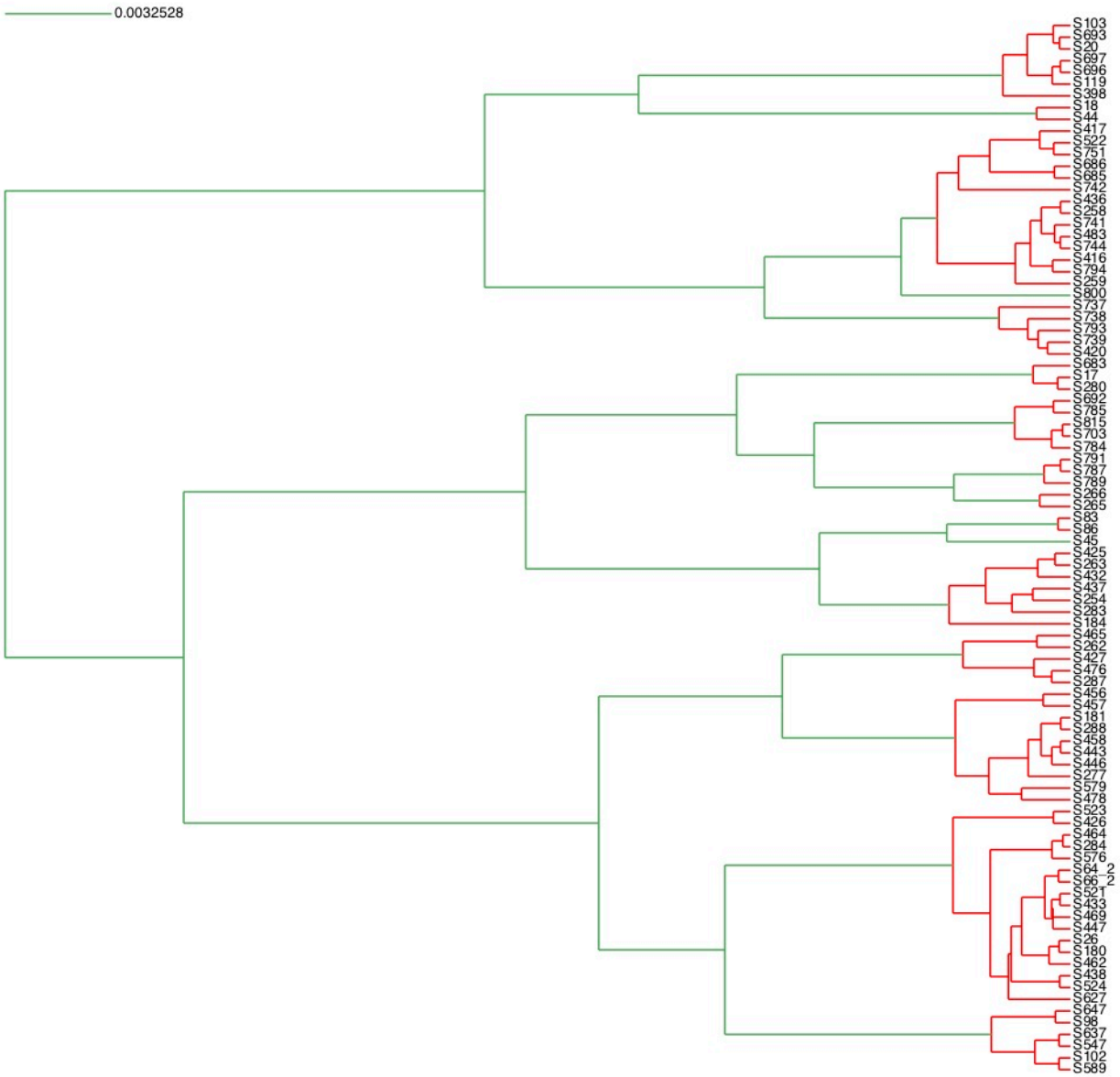

Figure S4. Results of the mPTP analysis based on the mtDNA.

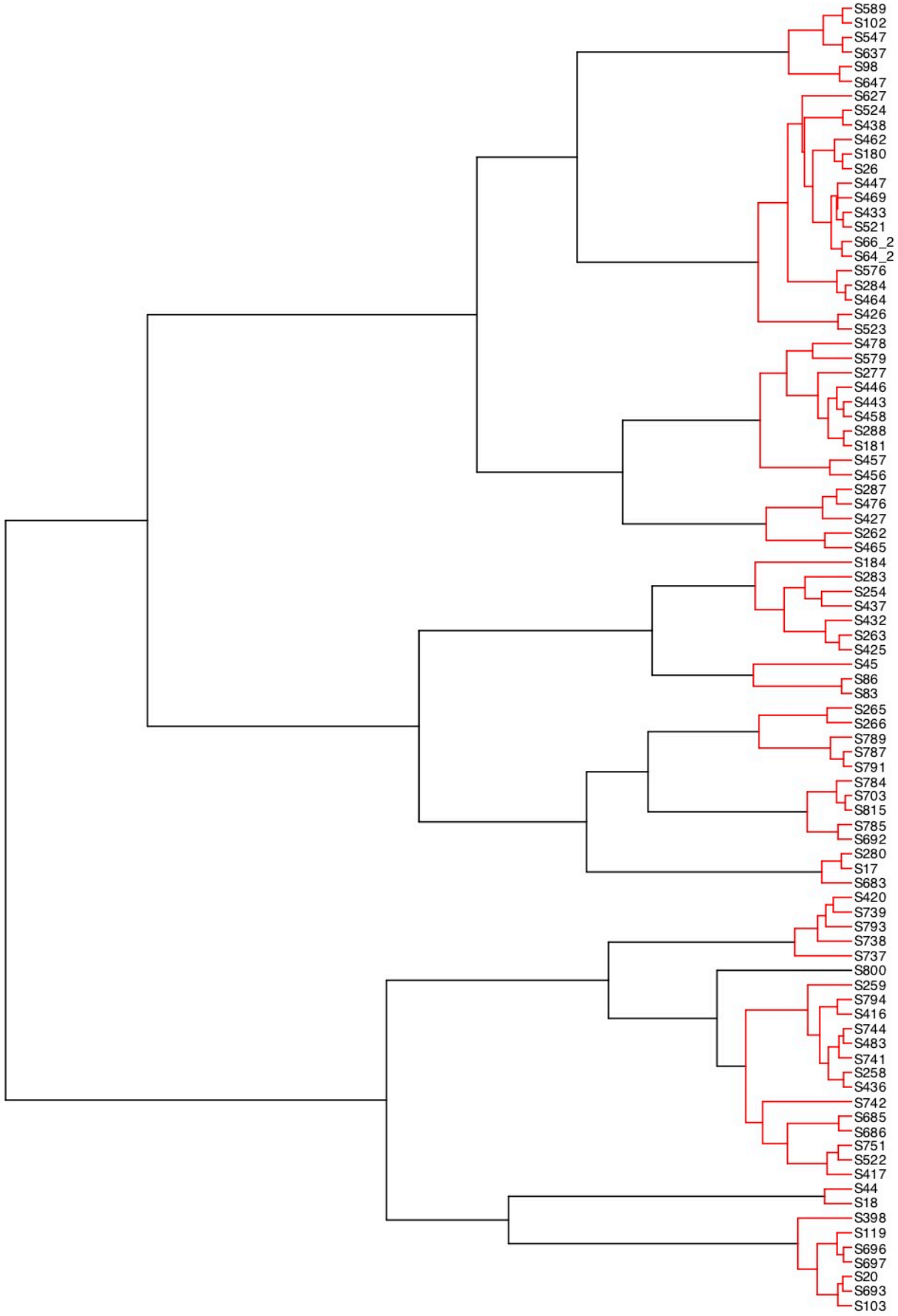

Figure S5. Results of the GMYC analysis based on the mtDNA.

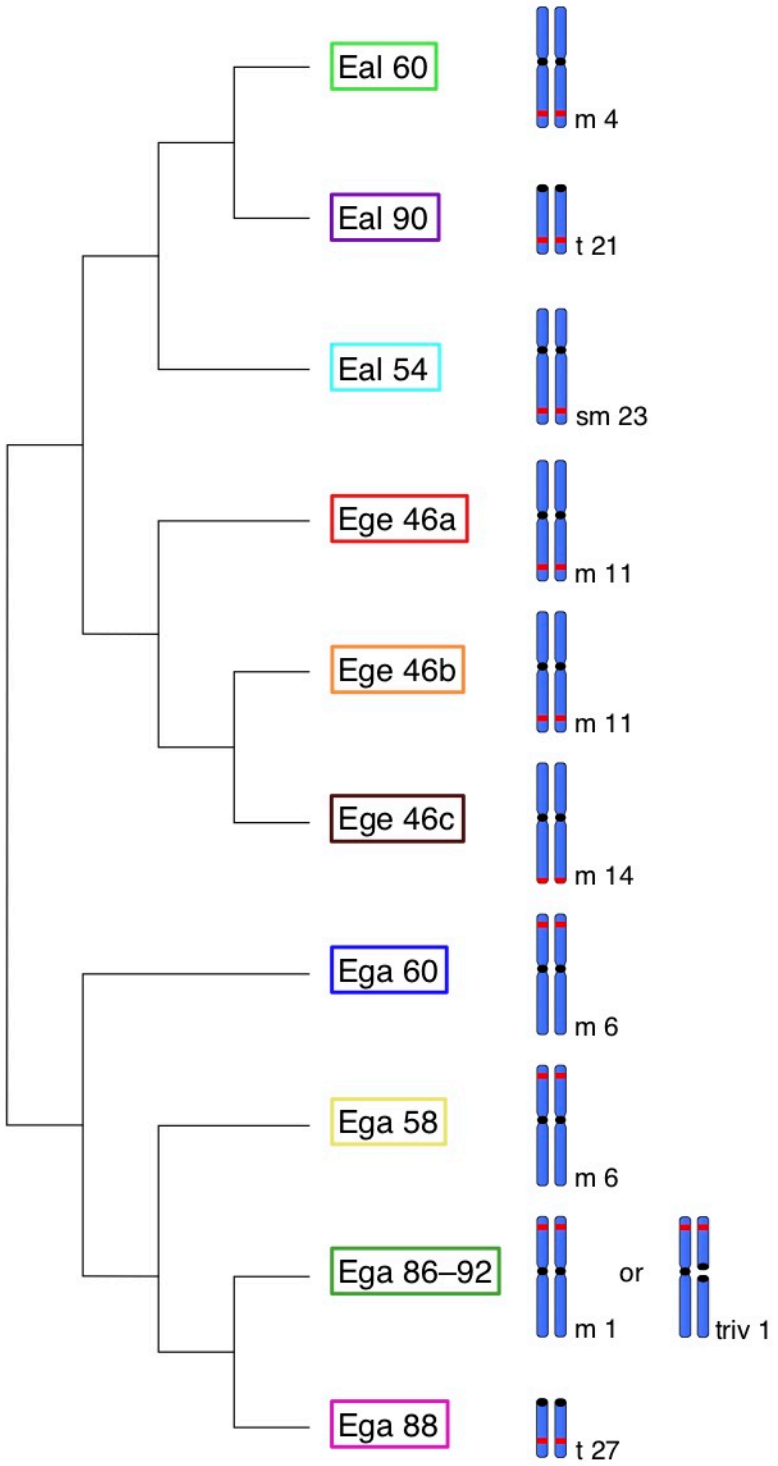

Figure S6. Scheme of the rDNA-bearing chromosome pair in a phylogenetic context (m - metacentric, sm - submetacentric, st - subtelocentric, t - telocentric, triv - trivalent). Numbers indicate the order of the chromosomal pair in the karyotype (see karyograms Figs. S1 and S2). Codes of the karyotypic races correspond to Fig. 1.

Table S1. Complete list of specimens used in cytogenetic and phylogenetic analyses. The specimen ID of given species are listed in numerical order and complemented with NM depository number, locality information, diploid number and GenBank accession numbers. Samples used in *BEAST analysis are marked *. (m – male, f – female, AT – Austria, CH – Switzerland, GR – Greece, IT – Italy, RO – Romania, SI – Slovenia, TR – Turkey, II – bivalent, III – trivalent, IV – quadrivalent).

Taxon/ Sample ID	Sex	Voucher		Locality	GPS	Karyotypic race	2n	Meiotic configuration	16S	COI	28S
E. (A.) alpha											
S098*	m	NMP6A 6898	IT:	Antey – St. André	45.788, 7.589	Eal 60	60	–	MK421820	MK421726	MK421654
S102	m	NMP6A 6899	CH:	Gondo	46.195, 8.137	Eal 60	60	30II	MK421821	MK421727	–
S547*	m	NMP6A 6900	CH:	Gondo	46.195, 8.137	Eal 60	60	30II	MK421867	MK421771	MK421684
S589*	m	NMP6A 6901	IT:	Loreglia	45.902, 8.370	Eal 60	60	30II	MK421870	MK421774	MK421686
S590	m	NMP6A 6902	IT:	Loreglia	45.902, 8.370	Eal 60	60	30II	–	–	–
S637*	f	NMP6A 6903	CH:	Gondo	46.195, 8.137	Eal 60	–	–	MK421872	MK421776	MK421688
S647	f	NMP6A 6904	IT:	Tavigliano	45.662, 8.072	Eal 60	–	–	MK421873	MK421777	–
S026*	m	NMP6A 6905	IT:	Nuova Olomio	46.161, 9.433	Eal 90	90	45II	MK421813	MK421719	MK421651
S064	m	NMP6A 6906	CH:	Castel San Pietro	45.860, 9.017	Eal 90	90	45II	MK421816	MK421722	–
S066*	m	NMP6A 6907	CH:	Castel San Pietro	45.860, 9.017	Eal 90	–	–	MK421817	MK421723	MK421652
S180*	m	NMP6A 6908	IT:	Mezzoldo	46.016, 9.665	Eal 90	90	45II	MK421824	MK421730	MK421656
S182	m	NMP6A 6909	IT:	Mezzoldo	46.016, 9.665	Eal 90	90	45II	–	–	–
S253	m	NMP6A 6910	IT:	Mezzoldo	46.016, 9.665	Eal 90	90	–	–	–	–
S284	m	NMP6A 6911	IT:	Sondrio	46.175, 9.857	Eal 90	90	45II	MK421838	MK421743	–
S426	m	NMP6A 6912	IT:	Domaso	46.157, 9.328	Eal 90	90	45II	MK421846	MK421750	–
S433*	m	NMP6A 6913	IT:	Teglio	46.182, 10.052	Eal 90	90	45II	MK421848	MK421752	MK421672
S438	m	NMP6A 6914	IT:	Teglio	46.182, 10.052	Eal 90	90	–	MK421851	MK421755	–
S439	m	NMP6A 6915	IT:	Puria	46.032, 9.048	Eal 90	90	–	–	–	–
S447*	m	NMP6A 6916	IT:	Teglio	46.182, 10.052	Eal 90	90	45II	MK421854	MK421758	MK421675
S462*	m	NMP6A 6917	IT:	Crespineto	46.155, 10.098	Eal 90	90	–	MK421857	MK421761	MK421677
S464	m	NMP6A 6918	IT:	Selvetta	46.157, 9.682	Eal 90	90	–	MK421858	MK421762	–
S469	m	NMP6A 6919	IT:	Puria	46.032, 9.048	Eal 90	90	–	MK421859	MK421763	–
S521*	m	NMP6A 6920	IT:	Puria	46.032, 9.048	Eal 90	90	45II	MK421863	MK421767	MK421681
S523*	m	NMP6A 6921	IT:	Domaso	46.157, 9.328	Eal 90	90	45II	MK421865	MK421769	MK421682
S524*	m	NMP6A 6922	IT:	Selvetta	46.157, 9.682	Eal 90	90	45II	MK421866	MK421770	MK421683
S576*	m	NMP6A 6923	CH:	Sottoponte	46.339, 9.555	Eal 90	–	–	MK421868	MK421772	MK421685
S580	m	NMP6A 6924	IT:	Olmo al Brembo	45.973, 9.650	Eal 90	90	–	–	–	–

S627*	f	NMP6A 6925	IT:	Olmo al Brembo	45.973, 9.650	Eal 90	–	–	MK421871	MK421775	MK421687
S846	m	NMP6A 6926	IT:	Varenna	46.009, 9.288	Eal 90	90	–	–	–	–
S1051	m	–	CH:	Somazzo	45.884, 8.991	Eal 90	90	45II	–	–	–
S1053	m	–	CH:	Somazzo	45.884, 8.993	Eal 90	90	45II	–	–	–
S1055	m	–	CH:	Somazzo	45.884, 8.994	Eal 90	90	45II	–	–	–
S1062	m	–	CH:	Somazzo	45.884, 8.995	Eal 90	90	45II	–	–	–
S1076	m	NMP6A 6927	CH:	Somazzo	45.884, 8.996	Eal 90	90	45II	–	–	–
S181	m	NMP6A 6928	IT:	Bad Salz	46.570, 10.790	Eal 54	54	27II	MK421825	MK421731	–
S183	m	NMP6A 6929	IT:	Sonvico	45.819, 10.131	Eal 54	54	27II	–	–	–
S262*	m	NMP6A 6930	IT:	Sonvico	45.819, 10.131	Eal 54	54	–	MK421806	MK421712	MK421646
S277*	m	NMP6A 6931	IT:	Pannone	45.871, 10.933	Eal 54	54	27II	MK421835	MK421740	MK421664
S282	m	NMP6A 6932	IT:	Sonvico	45.819, 10.131	Eal 54	54	27II	–	–	–
S287	m	NMP6A 6933	IT:	Pezzoro	45.763, 10.223	Eal 54	54	–	MK421802	MK421708	–
S288	m	NMP6A 6934	IT:	Lumini	45.623, 10.764	Eal 54	54	27II	MK421839	MK421744	–
S427*	m	NMP6A 6935	IT:	Monte Mondero	45.777, 10.183	Eal 54	54	27II	MK421803	MK421709	MK421644
S443	m	NMP6A 6936	IT:	Baslan	46.640, 11.135	Eal 54	54	27II	MK421852	MK421756	–
S446*	m	NMP6A 6937	IT:	Tret	46.468, 11.147	Eal 54	54	–	MK421853	MK421757	MK421674
S456*	m	NMP6A 6938	IT:	Ponte Caffaro	45.817, 10.515	Eal 54	54	27II	MK421855	MK421759	MK421676
S457	m	NMP6A 6939	IT:	Ponte Caffaro	45.817, 10.515	Eal 54	54	27II	MK421808	MK421714	–
S458	m	NMP6A 6940	IT:	Pannone	45.871, 10.933	Eal 54	54	27II	MK421856	MK421760	–
S465	m	NMP6A 6941	IT:	Sonvico	45.819, 10.131	Eal 54	54	27II	MK421805	MK421711	–
S466	m	NMP6A 6942	IT:	Sonvico	45.819, 10.131	Eal 54	54	27II	–	–	–
S467	m	NMP6A 6943	IT:	Sonvico	45.819, 10.131	Eal 54	54	27II	–	–	–
S476*	m	NMP6A 6944	IT:	Monte Mondero	45.777, 10.183	Eal 54	54	27II	MK421804	MK421710	MK421645
S478	m	NMP6A 6945	IT:	Pannone	45.871, 10.933	Eal 54	54	–	MK421807	MK421713	–
S579	m	NMP6A 6946	IT:	Vermiglio	46.290, 10.678	Eal 54	54	27II	MK421869	MK421773	–
S1056	m	–	IT:	Tablá	46.631, 10.971	Eal 54	54	27II	–	–	–
S1057	m	–	IT:	Tablá	46.631, 10.971	Eal 54	54	27II	–	–	–
S1058	m	–	IT:	Tablá	46.631, 10.971	Eal 54	54	27II	–	–	–
S1063	m	–	IT:	Tablá	46.631, 10.971	Eal 54	54	27II	–	–	–
S1075	m	NMP6A 6947	IT:	Tablá	46.631, 10.971	Eal 54	54	–	–	–	–
E. (A.) germanus											
S045	m	NMP6A 6948	AT:	Nassfeld Pass	46.560, 13.278	Ege 46a	46	–	MK421815	MK421721	–
S046	m	NMP6A 6949	AT:	Nassfeld Pass	46.560, 13.278	Ege 46a	46	–	–	–	–
S082	m	NMP6A 6950	IT:	Lestans	46.165, 12.880	Ege 46a	46	23II	–	–	–
S083	m	NMP6A 6951	IT:	Gerchia	46.247, 12.883	Ege 46a	46	23II	MK421818	MK421724	–

S086*	m	NMP6A 6952	IT:	Gerchia	46.247, 12.883	Ege 46a	46	23II	MK421819	MK421725	MK421653
S087	m	NMP6A 6953	IT:	Gerchia	46.247, 12.883	Ege 46a	46	23II	–	–	–
S088	m	NMP6A 6954	IT:	Lestans	46.165, 12.880	Ege 46a	46	–	–	–	–
S184*	m	NMP6A 6955	IT:	Soffranco	46.280, 12.244	Ege 46a	46	23II	MK421826	MK421732	MK421657
S185	m	NMP6A 6956	IT:	Montechiaro	46.632, 10.564	Ege 46a	46	–	–	–	–
S254*	m	NMP6A 6957	IT:	Schluderns	46.669, 10.593	Ege 46a	46	–	MK421827	MK421733	MK421658
S263	m	NMP6A 6958	IT:	Montechiaro	46.632, 10.564	Ege 46a	46	23II	MK421830	MK421736	–
S264	m	NMP6A 6959	IT:	Schluderns	46.669, 10.593	Ege 46a	46	–	–	–	–
S274	m	NMP6A 6960	IT:	Montechiaro	46.632, 10.564	Ege 46a	46	–	–	–	–
S283*	m	NMP6A 6961	IT:	Ometto	45.753, 11.117	Ege 46a	46	23II	MK421837	MK421742	MK421666
S425*	m	NMP6A 6962	AT:	Walchenbach	47.273, 10.782	Ege 46a	46	23II	MK421845	MK421749	MK421671
S432	m	NMP6A 6963	IT:	Quellenhof	46.747, 11.216	Ege 46a	46	23II	MK421847	MK421751	–
S437*	m	NMP6A 6964	IT:	Quellenhof	46.747, 11.216	Ege 46a	46	–	MK421850	MK421754	MK421673
S444	m	NMP6A 6965	IT:	Quellenhof	46.747, 11.216	Ege 46a	46	–	–	–	–
S445	m	NMP6A 6966	IT:	Quellenhof	46.747, 11.216	Ege 46a	46	–	–	–	–
S468	m	NMP6A 6967	IT:	Walchenbach	47.273, 10.782	Ege 46a	46	–	–	–	–
S527	m	NMP6A 6968	IT:	Walchenbach	47.273, 10.782	Ege 46a	46	–	–	–	–
S847	m	NMP6A 6969	IT:	Peaio	46.414, 12.277	Ege 46a	46	23II	–	–	–
S1010	m	NMP6A 6970	IT:	Soffranco	46.280, 12.244	Ege 46a	46	23II	–	–	–
S1011	m	NMP6A 6971	IT:	Soffranco	46.280, 12.244	Ege 46a	46	23II	–	–	–
S1023	m	–	IT:	Passo Rolle	46.293, 11.771	Ege 46a	46	23II	–	–	–
S1031	m	–	IT:	Passo Rolle	46.293, 11.771	Ege 46a	46	23II	–	–	–
S1032	m	–	IT:	Ponte Rio Jordao	46.322, 12.233	Ege 46a	46	23II	–	–	–
S1033	m	–	IT:	Ponte Rio Jordao	46.322, 12.233	Ege 46a	46	23II	–	–	–
S1061	f	NMP6A 6972	IT:	Passo Rolle	46.293, 11.771	Ege 46a	46	–	–	–	–
S1077	f	NMP6A 6973	IT:	Passo Rolle	46.293, 11.771	Ege 46a	46	–	–	–	–
S265*	m	NMP6A 6974	SI:	Srpenica	46.295, 13.495	Ege 46b	46	–	MK421831	–	MK421660
S266*	m	NMP6A 6975	SI:	Trdnajava Kluže	46.362, 13.589	Ege 46b	46	23II	MK421832	MK421737	MK421661
S278	m	NMP6A 6976	SI:	Srpenica	46.295, 13.495	Ege 46b	46	23II	–	–	–
S692*	m	NMP6A 6977	SI:	Laze I	45.860, 14.270	Ege 46b	46	–	MK421877	MK421781	MK421691
S703*	f	NMP6A 6978	SI:	Veliko Ubeljsko	45.775, 14.069	Ege 46b	46	–	MK421881	MK421785	MK421694
S784*	m	NMP6A 6979	SI:	Veliko Ubeljsko	45.775, 14.069	Ege 46b	46	23II	MK421889	MK421793	MK421701
S785*	m	NMP6A 6980	SI:	Laze II	45.867, 14.259	Ege 46b	46	23II	MK421890	MK421794	MK421702
S787	m	NMP6A 6981	SI:	Občina Kobarid I	46.251, 13.585	Ege 46b	46	23II	MK421891	MK421795	–
S788	m	NMP6A 6982	SI:	Občina Kobarid I	46.251, 13.585	Ege 46b	46	–	–	–	–
S789	m	NMP6A 6983	SI:	Občina Kobarid II	46.249, 13.587	Ege 46b	46	23II	MK421892	MK421796	–

S790	m	NMP6A 6984	SI:	Občina Kobarid II	46.249, 13.587	Ege 46b	46	23II	–	–	–
S791*	m	NMP6A 6985	SI:	Kamno	46.215, 13.639	Ege 46b	46	23II	MK421893	MK421797	MK421703
S792	m	NMP6A 6986	SI:	Kamno	46.215, 13.639	Ege 46b	46	23II	–	–	–
S815*	f	NMP6A 6987	SI:	Veliko Ubeljsko	45.775, 14.069	Ege 46b	–	–	MK421897	MK421801	MK421707
S017*	m	–	SI:	Bohinjska Bistrica	46.279, 13.962	Ege 46c	46	23II	MK421810	MK421716	MK421648
S280*	m	NMP6A 6988	SI:	Roče	46.108, 13.817	Ege 46c	46	23II	MK421836	MK421741	MK421665
S683	m	NMP6A 6989	SI:	Němški Rovt	46.275, 13.992	Ege 46c	46	23II	MK421874	MK421778	–
S695	m	NMP6A 6990	SI:	Němški Rovt	46.275, 13.992	Ege 46c	46	23II	–	–	–
S709	m	NMP6A 6991	SI:	Němški Rovt	46.275, 13.992	Ege 46c	46	23II	–	–	–
S712	m	NMP6A 6992	SI:	Němški Rovt	46.275, 13.992	Ege 46c	46	23II	–	–	–
E. (A.) gamma											
S018*	m	–	SI:	Podkoren	46.494, 13.749	Ega 60	60	28II + IV	MK421811	MK421717	MK421649
S044*	m	NMP6A 6993	IT:	Studena Bassa	46.516, 13.273	Ega 60	60	28II + IV	MK421814	MK421720	–
S020*	m	NMP6A 6994	SI:	Polje	46.270, 13.907	Ega 58	58	29II	MK421812	MK421718	MK421650
S103*	m	NMP6A 6995	SI:	Polje	46.270, 13.907	Ega 58	58	29II	MK421822	MK421728	MK421655
S119	m	NMP6A 6996	SI:	Polje	46.270, 13.907	Ega 58	58	–	MK421823	MK421729	–
S398*	m	NMP6A 6997	SI:	Bohinjska Bistrica	46.279, 13.962	Ega 58	58	29II	MK421841	–	MK421668
S693	m	NMP6A 6998	SI:	Lesno Brdo	46.011, 14.321	Ega 58	58	29II	MK421878	MK421782	–
S696*	f	NMP6A 6999	SI:	Nomenj I	46.272, 14.033	Ega 58	58	–	MK421879	MK421783	MK421692
S697*	m	NMP6A 7000	SI:	Nomenj II	46.283, 14.042	Ega 58	58	29II	MK421880	MK421784	MK421693
S704	m	NMP6A 7001	SI:	Lesno Brdo	46.011, 14.321	Ega 58	58	29II	–	–	–
S705	m	NMP6A 7002	SI:	Lesno Brdo	46.011, 14.321	Ega 58	58	29II	–	–	–
S706	m	NMP6A 7003	SI:	Lesno Brdo	46.011, 14.321	Ega 58	58	29II	–	–	–
S258*	m	NMP6A 7004	SI:	Kamnik	46.201, 14.602	Ega 88	87	42II + III	MK421828	MK421734	MK421659
S259	m	NMP6A 7005	SI:	Kamnik	46.201, 14.602	Ega 88	88	44II	MK421829	MK421735	–
S404	m	NMP6A 7006	SI:	Kebelj	46.399, 15.447	Ega 88	89	43II + III	–	–	–
S416	m	NMP6A 7007	SI:	Kamnik	46.201, 14.602	Ega 88	88	44II	MK421842	MK421746	–
S417*	m	NMP6A 7008	SI:	Kebelj	46.399, 15.447	Ega 88	88	44II	MK421843	MK421747	MK421669
S436	m	NMP6A 6993	SI:	Kamnik	46.201, 14.602	Ega 88	88	44II	MK421849	MK421753	–
S483*	m	NMP6A 7009	SI:	Nova Štifta	46.270, 14.768	Ega 88	87	42II + III	MK421860	MK421764	MK421678
S522	m	NMP6A 7010	SI:	Kebelj	46.399, 15.447	Ega 88	88	44II	MK421864	MK421768	–
S685*	m	NMP6A 7011	SI:	Ig	45.954, 14.531	Ega 88	88	44II	MK421875	MK421779	MK421689
S686*	f	NMP6A 7012	SI:	Gradež	45.880, 14.616	Ega 88	–	–	MK421876	MK421780	MK421690
S694	m	NMP6A 7013	SI:	Štefanja Gora	46.284, 14.496	Ega 88	88	44II	–	–	–
S740	m	NMP6A 7014	SI:	Vologa	46.262, 14.895	Ega 88	88	44II	–	–	–
S741*	m	NMP6A 7015	SI:	Kotedrež	46.158, 14.993	Ega 88	88	44II	MK421885	MK421789	MK421697

S742*	m	NMP6A 7016	SI:	Sklendrovec	46.120, 15.028	Ega 88	88	44II	MK421886	MK421790	MK421698
S743	m	NMP6A 7017	SI:	Podbočje	45.860, 15.458	Ega 88	88	44II	–	–	–
S744*	m	NMP6A 7018	SI:	Laško I	46.157, 15.231	Ega 88	88	44II	MK421887	MK421791	MK421699
S745	m	NMP6A 7019	SI:	Laško II	46.158, 15.237	Ega 88	88	44II	–	–	–
S751*	m	NMP6A 7020	SI:	Laško II	46.158, 15.237	Ega 88	88	44II	MK421888	MK421792	MK421700
S794*	m	NMP6A 7021	SI:	Vologa	46.262, 14.895	Ega 88	88	44II	MK421895	MK421799	MK421705
S799	m	NMP6A 7022	SI:	Vologa	46.262, 14.895	Ega 88	88	44II	–	–	–
S800*	m	NMP6A 7023	SI:	Podbočje	45.860, 15.458	Ega 88	88	–	MK421896	MK421800	MK421706
S1079	m	NMP6A 7024	SI:	Nova Štifta	46.270, 14.768	Ega 88	88	44II	–	–	–
S420*	m	NMP6A 7025	SI:	Kozjak	46.413, 15.175	Ega 86–92	92	46II	MK421844	MK421748	MK421670
S737*	m	NMP6A 7026	AT:	Rechberch	46.524, 14.580	Ega 86–92	92	46II	MK421882	MK421786	MK421695
S738*	m	NMP6A 7027	SI:	Potoče	46.302, 14.441	Ega 86–92	90	42II + 2III	MK421883	MK421787	MK421696
S739	m	NMP6A 7028	SI:	Studenca	46.246, 14.678	Ega 86–92	87	42II + III	MK421884	MK421788	–
S793*	m	NMP6A 7029	SI:	Studenca	46.246, 14.678	Ega 86–92	87	42II + III	MK421894	MK421798	MK421704
S1080	m	NMP6A 7030	SI:	Studenca	46.246, 14.678	Ega 86–92	86	43II	–	–	–
S1081	m	NMP6A 7031	SI:	Studenca	46.246, 14.678	Ega 86–92	86	43II	–	–	–
E. (*P.*) *italicus*											
S005	m		GR:	Vikos	39.949, 20.708				MK421809	MK421715	MK421647
E. (*T.*) *flavicaudis*											
S268	m		IT:	Ischia	40.729, 13.881				MK421833	MK421738	MK421662
E. (*E.*) *carpathicus*											
S270	m		RO:	Valea Orevita	44.727, 21.931				MK421834	MK421739	MK421663
E. (*E.*) *avcii*											
S346	f		TR:	Aydın	37.689, 27.225				MK421840	MK421745	MK421667
E. (*E.*) *celanus*											
S501	m		IT:	Abruzzo	42.404, 13.460				MK421861	MK421765	MK421679
E. (*E.*) *erymanthius*											
S504	m		GR:	Patra	38.227, 21.809				MK421862	MK421766	MK421680

Table S2. List of primer sequences and condition used for amplification.

Gene	Primers	Amplicon length	Sequence	Source	PCR conditions
16S	16Sbr 16SR	370 bp	CGATTTGAACTCAGATCA GTGCAAAGGTAGCATAATCA	Simon et al., 1994 Gantenbein et al., 1999	1. 95°C 3 min 2. 95°C 30s 3. 45°C 1min 4. 72°C 1,5 min 5. 72°C 10 min 35 cycles
COI	LCO1490 HCO2198	614 bp	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994	
28S	28SpsF1 28SpsR1	1100 bp	ATTACCCGCCGAATTTAAGC TCGGAGGGAACCAGCTAC	Murienne et al., 2008	1. 95°C 3 min 2. 95°C 30s 3. 56°C 30s 4. 72°C 1 min 5. 72°C 10 min 34 cycles

References:

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Gantenbein, B., Fet, V., Largiadèr, C.R., Scholl, A., 1999. First DNA phylogeny of *Euscorpius* Thorell, 1876 (Scorpiones, Euscorpiidae) and its bearing on taxonomy and biogeography of this genus. Biogeographica 75, 49–65.

Murienne, J., Harvey, M.S., Giribet, G., 2008. First molecular phylogeny of the major clades of Pseudoscorpiones (Arthropoda: Chelicerata). Mol. Phylogenet. Evol. 49, 170–184. https://doi.org/10.1016/j.ympev.2008.06.002

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701. https://doi.org/10.1093/aesa/87.6.651

Table S3. Geographical distribution of the karyotypic races (KR) of Alpine *Euscorpius* (*Alpiscorpius*) species based on the International Standardized Mountain Subdivision of the Alps (ISMSA) at section level (see Marazzi, 2005). (AT – Austria, CH – Switzerland, IT – Italy, SI – Slovenia).

Species	KR	Distribution
E. (*A.*) *alpha*		
	Eal 60	Pennine Alps (IT, CH)
	Eal 90	Lugano Prealps (IT, CH), Bergamasque Alps and Prealps (IT), Western Rhaetian Alps (IT, CH)
	Eal 54	Southern Rhaetian Alps (IT), Brescia and Garda Prealps (IT)
E. (*A.*) *germanus*		
	Ege 46a	Eastern Rhaetian Alps (AT, IT), Dolomites (IT), Venetian Prealps (IT), Carnic and Gailtal Alps (IT)
	Ege 46b	Julian Alps and Prealps (SI), central belt of Slovenian Dinaric Alps (SI)
	Ege 46c	Slovenian Prealps (SI)
E. (*A.*) *gamma*		
	Ega 60	border of the Carinthian-Slovenian Alps and Slovenian Prealps (IT, SI)
	Ega 58	Julian Alps and Prealps (SI), Slovenian Prealps (SI)
	Ega 86–92	Carinthian-Slovenian Alps (AT, SI)
	Ega 88	Carinthian-Slovenian Alps (SI), Slovenian Prealps (SI), high karstic plateau of the Dinaric Alps (SI)

Reference:

Marazzi, S., 2005. Atlante orografico delle Alpi: SOIUSA: suddivisione orografica internazionale unificata del sistema alpino. Priuli & Verlucca.

5. SHRNUTÍ A ZÁVĚR

V průběhu doktorského studia se našemu výzkumnému týmu podařilo významně rozšířit znalosti o cytogenetice štírů, které do současné doby byly k dispozici především pro intenzivně studovanou skupinu štírů s holokinetickými chromozomy, čeleď Buthidae. Předkládaná dizertační práce je kompilací unikátních komparativních studií, jež se zakládají na aplikaci různých metodických přístupů u skupin štírů s holokinetickými i monocentrickými chromozomy. Při řešení rozličných aspektů týkajících se diverzity a diferenciace karyotypu byl kladen důraz na propojení cytogenetických a sekvenčních dat analyzovaných druhů (viz *Gint* **Článek II**, *Alpiscorpius* **Článek VI**, *Pandinus s.l.* Příloha Obr. 5). Kombinace odlišných metodických přístupů nebyla při studiu mechanismů karyotypové diferenciace dosud u štírů rutinně implementována, ačkoliv vytváří účinný nástroj pro vysvětlení sledovaných fenoménů. Výsledky dílčích projektů této práce demonstrují, že znalost genetické struktury a příbuzenských vztahů studovaných taxonů je klíčovým prvkem pro interpretaci chromozomálního polymorfismu či polytypie (**Článek II** a **VI**). Současně tyto informace tvoří nezbytný rámec pro navržení evolučních trendů strukturních změn v uspořádání genomu zkoumaných druhů. Ve světle morfologické uniformity štírů, jež může maskovat skutečnou diverzitu současných taxonů, je význam syntézy cytogenetických a genetických znaků o to vyšší. V tomto ohledu naše výstupy komparativních analýz u alpských štírů také akcentují význam extenzivního vzorkování při studiu skryté diverzity a karyotypové variability u taxonů, jejichž populace jsou v důsledku nízké vagility a stenotopie senzitivní na prostorovou izolaci a akumulaci odlišných typů chromozomálních změn (**Článek VI**). Interdisciplinární studie, jež tvoří hlavní výstupy této práce, tak mohou představovat nový směr, jímž by se studium cytogenetiky štírů mohlo v budoucnu ubírat.

Souhrnné cytogenetické výsledky, jež byly získány pro zástupce obou evolučních linií štírů lišících se typem chromozomů, potvrdily předpoklad, že tyto odlišné systémy vykazují specifika karyotypových změn na různých strukturních úrovních. Štíři čeledi Buthidae mající holokinetické chromozomy se vyznačují nižší rozmanitostí v počtu chromozomů. Zástupci této čeledi jsou charakterističtí buď poměrně konzervativní nebo naopak vysoce polymorfní makrostrukturou karyotypů. Dle výskytu strukturních heterozygotů je patrné, že k reorganizaci chromozomů u buthidních štírů dochází s vysokou frekvencí. Děje se tak primárně skrze dva typy makrostrukturních přestaveb: převládající reciproké translokace a minoritní chromozomální fúze/rozpady. Naše výsledky mapování rDNA současně indikují možné zapojení inverzí v strukturní reorganizaci karyotypu buthidních štírů, nicméně výskyt tohoto

typu kryptických přestaveb je těžké odhalit, což může způsobit podhodnocení jejich významu v evoluci karyotypu. Časté chromozomální změny nejsou u buthidních druhů překvapivě provázeny zachováním telomerických (TTAGG)*n* repetic ve formě ITS, a pravděpodobně se také nepodílí velkou měrou ani na změnách distribuce a disperze rDNA, jak by se předpokládalo.

Štírům s monocentrickými chromozomy se nedostává velké pozornosti, ačkoliv se jedná se o cytogeneticky velice zajímavou skupinu. Tyto evoluční linie štírů zpravidla vykazují na mezidruhové úrovni vysoce diferencované karyotypy, což podporují i námi získané výsledky. Makrostrukturní změny v uspořádání genomu zde pravděpodobně nastávají převážně v důsledku centrických (resp. Robertsonovských) fúzí či rozpadů. Tomu napovídají nejen avizované zřetelné rozdíly ve 2*n*, ale také vysoký podíl strukturních heterozygotů právě pro tento typ přestaveb, či přítomnost centromerických ITS (Příloha Obr. 3). Zapojení tandemových fúzí v chromozomálních změnách také není zcela vyloučeno, jak naznačuje např. specifická distribuce ITS u *Pandinus sensu lato*. Reciproké translokace, jež byly v heterozygotním stavu identifikovány pouze u jednoho námi studovaného druhu, se pravděpodobně v diferenciaci karyotypu štírů s monocentrickými chromozomy uplatňují v menší míře. U těchto evolučních linií štírů není ovšem vyloučeno ani zapojení para- i pericentrických inverzí, což dokazují jak publikované studie (Shanahan 1989b, Shanahan & Hayman 1990), tak naznačují i naše výsledky odlišné distribuce rDNA mezi blízce příbuznými druhy (**Článek VI**).

Předkládaná dizertační práce významně přispěla k rozšíření poznatků o karyotypové rozmanitosti na různých strukturních úrovních u zástupců štírů s holokinetickými a monocentrickými chromozomy. Nicméně pro řadu sledovaných fenoménů, např. multiplikace rDNA u buthidních štírů (**Článek I**), velikostní heteromorfie lokusů rDNA mezi homologickými chromozomy u *Pandinurus*, či distribuce ITS u *Pandinus s.l.* (Příloha Obr. 2–3), prozatím nemáme dostatek informací, jež byl indikovaly skutečné pozadí mechanismů způsobujících tyto změny na mikrostrukturní úrovni. Výsledky práce tak současně otevírají řadu otázek, jež mohou být předmětem řešení budoucích projektů zaměřených na objasnění podstaty kryptických chromozomálních změn, které mohou hrát důležitou roli v diferenciaci a evoluci karyotypů štírů.

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7. PŘÍLOHY

Tabulka 1. Přehledová tabulka všech cytogeneticky analyzovaných druhů čeledi Buthidae s příslušnými karyotypovými charakteristikami. Hodnoty v závorkách uvedené u každého rodu udávají počet analyzovaných taxonů/druhovou rozmanitost rodu. (2*n* – diploidní počet chromozomů, * sporné údaje 2*n*, M – meióza I bez strukturních heterozygotů, m – mitotické metafáze bez možnosti identifikace struktur. heterozygotů, INvar – intraindividuální variabilita, N – počet analyzovaných jedinců, NOR – organizátor jadérka, T – terminální, INT – intersticiální, ST – subterminální)

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
AEGAEOBUTHUS (1/4)							
A. gibbosus	Turecko	28	M	3♂♀	–	–	Karataş et al. 2019
ANANTERIS (1/85)							
A. balzanii	Brazílie	12	m	embrya	–	–	Piza 1947a
″	Brazílie	14	5II + ***IV*** (1♂)	3♂	AgNO3	2/T	Mattos et al. 2013
ANDROCTONUS (11/29)							
A. amoreuxi	Egypt	24	ano (nespecifikováno)	?	–	–	Moustafa et al. 2005
	Maroko	24	9II + ***VI*** (1♂); 8II + ***VIII*** (1♂)	4♂	18S	2/INT	**Článek I**
A. australis	Egypt	24	ano (nespecifikováno)	?	–	–	Moustafa et al. 2005
″	Egypt	24	M	1♂	18S	2/INT	Sadílek et al. 2015
A. bicolor	Egypt	24	ano (nespecifikováno)	?	–	–	Moustafa et al. 2005
″	Izrael	24	M	1♂	18S	2/INT	**Článek I**
A. bourdoni	Maroko	24	m	1♂	18S	2/INT	Sadílek et al. 2015
″	Maroko	24	M	2♂	18S	2/INT	**Článek I**
A. crassicauda	Egypt	24	ano (nespecifikováno)	?	–	–	Moustafa et al. 2005
″	Turecko	24	1♂ Invar: 10II + ***IV*** nebo 8II + ***VIII***	2♂	18S	2/INT	Sadílek et al. 2015
A. gonneti	Maroko	24	9II + ***VI*** (1♂)	2♂	18S	2/INT	**Článek I**
A. liouvillei	Maroko	24	M	2♂	18S	2/INT	**Článek I**
A. maelfaiti	Pákistán	24	M	1♂	18S	2/INT	Sadílek et al. 2015
A. mauritanicus	Maroko	24	m	?	–	–	Chovet et al. 1971
″	Maroko	24	M	1♂	18S	2/INT	**Článek I**
A. sergenti	Maroko	24	10II + ***IV*** (1♂)	2♂	18S	2/INT	**Článek I**
A. tenuissimus	Egypt	24	m	2♂	18S	2/INT	**Článek I**
APISTOBUTHUS (1/2)							
A. pterygocercus	Omán	22	M	1♂	18S	2/T	**Článek I**
BABYCURUS (3/14)							
B. jacksoni	Tanzánie	16	M	1♂	–	–	Kovařík et al. 2018b
B. buettneri	Kamerun	28	M	1♂	–	–	Kovařík et al. 2018b

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
B. gigas	Tanzánie	30	m	1♂	–	–	Kovařík et al. 201ba
BARBARACURUS (3/10)							
B. sofomarensis	Etiopie	22	6II + ***X*** (2♂)	2♂	–	–	Kovařík et al. 2015a
B. somalicus	Somaliland	36	M	1♂	–	–	Kovařík et al. 2018b
B. zambonelli	Eritrea	26	m	1♂	–	–	Kovařík et al. 2018b
BUTHACUS (3/23)							
B. macrocentrus	Turecko	28	m	3♂	18S	2/T	**Článek I**
B. nigroaculeatus	?	26	m	1♂	18S	2/INT	**Článek I**
B. stockmanni	Maroko	20	m	1♂	–	–	Kovařík et al. 2016d
″	″	″	″	″	18S	2/INT	**Článek I**
BUTHOSCORPIO (1/5)							
B. sarasinorum	Srí Lanka	14	m	1♂	–	–	Kovařík et al. 2016a
BUTHUS (5/55)							
B. atlantis	Maroko	22	M	2♂	18S	2/INT	**Článek I**
B. awashensis	Etiopie	22	6II + ***X*** (1♂)	1♂	18S	2/INT	**Článek I**
B. berberensis	Somaliland	22	M	3♂	18S	2/INT	**Článek I**
B. occitanus	Francie	22	?	?	–	–	Guénin 1961
″	?	44*	?	?	–	–	Carnoy 1885
B. maroccanus	Maroko	22	M	1♂	18S	2/INT	**Článek I**
CENTRUROIDES (4/92)							
C. exilicauda	?	26	–	?	–	–	Wilson 1931
C. baracoae	Kuba	26	M	2♂	18S	2/T	**Článek I**
C. gracilis	Kuba	24	10II + ***IV*** (1♂)	1♂	18S	2/T	**Článek I**
C. vittatus	USA	26	–	–	–	–	Riess et al. 1978
CHARMUS (1/5)							
C. laneus	Srí Lanka	9	m	1♂	–	–	Kovařík et al. 2016a
COMPSOBUTHUS (6/47)							
C. acutecarinatus	Omán	22	M	1♂, 1♀	18S	2/ST	**Článek I**
C. arabicus	Omán	22	m	1♂	18S	2/INT	**Článek I**
C. eritreaensis	Eritrea	22	M	1♂	–	–	Kovařík et al. 2016b
″	″	″	″	″	18S	2/INT	**Článek I**
C. maindroni	Omán	22	9II + ***IV*** (2♂)	3♂	18S	2/INT	**Článek I**

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
C. matthiesseni	Turecko	22	7II + ***VII*** (1♂)	3♂, 1♀	–	–	Stahlavsky et al. 2014
″	″	″	″	″	18S	2/INT	**Článek I**
C. schmiedeknechti	Turecko	22	M	1♂	18S	2/INT	**Článek I**
GINT (5/12)							
G. amoudensis	Somaliland	35, 36	m	4♂	–	–	Kovařík et al. 2018c
″	Somaliland	35, 36	14II + ***III*** + ***IV*** (1♂); 13II + ***III*** + ***VI*** (1♂); 15II + ***VI*** (1♂); 16II + ***IV*** (1♂)	4♂	18S	2–3/T, ST, INT	**Článek II**
G. banfasae	Somaliland	18, 19	7II + ***IV*** (2♂); 6II + ***VI*** (1♂); 5II + ***III*** + ***VI*** (2♂)	5♂	18S	2/ST	**Článek II**
G. dabakalo	Somaliland	23	m	1♂	–	–	Kovařík et al. 2018c
″	Somaliland	23, 24, 27	9II + ***V*** (1♂); 8II + 2***III*** + ***V*** (1♂); 8II + ***III*** + ***V*** (1♂)	3♂	18S	2/ST	**Článek II**
G. gaitako	Etiopie	30	13II + ***IV*** (1♂)	1♂	–	–	Kovařík et al. 2013a
″	Etiopie	30	13II + ***IV*** (6♂)	6♂	18S	2T	**Článek II**
G. gubanensis	Somaliland	45	21II + ***III*** (1♂)	1♂	18S	2T	**Článek II**
G. maidensis	Somaliland	34	m	3♂	–	–	Kovařík et al. 2018c
″	Somaliland		M	3♂	18S	2/T	**Článek II**
GROSPHUS (2/10)							
Grosphus cf. *darainensis*	Madagaskar	14	5II + ***IV*** (1♂)	1♂	18S	2/?	**Článek I**
Grosphus sp.	Madagaskar	13	2II + ***VIII*** + I (1♂)	1♂	18S	2/T	**Článek I**
HEMILYCHAS (1/1)							
H. alexandrinus	Australia	14	m	2♀	–	–	Shanahan 1989a
HETEROCTENUS (3/9)							
H. abudi	Dominikánská republika	16	M	1♂	18S	2/T	**Článek I**
H. garridoi	Kuba	23	10II + ***III*** (1♂)	1♂	18S	2/T	**Článek I**
H. princeps	Dominikánská republika	16	M	3♂	18S	2/T	**Článek I**
HOTTENTOTTA (12/55)							
H. arenaceus	Namibie	14	m	1♂	18S	2/INT*	**Článek I**
H. conspersus	Namibie	16	M	1♂	18S	2/INT	**Článek I**
H. flavidulus	Pákistán	24	5II + ***XIV*** (1♂)	1♂	18S	2/T	**Článek I**
H. hottentotta	Mali/Benin	16	m	1♂, 1♀	18S	2/INT	**Článek I**
H. judaicus	Palestina	16	M	6♂	–	–	Qumsiyeh et al. 2013

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
H. minusalta	Afghánistán	16	M	3♂	18S	2/INT	**Článek I**
H. saulcyi	Turecko	14	m	1♂	18S	2/INT	**Článek I**
H. sousai	Maroko	14	M	1♂	18S	2/INT	**Článek I**
H. tamulus	Indie	22	9II + ***IV*** (1♂)	1♂	–	–	Sharma et al. 1959
″	Indie	20-28			–	–	Venkatanarasimhiah & Rajasekarasetty 1964a
″	Indie	24			–	–	Gupta & Sarker 1965
H. trilineatus	Zimbabwe	24	M	1♂	–	–	Newlands & Martindale 1980
H. ugandaensis	Uganda	22	m	1♀	18S	2/T	**Článek I**
Hottentotta sp.	Pákistán	22	8II + ***VI*** (1♂)	1♂	18S	2/T	**Článek I**
ISCHNOTELSON (3/2)							
I. guanambiensis	Brazílie	25	11II + ***III*** (1♂)	1♂	AgNO3/28S	2/T	Ubinski et al. 2018
I. peruassu	Brazílie	26	M	2♂2♀	AgNO3/28S	2/T	Ubinski et al. 2018
Ischnotelson sp.	Brazílie	26	INvar: 13II nebo 11II + ***IV*** (1 jádro) (1♂)	2♂2♀	AgNO3/28S	2/T	Ubinski et al. 2018
ISOMETROIDES (1/1)							
I. vescus	Austrálie	14	5II + ***IV*** (3♂)	6♂	–	–	Shanahan 1989a
ISOMETRUS (3/7)							
I. maculatus	Brazílie	12	2II + ***VIII*** (2♂); 3III + ***VI*** (2♂)	4♂	–	–	Piza 1947b, 1950b, 1955
″	Austrálie	14	5II + ***IV*** (1♂)	1♂	–	–	Shanahan 1989a
I. melanodactylus	Austrálie	14	m	4♂, 1♀	–	–	Shanahan 1989a
I. thwaitesi	Srí Lanka	8	m	1♂	–	–	Kovařík et al. 2016a
″	″	″	2II + ***IV***	9♂	18S	2/T	**Článek I**
JAGUAJIR (3/3)							
J. agamemnon	Brazílie	28	***XXVIII*** (3♂)	4♂, 2♀	AgNO3	2/T	Mattos et al. 2013
″	Brazílie	28	M	2♂	28S	2/T	Ubinski et al. 2018
J. pintoi	Brazílie	28	INvar: 14II nebo 13II + ***IV*** (2 jádra) (2♂)	4♂	AgNO3/28S	2/T	Ubinski et al. 2018
J. rochae	Brazílie	28	M	1♂	–	–	Piza 1957
″	Brazílie	28	8II + ***IV*** + ***VIII*** (2♂); 9II + ***X*** (1♂); 10II + ***VIII*** (1♂)	4♂, 2♀	AgNO3	2/T; 3/T, INT	Mattos et al. 2013
″	Brazílie	28	m	9♂	28S	2/T	Ubinski et al. 2018
JANALYCHAS (1/7)							
J. srilankensis	Srí Lanka	16	m	1♂	–	–	Kovařík et al. 2016a
LEIURUS (4/12)							
L. abdullahbayrami	Turecko	22	9II + ***IV*** (1♂)	3♂, 3♀	–	–	Stahlavsky et al. 2014

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
L. abdullahbayrami	Turecko	22	9II + ***IV*** (1♂)	2♂	18S	2/INT	**Článek I**
L. hebraeus	Západní břeh Jordánu	22	m	1♂	18S	2/INT	**Článek I**
L. quinquestriatus	Palestina	22	M	♂♀	–	–	Qumsiyeh et al. 2013
Leiurus sp.	Afrika	22	M	1♂	18S	2/INT	**Článek I**
LYCHAS (7/43)							
L. krali	Thajsko	16	M	2♂	18S	4/T	**Článek I**
L. marmoreus	Austrálie	12, 14, 15	5II + ***IV*** (13♂); 4II + ***VI*** (4♂); 3II + ***VIII*** (2♂); 2II + ***X*** (3♂)	90♂, 39♀	–	–	Shanahan 1989a
L. mucronatus	Thajsko	10	2II + ***VI*** (1♂)	1♂	18S	4/T	**Článek I**
L. obsti	Tanzánie/Etiopie	10	1II + ***VIII*** (1♂)	2♂	18S	2/T	**Článek I**
L. scutilus C. L.	Thajsko	14	M	2♂	18S	2/T	**Článek I**
L. variatus	Austrálie	14, 16	5II + ***IV*** (1♂); II + ***XII*** (7♂); 6II + ***IV*** (1♂)	15♂, 18♀	–	–	Shanahan 1989a
MESOBUTHUS (4/25)							
M. eupeus	?	22	?	?	–	–	Sokolow 1913
″	Turecko	22	M	2♂	18S	2/INT	**Článek I**
″	Turecko	20	M	3♂♀	–	–	Karataş et al. 2019
M. macmahoni	Indie	24	M	1♂	–	–	Sharma et al. 1959
MICROBUTHUS (3/7)							
M. gardneri	Omán	26	M	1♂	–	–	Lowe et al. 2018
M. kristensenorum	Omán	26	M	2♂	–	–	Lowe et al. 2018
M. satyrus	Omán/Jemen	26	M	1♂	–	–	Lowe et al. 2018
ODONTOBUTHUS (1/6)							
O. doriae	Indie	22	INvar: 6II + ***X*** nebo 6II + ***VI*** + ***IV*** (1♂)	5♂	–	–	Sharma et al. 1959
OLIVIERUS (1/18)							
O. martensii	Čína	24	?	12♂	–	–	Sato 1940
ORTHOCHIRUS (3/41)							
O. afar	Somaliland	24	M	2♂	18S	2/INT	**Článek I**
O. glabrifrons	Omán	22	M	2♂	18S	4/INT	**Článek I**
O. innesi	Maroko/Omán	22	M	9♂	18S	2/INT	**Článek I**
PARABUTHUS (9/33)							
P. abyssinicus	Etiopie/Eritrea	16	M	2♂	–	–	Kovařík et al. 2016c

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
P. abyssinicus	Etiopie/Eritrea	16	M	2♂	18S	2/T	**Článek I**
P. capensis	JAR	20	m	1♂	18S	2/T	**Článek I**
P. glabrimanus	Namibie	20	M	1♂	18S	2/T	**Článek I**
P. kabateki	Somaliland	16	6II + ***IV*** (1♂); 5II + ***VI*** (1♂)	2♂	–	–	Kovařík et al. 2019
P. kajibu	Etiopie	18	M	1♂	–	–	Kovařík et al.2016c
″	″	″	″	″	18S	2/T	**Článek I**
P. mossambicensis	Zimbabwe	36*	M	1♂	–	–	Newlands & Martindale 1980
″	Mozambik	18	m	1♂	18S	2/T	**Článek I**
P. pallidus	Etiopie	20	8II + ***IV*** (1♂)	1♂	–	–	Kovařík et al. 2016c
″	″	″	″	″	18S	2/T	**Článek I**
P. planicauda	JAR	18	M	2♂	18S	2/T	**Článek I**
P. raudus	Zimbabwe	18	M	1♂	–	–	Newlands & Martindale 1980
″	Namibie	20	M	1♂	18S	2/T	**Článek I**
P. robustus	Somaliland	16	M	1♂	–	–	Kovařík et al. 2019
P. somalilandus	Somaliland	16	M	1♂	–	–	Kovařík et al. 2019
P. transvaalicus	Zimbabwe	20	M	2♂	–	–	Newlands & Martindale 1980
PHYSOCTONUS (1/3)							
P. debilis	Brazílie	26	m	3♀	AgNO3/28S	2/T	Ubinski et al. 2018
REDDYANUS (6/27)							
R. basilicus	Srí Lanka	15, 16	m	2♂	–	–	Kovařík et al. 2016a
″	″	″	7II + ***III*** (1♂)	2♂	18S	4/T	**Článek I**
R. ceylonensis	Srí Lanka	16	m	1♂	–	–	Kovařík et al. 2016a
″	″	″	M	2♂	18S	6/T	**Článek I**
R. loebli	Srí Lanka	17	m	1♂	–	–	Kovařík et al. 2016a
″	″	″	2II + ***XIII*** (1♂); 3II + ***XI*** (1♂)	2♂	18S	4/T	**Článek I**
Reddyanus sp. 1	Vietnam	14	M	1♂	18S	4/T	**Článek I**
Reddyanus sp. 2	Thajsko	11	II + ***IX***	3♂	18S	2/T	**Článek I**
RHOPALURUS (1/3)							
R. laticauda	Brazílie	22	9II + ***IV*** (17♂)	19♂	AgNO3/28S	2/T	Ubinski et al. 2018
SOMALICHARMUS (1/1)							
S. whitmanae	Etiopie	20, 21	8II + ***IV*** (1♂); 7II + ***VI*** (1♂); 9II + ***III*** (1♂)	8♂	–	–	Kovařík et al. 2016e

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
THAICHARMUS (1/4)							
Thaicharmus sp.	Vietnam	16	M	1♂	18S	2/T	**Článek I**
TITYUS (29/220)							
T. bahiensis	Brazílie	5, 6, 7, 9, 10, 12, 16, 17, 18, 19	II + ***VII***; 6II + 2***III***; II + ***VI***; II + ***IV***; II + ***III***; 3II + ***IV***; II + 2***IV***; II + ***IV***; ***IX*** (1♂); ***VII*** (1♂); 6II + ***V***; 5II + ***III*** + V; 7II + ***V***; 5II + ***VII***; 5II + ***III*** + ***IV***; 3II + ***IV***; 3II + ***III***; II + ***IV*** + ***III*** (1♂), INvar: 3II + ***IV*** nebo 3II + ***VI*** (1♂)	? ♂, ♀, embrya	–	–	Piza, 1939a,b, 1940, 1943a,b, 1944, 1947a,c, 1948b,c, 1950a,b
″	Brazílie	6	M	? ♂	–	–	Brieger & Graner 1943
″	Brazílie	10	2II + ***VI***; 3II + ***IV***; II + ***VIII***; ***X***; komplexní konfigurace bez možnosti analýz	? ♂	–	–	Takahashi 1976
″	Brazílie	10			–	–	Cunha & Pavan 1954
″	Brazílie	5, 6, 9, 10	II + ***VII*** (2♂); ***VI***; II + ***III***	16♂, 9♀	AgNO3	2/T; 2/ST; 3/T, ST	Schneider et al. 2009b
″	Brazílie	6	VI (7♂)	7♂, 5♀	AgNO3	2/T	Mattos et al. 2013
T. clathratus	Brazílie	19, 20	4II + ***XI*** (2♂); INvar: II + ***XIII*** nebo 3II + ***XIII*** (1♂); 2II + ***XI***, 3II + ***XI*** nebo 4II + ***XI*** (1♂); 3II + ***XI***, 4II + ***X*** nebo 4II + ***XI*** (1♂); 4II + ***X*** nebo 4II + ***XI*** (1♂); 5II + ***IX***, 5II + ***X*** nebo 5II + ***XI*** (1♂); 3II + ***X***, 4II + ***X*** nebo 5II + ***X*** (1♂); 4II + *IX* nebo 4II+***X*** (1♂)	15♂, 1♀	AgNO3/28S	2/T	Mattos et al. 2018
T. confluens	Brazílie	13	5II + ***III*** (2♂)	2♂	AgNO3	2/T	Mattos et al. 2013
″	Argentina	5, 6	II + ***IV*** (8♂); ***V*** (3♂); ***V*** (1♂); ***VI*** (1♂)	21♂, 6♀, 11 embryí	28S	2/T, INT	Adilardi et al. 2016
T. costatus	Brazílie	16	m	1♀	AgNO3	2/T	Mattos et al. 2013
T. curupi	Argentina	31, 32	14II + ***III*** (1♂)	3♂	28S	2/T	Ojanguren-Affilastro et al. 2017
T. crassimanus	Dominikánská republika	29	12II + ***V*** (2♂)	2♂	18S	2/T	**Článek I**
T. ecuadorensis	Ekvádor	24	10II + ***IV*** (1♂)	3♂	18S	2/T	**Článek I**
T. elii	Dominikánská republika	22	7II + ***VIII*** (1♂)	1♂	18S	2/T	**Článek I**
T. fasciolatus	Brazílie	14	M	10♂, 6♀	AgNO3	2/T	Mattos et al. 2013
T. fuhrmanni	Kolumbie	22	M	1♂	18S	2/T	**Článek I**
T. gaffini	Kolumbie	22	M	3♂	18S	4/T	**Článek I**
T. magnimanus	?	20	M	?	–	–	Kovařík et al. 2009
T. mana	Fr. Guayana	20	7II + ***VI*** (1♂)	1♂	18S	2/T	**Článek I**

Tabulka 1. ***pokračování***

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
T. maranhensis	Brazílie	20	INvar: 9II, 10II, nebo 8II + ***IV*** (2♂)	5♂, 7♀	AgNO3	2/T	Mattos et al. 2013
″	Brazílie	20	–	5♂	28S	2/T	Mattos et al. 2018
T. martinpaechi	Brazílie	6	***VI*** (2♂)	2♂, 1♀	AgNO3	2/T	Mattos et al. 2013
T. matogrossensis	Brazílie	20	INvar: 10II nebo 8II + IV nebo 9II	1♂	–	–	Piza 1947a
″	Brazílie	19, 20	8II + ***IV*** (3♂); 8II + ***III*** (1♂); 6II + ***III*** + ***IV*** (1♂)	41♂, 3♀	AgNO3/28S	2/T	Mattos et al. 2018
T. metuendus	Brazílie	15, 16		3♂, 1♀	–	–	Piza 1952
T. neglectus	Brazílie	26, 27	12II + ***III*** (1♂)	1♂, 2♀	–	–	Piza 1950b
T. neibae	Dominikánská republika	32	10II + 2***III*** + ***VI*** (1♂)	1♂	18S	2/T	**Článek I**
T. obscurus	Brazílie	11, 12, 13, 14, 16	5II + ***III*** (2♂); 3II + 2***III*** (1♂); 4II + ***III*** (1♂); 6II + ***IV*** (1♂)	15♂, 22♀	45S	4/T	Almeida et al. 2017
T. paraguayensis	Brazílie	16	INvar: 18II, 5II + 3***IV***, nebo 5II + 3***IV*** + ***VIII*** (1♂)	4♂, 8♀	AgNO3	2/T	Mattos et al. 2013
″	Brazílie	16, 17, 18	5II + ***VII*** (5♂); INvar: 5II + ***VII*** nebo 5II + ***VIII*** (1♂)	29♂	AgNO3/28S	2/T	Mattos et al. 2018
T. pusillus	Brazílie	19, 20	7II + ***VI*** (4♂); INvar: 7II + ***IV*** nebo 8II + ***III*** (1♂), 7II + ***IV*** nebo 7II + ***V*** (2♂)	23♂, 1♀	AgNO3/28S	2/T	Mattos et al. 2018
T. serrulatus	Brazílie	12		?	–	–	Piza 1947a
″	Brazílie	12	m	P♀	AgNO3/pDm 238 rDNA	1/T	Schneider & Cella 2010
T. silvestris	Brazílie	16, 24	2II + ***XII*** (1♂)	16♂, 3♀	AgNO3/28S	2/T	Mattos et al. 2018
T. smithii	Grenada	6	M	2♂	18S	6/T	**Článek I**
T. stigmurus	Brazílie	16	m	2♀	–	–	Piza 1950b
″	Brazílie	16	m	3♀	AgNO3	2/T	Mattos et al. 2013
T. trivittatus	Brazílie	14	5II + ***IV*** (1♂)	? ♂, ♀, embrya	–	–	Piza 1948a
″	Argentina	6	m	P♀	28S	2/T	Adilardi et al. 2014
T. uruguayensis	Argentina	31	12II + ***VII*** (7♂); 10II + ***IV*** + ***VII*** (2♂)	9♂	28S	4/T	Ojanguren-Affilastro et al. 2017
T. ythieri	?	20	M	?	–	–	Kovařík et al. 2009
TROGLORHOPALURUS (2/2)							
T. lacrau	Brazílie	22	m	10♀	AgNO3	2/T	Ubinski et al. 2018
T. translucidus	Brazílie	20	M	2♀	AgNO3	2/T	Ubinski et al. 2018
UROPLECTES (10/34)							
U. carinatus	Zimbabwe	20, 48*	M	3♂	–	–	Newlands & Martindale 1980

ROD/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
U. chubbi	Zimbabwe	20	M	2♂	–	–	Newlands & Martindale 1980
U. emiliae	?	28	M	1♂	18S	2/T	**Článek I**
U. fischeri	Etiopie	28	12II + ***IV*** (1♂)	1♂	–	–	Kovařík et al. 2016c
U. flavoviridis	Zimbabwe	26	M	3♂	–	–	Newlands & Martindale 1980
U. formosus	JAR	16	M	2♂	18S	2/T	**Článek I**
U. olivaceus	Zimbabwe	24	10II + ***IV*** (1♂)	1♂	–	–	Newlands & Martindale 1980
U. otjimbinguensis	Namibie	26	8II + ***X*** (1♂); 9II + ***VIII*** (2♂)	3♂	18S	4/T; 6/T	**Článek I**
U. planimanus	Zimbabwe	22	M	4♂	–	–	Newlands & Martindale 1980
U. vittatus	Zimbabwe	24	M	1♂	–	–	Newlands & Martindale 1980
ZABIUS (1/3)							
Z. fuscus	Argentina	18	7II + ***IV*** (1♂)	10♂, 12♀, 11 embryí	28S	2/T	Adilardi et al. 2015

Tabulka 2. Přehledová tabulka všech cytogeneticky analyzovaných druhů čeledí štírů s monocentrickými chromozomy s příslušnými karyotypovými charakteristikami. (2*n* – diploidní počet chromozomů, M – meióza I bez strukturních heterozygotů, m – mitotické metafáze bez možnosti identifikace struktur. heterozygotů, N – počet analyzovaných jedinců, NOR – organizátor jadérka, *p* – krátká chromozomální raménka, *q* – dlouhá chromozomální raménka, T – terminální, INT – intersticiální, ST – subterminální)

ČELEĎ/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
BOTHRIURIDAE							
***Bothriurus** araguayae*	Brazílie	44	–	6	–	–	Ferreira 1968
B. araguayae	Brazílie	42	19II + ***IV*** (1♂)	8♂, 1♀	AgNO3	6/*p*,T	Schneider et al. 2009b
B. flavidus	Argentina	48	–	?	–	–	Giacomozzi 1977
B. prospicuus	Argentina	50	–	?	–	–	Giacomozzi 1977
B. rochensis	Brazílie	46	M	2♂, 2♀	AgNO3	6/*p*,T	Schneider et al. 2009b
Bothriurus sp.	Brazílie	36	M		–	–	Piza 1947a
***Brachistosternus** alienus*	Argentina	28	–	?	–	–	Giacomozzi 1977
B. alienus	Argentina	46	M	2♂	–	–	Adilardi et al. 2013
B. ferrugineus	Argentina	46	M	6♂	–	–	Rodríguez-Gil et al. 2009
B. montanus	Argentina	46	M	4♂	–	–	Rodríguez-Gil et al. 2009
B. pentheri	Argentina	46, 42	M	9♂	–	–	Giacomozzi 1977, Rodríguez-Gil et al. 2009
***Timogenes** elegans*	Argentina	48	–	?	–	–	Giacomozzi 1977
CARABOCTONIDAE							
***Hadrurus** hirsutus*	?	100	–	?	–	–	Wilson 1931
CHACTIDAE							
***Brotheas** amazonicus*	Brazílie	50	M	4♂	–	–	Ferreira, 1968
***CHAERILIDAE**							
***Chaerilus** cimrmani*	Thajsko	104	M	2♂	–	–	Kovařík et al. 2018a
C. granulatus	Vietnam	96	M	1♂	–	–	Kovařík et al. 2015c
C. hofereki	Vietnam	90	M	1♂	–	–	Kovařík et al. 2014
C. majkusi	Malajsie	76	M	4♂	–	–	Kovařík et al. 2018a
C. neradorum	Thajsko	106/108	M	1♂	–	–	Kovařík et al. 2018a
C. sejnai	Malajsie	124/126	M	1♂	–	–	Kovařík et al. 2018a

Tabulka 2. ***pokračování***

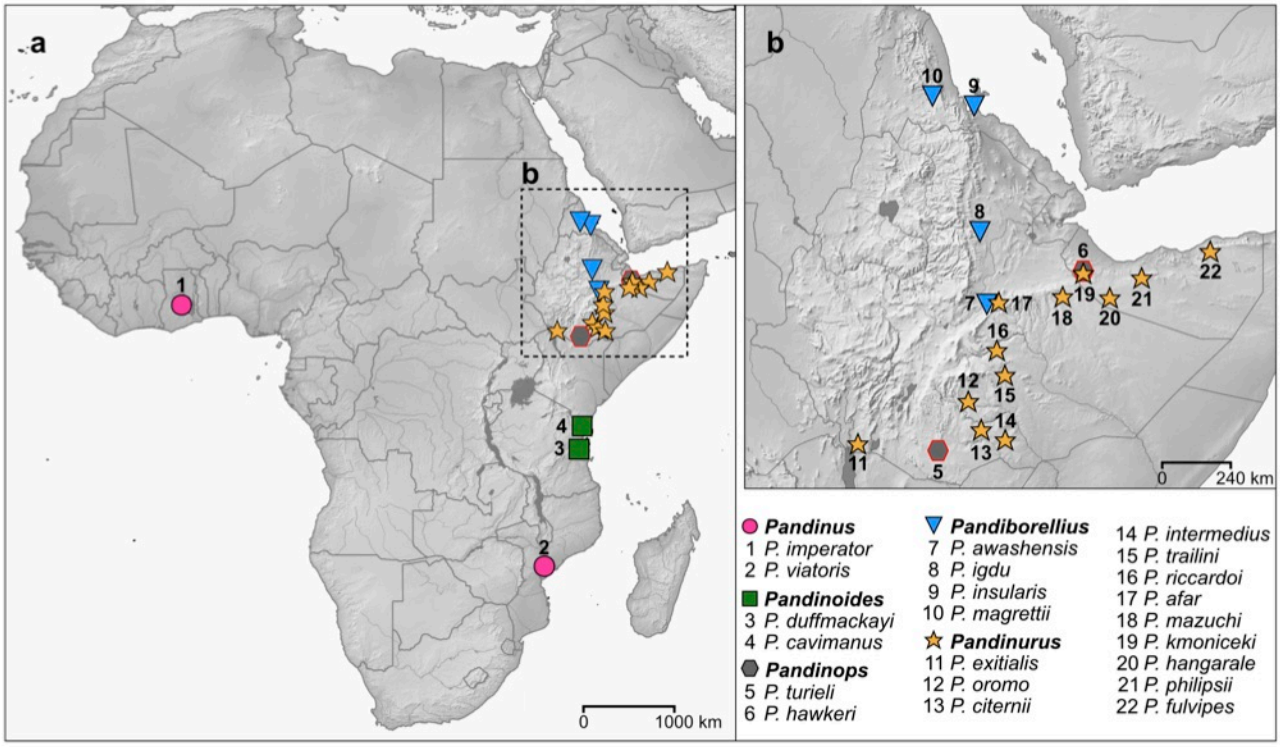
ČELEĎ/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
C. stockmannorum	Thajsko	186	89II + ***III*** + ***VI*** (1♂)	1♂	–	–	Kovařík et al. 2018a
C. tichyi	Malajsie	136	M	1♂	–	–	Kovařík et al. 2018a
EUSCORPIIDAE							
Alpiscorpius *delta*	Itálie	54	M	24♂	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. beta	Itálie, Švýcarsko	60	M	5♂	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. alpha	Itálie, Švýcarsko	90	M	24♂	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. germanus	Itálie, Rakousko, Slovinsko	46	M	28♂, 2♀	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. lambda	Slovinsko	46	M	12♂, 1♀	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. kappa	Slovinsko	46	M	6♂	18S	2/*q*,T	**Článek VI**, Kovařík et al. 2019b
A. omikron	Slovinsko	58	28II + ***IV*** (2♂)	9♂, 1♀	18S	2/*p*,ST	**Článek VI**, Kovařík et al. 2019b
A. sigma	Itálie, Slovinsko	60	M	2♂	18S	2/*p*,ST	**Článek VI**, Kovařík et al. 2019b
A. omega	Slovinsko	88	42II + ***III*** (2♂); 43II + ***III*** (1♂)	21♂	18S	2/*q*,ST	**Článek VI**, Kovařík et al. 2019b
A. ypsilon	Rakousko, Slovinsko	86–92	42II + ***III*** (2♂); 42II + 2***III*** (1♂)	7♂	18S	2/*p*,ST	**Článek VI**, Kovařík et al. 2019b
A. gamma	Slovinsko	78	–	–	–	–	Kovařík et al. 2019b
Euscorpius *aladaglarensis*	Turecko	88	ano (nespecifikováno)	1♂, 1 embryo	–	–	Karataş et al. 2019
E. carpathicus	Itálie	70–84	M	?	–	–	Sokolow 1913
SCORPIOPIDAE							
Euscorpiops *neradi*	Thajsko	48	m	1♂	–	–	Kovařík et al. 2013b
E. orioni	Thajsko	103	50II + ***III*** (2♂)	2♂	–	–	**Článek III**
Alloscorpiops *wongpromi*	Thajsko	95	46II + ***III*** (1♂)	1♂	–	–	Kovařík et al. 2015b
HORMURIDAE							
Hadogenes *bicolor*	Jihoafrická republika	96	–	?	–	–	Newlands & Cantrell 1985
H. gracilis	Jihoafrická republika	80	–	?	–	–	Newlands & Cantrell 1985
H. granulatus	Zimbabwe	96	–	?	–	–	Newlands & Cantrell 1985
H. gunningi	Jihoafrická republika	88	–	?	–	–	Newlands & Cantrell 1985
H. lawrencei	Namibie	132	–	?	–	–	Newlands & Cantrell 1985
H. minor	Jihoafrická republika	106	–	?	–	–	Newlands & Cantrell 1985
H. phyllodes	Jihoafrická republika	72	–	?	–	–	Newlands & Cantrell 1985
H. hahnii	Namibie	100	–	?	–	–	Newlands & Cantrell 1985
H. tityrus	Namibie	168	–	?	–	–	Newlands & Cantrell 1985

Tabulka 2. ***pokračování***

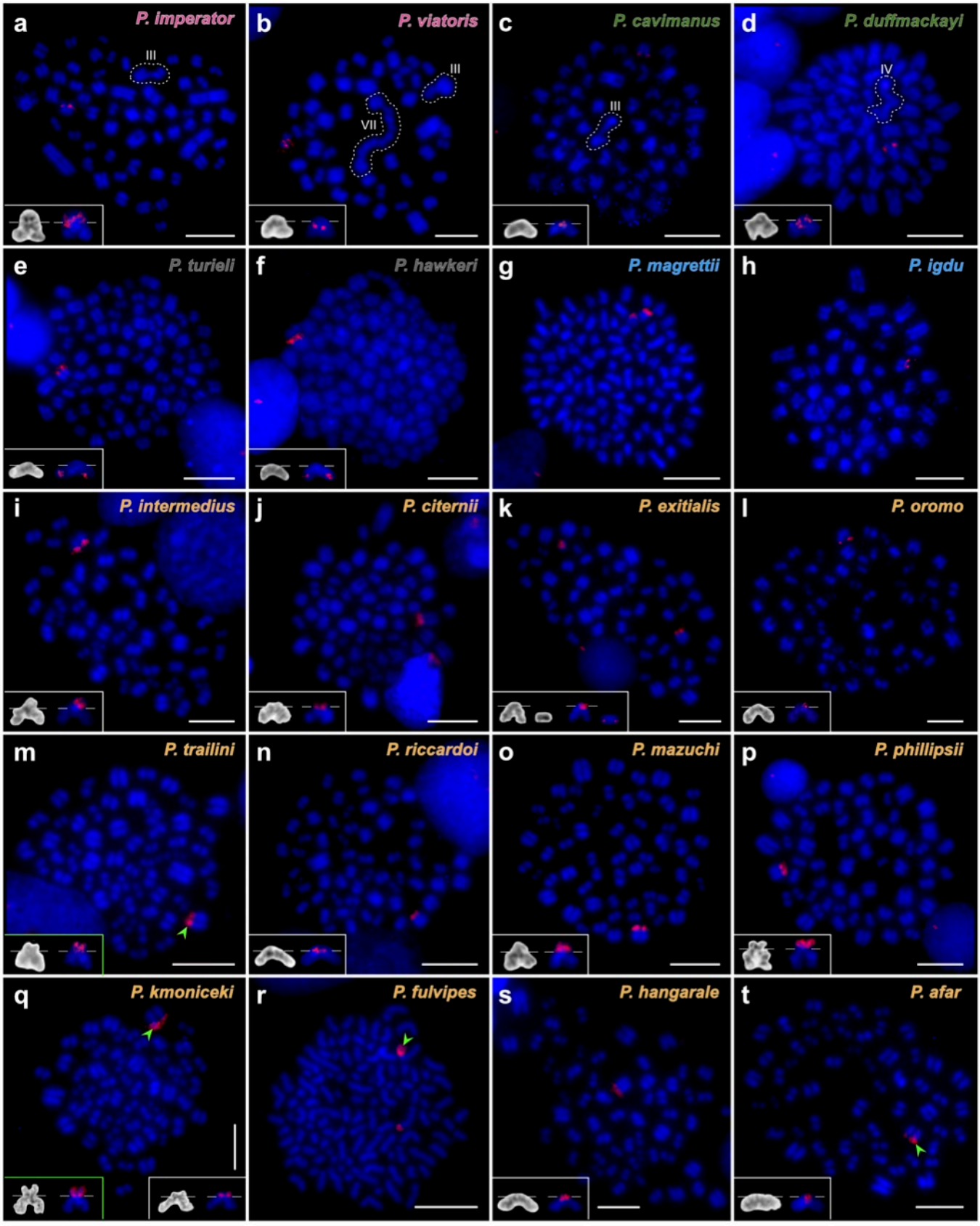
ČELEĎ/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
H. trichiurus	Jihoafrická republika	48	m	1♀	18S	2/T	**Článek V**
H. troglodytes	Jihoafrická republika	84	–	?	–	–	Newlands & Cantrell 1985
H. weygoldti	Jihoafrická republika	113	m	1♂	18S	2/INT	**Článek V**
H. zuluanus	Jihoafrická republika	80	M	1♂	18S	2/*p*,T	**Článek V**
H. zumpti	Jihoafrická republika	60	–	?	–	–	Newlands & Cantrell 1985
Hadogenes sp.	Jihoafrická republika	174	–	?	–	–	Newlands & Cantrell 1985
Liocheles *australasiae*	Japonsko	54–64	m	79♀	–	–	Yamazaki et al. 2001
Opisthacanthus *elatus*	–	60–62	M	?	–	–	Wilson 1931
SCORPIONIDAE							
Heterometrus *bengalensis*	Indie	64	M	♂	–	–	Sharma et al. 1962
H. fulvipes	Indie	86	M	♂	–	–	Sharma et al. 1962
″	Indie	86, 88	M	2♂	–	–	Venkatanarasimhiah & Rajasekarasetty 1964a
H. gravimanus	Indie	112		?	–	–	Venkatanarasimhiah & Rajasekarasetty 1964b
H. longimanus	Indie	64		?	–	–	Srivastava & Agrawal 1961
H. minotaurus	Thailand	54	19II + ***XIII*** + ***III*** (1♂)	1♂	–	–	**Článek IV**
H. scaber	Indie	96	M		–	–	Venkatanarasimhiah & Rajasekarasetty 1964a
H. spinifer	–	56	m	embryo	–	–	Vitková et al. 2005
H. swammerdami	Indie	60	M	?	–	–	Venkatanarasimhaiah 1965
H. yaleensis	Srí Lanka	99	46II + ***III*** + ***IV***	1♂	–	–	Kovařík et al. 2019c
Heterometrus sp.	Indie	62	ano (nespecifikováno)	?♂	–	–	Rajasekarasetty et al. 1979
Nebo *hierichonticus*	Palestina	50	M	?	–	–	Qumsiyeh et al. 2014
Pandiborellius *igdu*	Etiopie	101	49II + ***III*** (1♂)	1♂	18S	2/*p**, T	**Příloha Obr. 1–5**
P. magrettii	Eritrea	102	M	1♂	18S	2/*p**, T	**Příloha Obr. 1–5**
Pandinoides *cavimanus*	Tanzánie	109	53II + ***III*** (4♂)	4♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. duffmackayi	Tanzánie	108	52II + ***IV*** (1♂)	1♂	18S	2/*p*, ST	**Příloha Obr. 1–5**
Pandinops hawkeri	Somaliland	168	M	3♂	18S	2/*q*, T	**Příloha Obr. 1–5**
P. turieli	Etiopie	166	M	1♂	18S	2/*q*, T	**Příloha Obr. 1–5**
Pandinurus *afar*	Etiopie	116	M	1♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. citernii	Etiopie	120	M	5♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. exitialis	Etiopie	116	M	2♂	18S	4/*p*,*q*, T	**Příloha Obr. 1–5**

Tabulka 2. ***pokračování***

ČELEĎ/druh	Stát	2*n*	Heterozygozita	N	Detekce NOR/rDNA	počet/pozice NOR/rDNA	Reference
P. fulvipes	Somaliland	112–113	m	1♂	18S	2/*p**, T	**Příloha Obr. 1–5**
P. hangarale	Somaliland	120	M	1♂	–	–	Kovařík et al. 2017b
″	″	″	M	2♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. intermedius	Etiopie	110	M	5♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. kmoniceki	Somaliland	120		1♂	–	–	Kovařík et al. 2017a
″	″	″	M	1♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. mazuchi	Etiopie	120	M	1♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. oromo	Etiopie	118	M	3♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. phillipsii	Somaliland	120	M	2♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. riccardoi	Etiopie	120	M	1♂	18S	2/*p*, T	**Příloha Obr. 1–5**
P. trailini	Etiopie	118	M	4♂	18S	2/*p*, T	**Příloha Obr. 1–5**
Pandinus *imperator*	–	120	–	–	–	–	Guénin 1957
″	Ghana	117	57II + ***III*** (1♂)	1♂	18S	2/*p*, ST	**Příloha Obr. 1–5**
P. viatoris	Mozambik	93, 94	42II + ***III*** + ***VII*** (1♂); 40II + 2***III*** + ***VII*** (1♂)	2♂	18S	2/*q*, INT	**Příloha Obr. 1–5**
Scorpio *marus fuscus*	Západní břeh Jordánu	52	M	1♂	–	–	Qumsiyeh et al. 2013
URODACIDAE							
Urodacus *armatus*	Austrálie	124, 144	M	5♂	–	–	Shanahan 1989b
U. elongateus	Austrálie	56	M	1♂	–	–	Shanahan 1989b
U. novaehollandiae	Austrálie	66, 68, 72, 126, 130, 175	M	13♂	–	–	Shanahan 1989b
U. planimanus	Austrálie	68, 70	M	3♂	–	–	Shanahan 1989b
U. yaschenkoi	Austrálie	94, 114, 116	M	12♂	–	–	Shanahan 1989b
U. manicatus	Austrálie	29–64	komplexní inter- a intrapopulační polymorfismus	163♂	–	–	Shanahan 1989b
VAEJOVIDAE							
Vaejovis *boreus*	–	±100	–	–	–	–	Wilson 1931

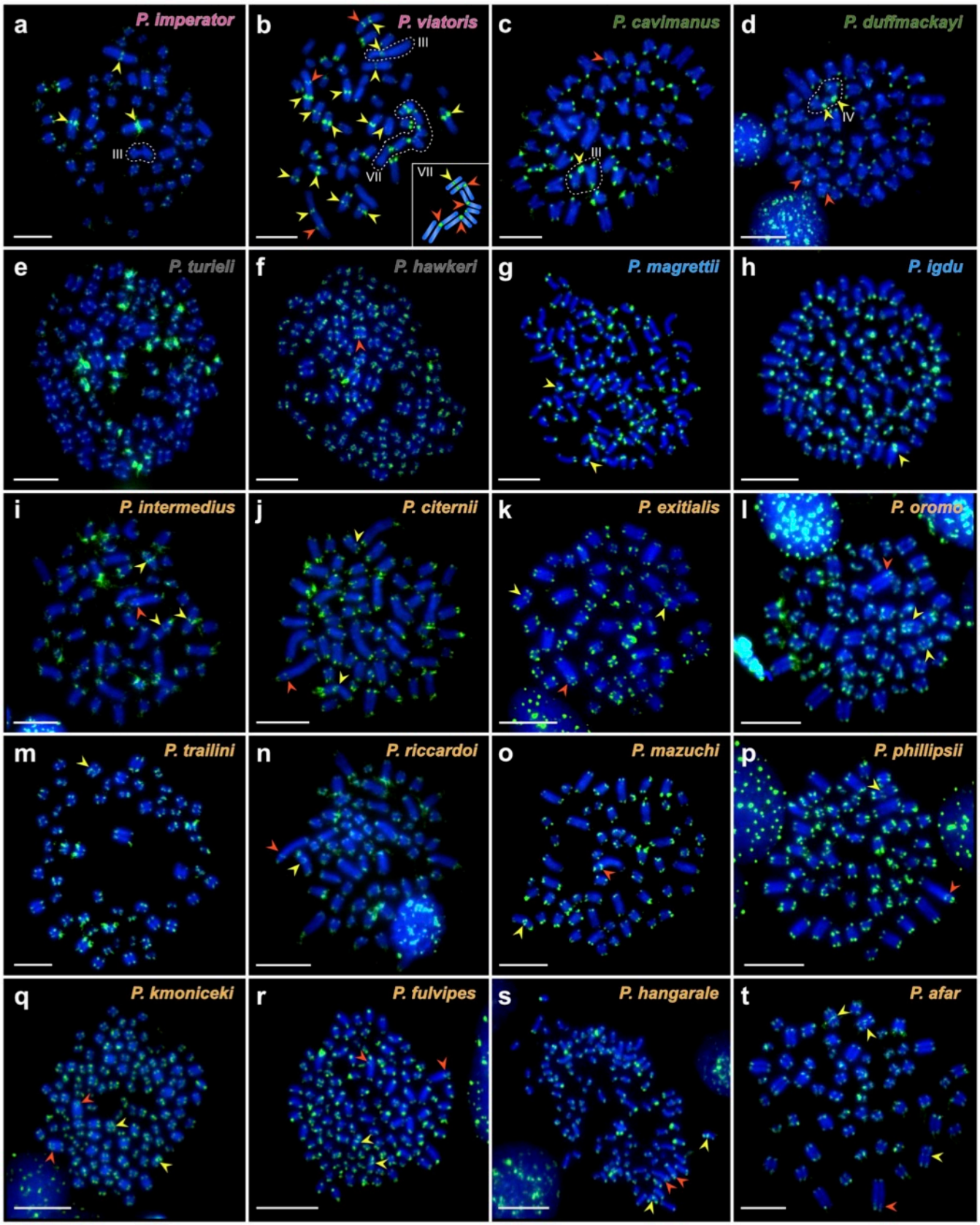


Obrázek 1. Mapa s vyznačenými lokalitami cytogeneticky analyzovaných druhů *Pandinus sensu lato*.



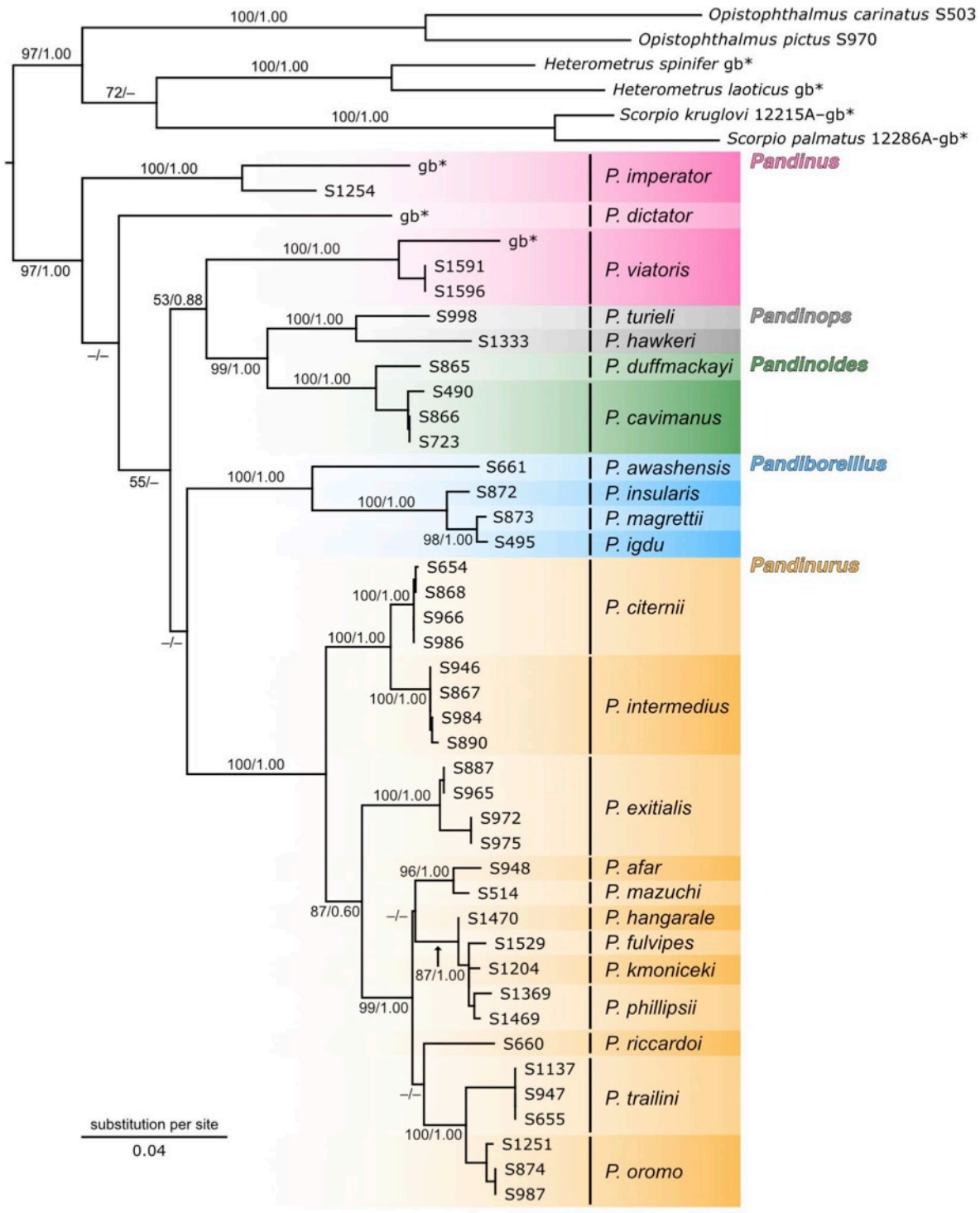
Obrázek 2. *popisek pokračuje na následující straně.*

Obrázek 2. Lokalizace 18S rDNA (červený signál) na chromozomech metafáze I (a-f, h-q, s-t) nebo mitotické metafáze (g, r) druhů *Pandinus sensu lato*. **(a)** *Pandinus imperator* [2*n* = 117 – 57II + III]; **(b)** *Pandinus viatoris* [2*n* = 94 – 42II + III + VII]; **(c)** *Pandinoides cavimanus* [2*n* = 109 – 53II + III]; **(d)** *Pandinoides duffmackayi* [2*n* = 108 – 52II + IV]; **(e)** *Pandinops turieli* [2*n* = 166]; **(f)** *Pandinops hawkeri* [2*n* = 168]; **(g)** *Pandiborellius magrettii* [2*n* = 102]; **(h)** *Pandiborellius igdu* [2*n* = 101 – 49II + III]; **(i)** *Pandinurus intermedius* [2*n* = 110]; **(j)** *Pandinurus citernii* [2*n* = 120]; **(k)** *Pandinurus exitialis* [2*n* = 116]; **(l)** *Pandinurus oromo* [2*n* = 118]; **(m)** *Pandinurus trailini* [2*n* = 118]; **(n)** *Pandinurus riccardoi* [2*n* = 120]; **(o)** *Pandinurus mazuchi* [2*n* = 120]; **(p)** *Pandinurus phillipsii* [2*n* = 120]; **(q)** *Pandinurus kmoniceki* [2*n* = 120]; **(r)** *Pandinurus fulvipes* [2*n* = 112–113]; **(s)** *Pandinurus hangarale* [2*n* = 120]; **(t)** *Pandinurus afar* [2*n* = 118]. Heterozygotní asociace jsou orámovány přerušovanou čarou (III – trivalent, IV – kvadrivalent, VII – heptavalent). V rámečku se nachází výřez chromozomu metafáze II nesoucího rDNA barveného Giemsou a po aplikaci 18S rDNA-FISH; bílé linky indikují pozici centromery. Hrot šipky (zelený) ukazuje větší lokus rDNA na homologickém páru chromozomů. Měřítko = 10 μm.



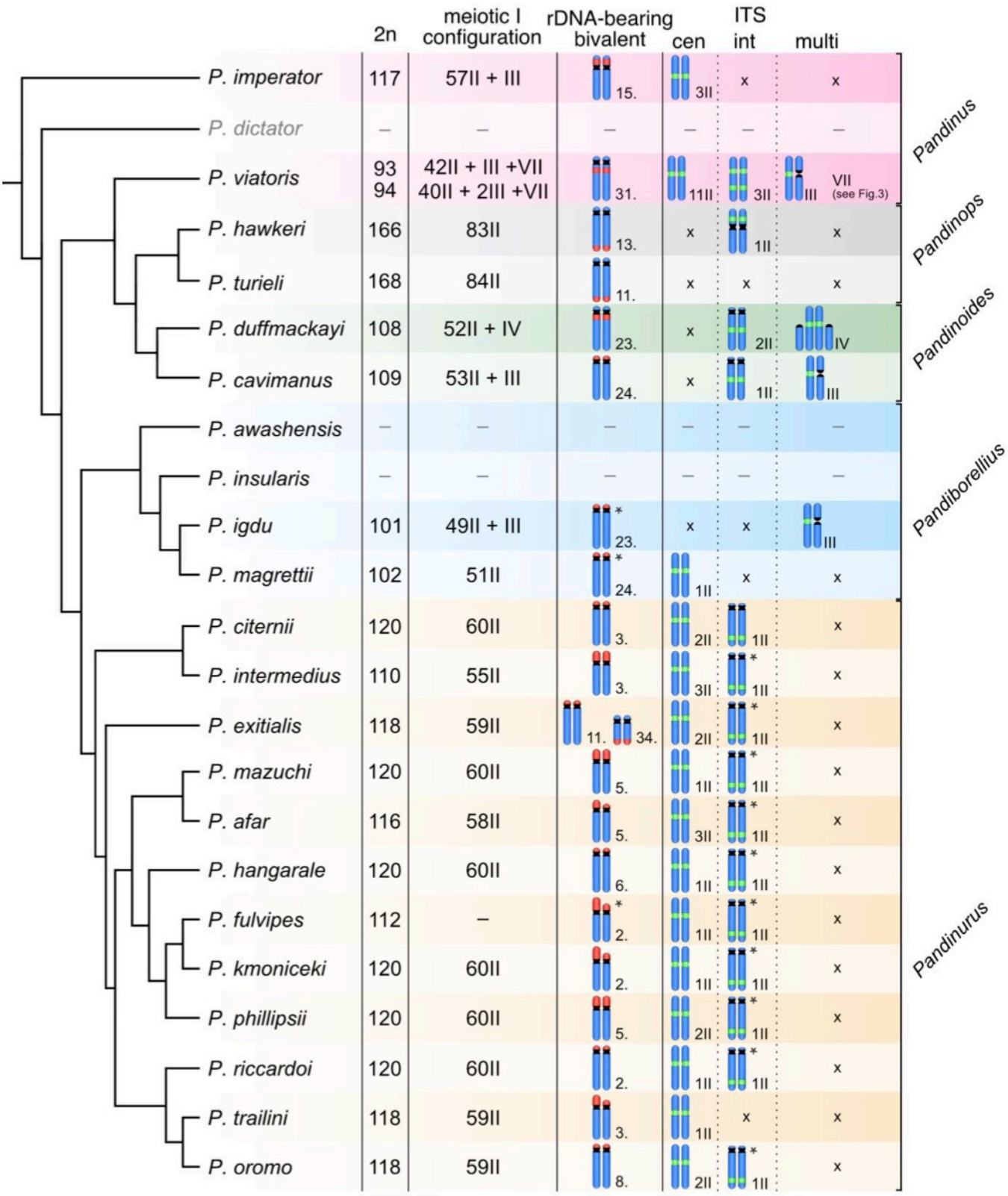
Obrázek 3. *popisek pokračuje na následující straně.*

Obrázek 3. Lokalizace (TTAGG)*n* motivu (zelený signál) na chromozomech metafáze I (a-f, i-p, t) nebo mitotické metafáze (g-h, q-s) druhů *Pandinus sensu lato*. **(a)** *Pandinus imperator* [2*n* = 117 – 57II + III]; **(b)** *Pandinus viatoris* [2*n* = 94 – 42II + III + VII]; **(c)** *Pandinoides cavimanus* [2*n* = 109 – 53II + III]; **(d)** *Pandinoides duffmackayi* [2n = 108 – 52II + IV]; **(e)** *Pandinops turieli* [2*n* = 166]; **(f)** *Pandinops hawkeri* [2*n* = 168]; **(g)** *Pandiborellius magrettii* [2*n* = 102]; **(h)** *Pandiborellius igdu* [2*n* = 101]; **(i)** *Pandinurus intermedius* [2*n* = 110]; **(j)** *Pandinurus citernii* [2*n* = 120]; (k) *Pandinurus exitialis* [2*n* = 116]; (l) *Pandinurus oromo* [2*n* = 118]; **(m)** *Pandinurus trailini* [2*n* = 118]; **(n)** *Pandinurus riccardoi* [2*n* = 120]; **(o)** *Pandinurus mazuchi* [2*n* = 120]; **(p)** *Pandinurus phillipsii* [2*n* = 120]; **(q)** *Pandinurus kmoniceki* [2*n* = 120]; (r) *Pandinurus fulvipes* [2*n* = 112–113]; (s) *Pandinurus hangarale* [2*n* = 120]; **(t)** *Pandinurus afar* [2*n* = 118]. Heterozygotní asociace jsou lemovány přerušovanou čarou (III – trivalent, IV – kvadrivalent, VII – heptavalent). Žlutý hrot šipky ukazuje centromerické ITS, červený hrot špičky ukazuje subterminální nebo intersticiální ITS. Měřítko = 10 μm.



Obrázek 4. *popisek pokračuje na následující straně.*

Obrázek 4. Fylogenetický strom *Pandinus sensu lato* sestrojený na základě konkatenovaného datasetu (16S rRNA, cytochrom c oxidáza podjednotky I (COI), cytochrom B (Cytb), 28S rDNA). Strom byl vypočítaný metodou maximální věrohodnosti (ML) pomocí substitučního modelu GTR+G (program raxmlGUI 1.3). Statistická podpora jednotlivých uzlů byla zjištěna pomocí bootstrapu s 1000 pseudoreplikáty. Čísla v uzlech představují hodnotu bootstrapové podpory/Bayesovské posteriorní pravděpodobnosti. Hodnoty <50/0.50 jsou označeny pomlčkou (–). Bayesovská analýza byla provedena v programu MrBayes 3.2.2 (25 000 000 generací) s využitím substitučních modelů zjištěných pomocí PartitionFinder v.1.1 (16S – GTR+G; COI, Cytb, 28S – GTR+I+G). gb* označuje vzorky, pro něž byla data získaná z databáze GenBank.



Obrázek 5. *popisek pokračuje na následující straně.*

Obrázek 5. Schéma propojující výsledky cytogenetických analýz a rekonstrukce příbuzenských vztahů u druhů *Pandinus s.l.* Číslo u bivalentu nesoucího rDNA indikuje pozici příslušného chromozomálního páru v karyotypu na základě měření. (Vysvětlivky: 2n – diploidní počet chromozomů, II – bivalent, III – trivalent, IV – kvadrivalent, VII – heptavalent).