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Fenotypové a molekulární přístupy v systematice palearktických a neotropických rosniček rodů *Hyla* a *Osteocephalus* (Amphibia: Hylidae)

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Dizertační práce

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V Praze, 25. září 2009 ….....

Václav Gvoždík

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Popření podle článků 8.2 a 8.3 Mezinárodních pravidel zoologické nomenklatury

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"Taxonomie je záležitostí evidence..."

Wolfgang Wüster (2002)

Summary

Gvoždík V., 2009. [Phenotypic and molecular approaches in the systematics of the Palearctic and Neotropic tree frogs, *Hyla* and *Osteocephalus* (Amphibia: Hylidae)]. PhD Thesis. Faculty of Science, Charles University, Prague, Czech Republic, 125 + i–xxiii pp.

The systematics and taxonomy of tree frogs of the family Hylidae has undergone turbulent changes both at higher (subfamilies, genera) and lower (species) level during the last decades. The original approach used morphological characters to distinguish tree frog. It has been completed by bioacoustics, since the advertisement calls of the males were found to represent an important behavioural reproductive barrier and therefore useful for systematic evaluations. However, a completely new impulse was given to systematics by the introduction of modern genetic methods. These methods have allowed to distinguish not only morphologically but even acoustically cryptic taxa. The most reliable method to distinguish tree frog at present is a combination of data from the different disciplines.

The present dissertation thesis aims to evaluate the systematics of tree frogs of the genus *Hyla* from Eastern Europe and the Middle East as well as the systematics of the Amazonian genus *Osteocephalus* using a combination of morphological, bioacoustic and molecular approaches. The thesis is composed of a general introduction, three published papers, two manuscripts under reviews and a conclusion chapter.

The first three papers deal with the phenotypic (morphological plus bioacustic) approach in systematic research. The first paper describes the variation in the colour pattern between Cypriote and adjacent mainland (Turkish, Syrian, Lebanese) populations of *Hyla savignyi*. It quantifies discovered differences in frequencies of different colour patterns and discusses their possible importance for taxonomy. The second paper attempts to solve a question of geographic morphological variation in parapatric tree frogs *H. savignyi* and *H. arborea* (and also *H. orientalis* according to the current taxonomy). It aims to test the hypothesis that climatic factors influence the body shape of the frogs. Originally, this project aimed to present a morphological intraspecific taxonomic evaluation of different tree frogs' populations, but morphology alone turned out to be inconvenient for that purpose. In contrary, the project brought up the new hypothesis about evolutionary-ecological interpretations of the morphological data in the similar, but acoustically and genetically distant Mediterranean populations of *H. savignyi* and *H. arborea* (and *H. orientalis*). The third paper provides the first record of a second species of tree frog from Iran, from where only *H. savignyi* had been reported before using acoustic methods.

The fourth paper is a phylogeographic study of tree frogs from the Middle East using sequences of mitochondrial and nuclear DNA. Using the molecular approach, a new species was discovered from the region from Yemen to southern Levant. Subsequently, this species was distinguished also by distinct acoustic characteristics and to a lesser degree also by morphology. The new species will be described in the forthcoming paper in the official way (validity of the description within the thesis is disclaimed according to the ICZN). Beside this result, the fourth paper is also discussing the validity of the taxa *H. arborea schelkownikowi* and *H. arborea gumilevskii* on the basis of a phylogenetic approach. Demographic analyses of particular populations of the three species (*H. savignyi*, *H. orientalis*, and the new species) were carried out to gain insights into the evolutionary history of the species.

The fifth paper is a description of a new species of Amazonian tree frog of the genus *Osteocephalus* based on a combination of morphological, ecological and genetic approaches. The paper also brings a preliminary report on the phylogeny of the genus, which is briefly commented with respect to the evolution of different modes of reproductive strategies within the genus.

Obsah

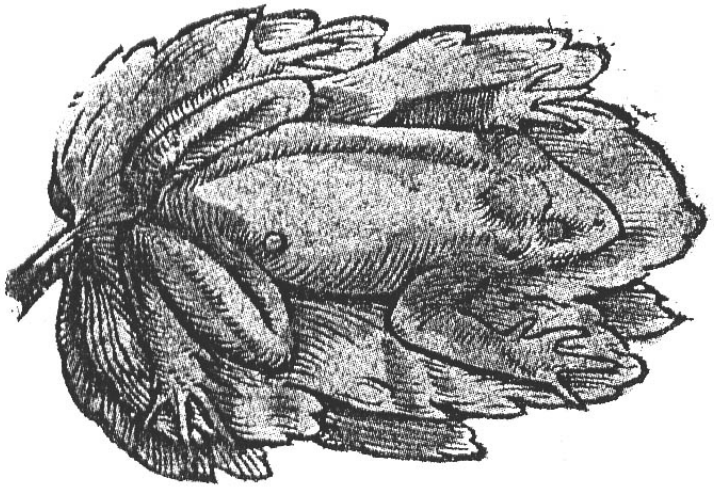
Úvod

Systematika a biogeografie rosničkovitých žab na úrovni čeledi Hylidae

Rosničky patří se svými v současnosti více než 850 druhy mezi nejpočetnější čeledi žab a dělí se do tří podčeledí, neotropické Phyllomedusinae, australo-papuánské Pelodryadinae a nejpočetnější Hylinae rozšířené v neotropické a nearktické oblasti, ve východní a západní části Palearktu a okrajově v orientální oblasti (Frost 2009). Typovým rodem čeledi je rod *Hyla* Laurenti, 1768 a typovým druhem rodu *Hyla* je *Rana arborea* Linnaeus, 1758, což není nikdo jiný než nejběžnější evropská rosnička zelená (*Hyla arborea*). Nicméně již samotná historie popisu této všeobecně známé žabky byla po dlouhá léta zahalená mlhou, neb Carl von Linné použil pro popis druhu mimo evropské rosničky zelené také celou řadu druhů amerických s uvedením „habitatu" „*Sub foliis arborum Europae, Americae*" (Linnaeus, 1758). Všechny tyto žáby patřily formálně mezi syntypy, ačkoliv bylo jasné, že právoplatným nositelem jména je jen evropská rosnička. Této mlze učinili konec až Dubois & Ohler (1996), když stanovili po téměř detektivním pátrání v historických pramenech jako lektotyp nominálního taxonu *Rana arborea* jedince vyobrazeného Gesnerem již v roce 1554 (Hist. Animal.: 55) s určením typové lokality okolí Curychu, Švýcarsko (obr. 1). Až tímto činem bylo po 238 letech formálně stabilizováno jméno pro všem známou rosničku zelenou (*Hyla arborea*), typový druh rodu a tím i čeledi.

Podobně jako celá čeleď i rod *Hyla* donedávna představoval jeden z nejpočetnějších rodů v žabí říši čítající přes 250 druhů s centrem rozšíření v neotropické oblasti (Duellman & Trueb 1994). Rod byl po celou dobu udržován jednotný na základě morfologických podobností, ačkoliv pochybnosti o jeho monofylii existovaly. Teprve nedávno došlo na základě molekulárních studií k přehodnocení systematiky rodu na základě evoluční příbuznosti různých linií a rod *Hyla* byl rozdělen do řady méně početných rodů, všechny z neotropické oblasti, jako např. *Bokermannohyla*, *Dendropsophus*, *Hyloscirtus*, *Hypsiboas* aj. (Faivovich et al. 2005). Podobných výsledků publikovaných téměř ve stejnou dobu, ale bez taxonomických implikací, dosáhla také studie Wiens et al. (2005) a následně vše bylo potvrzeno ve velkolepém díle *The Amphibian Tree of Life* (Frost et al. 2006). V samotném rodu *Hyla* pak zůstalo pouze něco přes 30 druhů vyskytujících se pouze v holarktické a okrajově v orientální oblasti (Frost 2009). Jak již bylo řečeno, centrem rozšíření a zároveň předpokládaným místem vzniku rosničkovitých žab je neotropická oblast, kde rosničky radiovaly do obrovské druhové diverzity (Wiens et al. 2006). Z této oblasti se pak postupně

rozšířily do oblasti nearktické a následně palearktické, přičemž je na tomto transektu (Nearktis, V Palearkt, Z Palearkt) evidentní úbytek druhové diverzity (Smith et al. 2005), což je vysvětlováno především kratším časem pro speciační události daným pozdější kolonizaci daných regionů (Wiens et al. 2006).

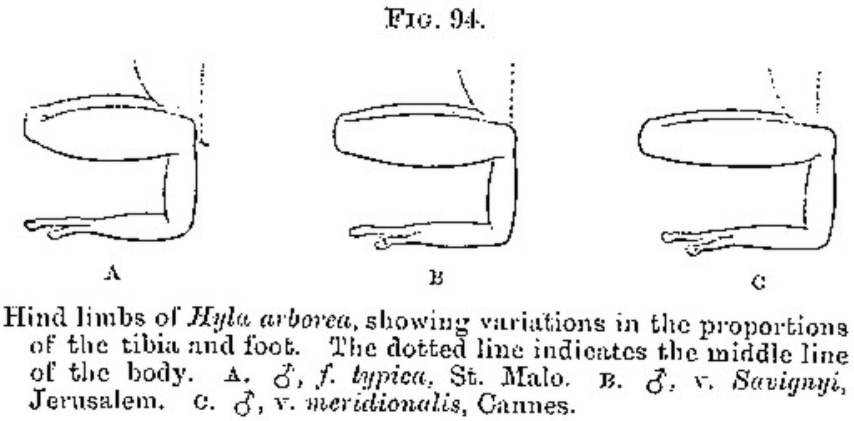


Obr. 1. Vyobrazení exempláře, který představuje lektotyp *Rana arborea* Linnaeus, 1758. Originál Gesnerus, 1554, Hist. Animal.: 55; převzato z Dubois & Ohler (1996).

Palearktické rosničky – tradiční systematický přístup

V palearktické oblasti mají rosničky disjunktní rozšíření – v západní a východní části, což je zřejmě způsobeno přítomností Tibetské náhorní plošiny, kde nepanují pro rosničky vhodné klimatické podmínky (Smith et al. 2005). Dříve byly všechny rosničky palearktické oblasti považovány za druhy ze skupiny (někdy též komplexu) *Hyla arborea*. Studie autorů Faivovich et al. (2005) a Smith et al. (2005) však překvapivě ukázaly, že *Hyla japonica* z Dálného východu nepatří do linie palearktických rosniček, nýbrž do jedné ze skupin severoamerických druhů, jmenovitě do skupiny *H. eximia* (Faivovich et al. 2005). Z biogeografického hlediska to znamená, že tento druh (nebo respektive taxonomicky zatím nedořešený druhový komplex) expandoval do palearktické oblasti nezávisle na starší předchozí invazi rosniček, která dala vznik druhové skupině *H. arborea* (Smith et al. 2005). Toto zjištění bylo o to zajímavější, že *H. japonica* byla po dlouhou část 20. století považována za pouhý poddruh *H. arborea japonica* (např. Těrěntěv & Černov 1949) nebo někdy dokonce jen za dálnovýchodní populaci blízkovýchodního poddruhu *H. arborea savignyi* (Flower 1933). Že však nemá blízkou příbuznost se západopalearktickými rosničkami, ale ukázali již Daito (1968) na základě hybridizačních pokusů a Kuramoto (1980, 1984) na podstatě

akustických dat a dalších rozšířených hybridizačních experimentů. Z těchto prací bylo patrné, že morfologická uniformita rosniček tedy nemusí odrážet jejich příbuznost.



Obr. 2. Tradiční morfologický přístup v taxonomii západopalearktických rosniček. Velký význam se kladl např. na vzájemný poměr délky stehna a holeně. Převzato z Boulenger (1898). Tento znak však může podléhat pod vlivem vnějších podmínek výrazné fenotypové plasticitě.

Podobně „zmatená“ situace panovala po dlouhou dobu 20. století také v systematice západopalearktických rosniček, kdy všechny byly až do konce 60. let považované za sice polytypický, ale jediný druh *Hyla arborea*. Systematika rosniček byla tehdy založena na morfologických znacích přejímaných ještě z 19. století (obr. 2). Nicméně nutno podotknout, že již Boscá v roce 1880 a Herón-Royer v roce 1884 si všimli významu oznamovacích hlasů žab (hlasy samců v období rozmnožování, tzv. *advertisement calls*), a konkrétně rosniček, když poukázali na odlišnosti v hlasových projevech rosniček z jižního Španělska, respektive jižní Francie, které zmíněni autoři popsali jako *Hyla perezii* a *Hyla barytonus* (Schneider & Sinsch 2007). Druhá z nich nese ukazatel na svůj specificky hlasový projev přímo v názvu. Obě však byly synonymizovány s dříve popsanou *H. meridionalis*, respektive toho času *H. arborea* (var.) *meridionalis*. První práce znovu poukazující na její druhový statut, a vůbec první práce poukazující na samostatný druhový statut některé z evropských populací rosniček, byly opět studie založené na bioakustice, tedy nemorfologických znacích (Paillette 1967a,b, Schneider 1968). Další pak byla zaměřena na blízkovýchodní rosničky, také bioakustická studie, rovněž poukazující na druhový statut těchto populací, nyní *H. savignyi* (Schneider & Nevo 1972). Tím byl definitivně prokázán význam hlasových projevů rosniček pro studium jejich systematiky. Nicméně tyto první práce si přesto musely počkat na své uznání ještě řadu let, než byly všeobecně akceptovány. Mezitím se dostlaly ke slovu také genetické metody. Tyrhénská *H. sarda* byla povýšena na druhovou úroveň na základě analýz allozymů (Lanza 1983). Velkým překvapením posledních let pak bylo povýšení italských populací rosniček, které byly do té doby považované za nominotypický poddruh *H. arborea*, na samostatný druh

H. intermedia. Tento krok byl učiněn rovněž na základě allozymové analýzy (Nascetti et al. 1995). Autoři tehdy mylně rosničky popsali jako nový druh *H. italica*, který však byl posléze synonymizován a nahrazen starším dostupným jménem *H. intermedia* (cf. Dubois 1995). Tím bylo v posledních letech docíleno dočasné „stability" v taxonomii evropských rosniček. Pro doplnění je vhodné uvést, že „zbylé" taxony zůstaly na poddruhové úrovni druhu *H. arborea*: *H. a. molleri* (Iberský poloostrov), *H. a. schelkownikowi* (Kavkaz), *H. a. kretensis* (Kréta, někdy též Peloponés a západní Malá Asie), ačkoliv jejich validita byla některými autory zpochybňována (např. Egiasarian & Schneider 1990; Schneider 1974, 2004b; Scheider & Sinsch 2007).

Západopalearktické rosničky – fylogeografický přístup

Nové oživení systematiky západopalearktických rosniček přinesla až studie popisující nový poddruh *H. arborea* z jihovýchodního Ázerbajdžánu nazvaný *H. a. gumilevskii*, podložený nalezenými rozdíly ve velikosti genomu a částečně i allozymech a morfologii (Litvinchuk et al. 2006). Zajímavou a poněkud zvláštní novinkou pak byl také popis nového druhu rosničky *H. heinzsteinitzi* z centra Jeruzaléma a dvou lokalit v okolí, popsaný na základě tradičních morfologických a bioakustických přístupů (Grach et al. 2007)[1]. Zcela nový vítr však zavál až s aplikacemi fylogeografických metod, přístupu studujícího fylogenetické vztahy mezi populacemi téhož nebo blízce příbuzných druhů metodami analýzy variability sekvencí DNA (Avise et al. 1987). V roce 2007 se objevily hned tři studie na populacích dvou druhů západopalearktických rosniček, *H. meridionalis* (Recuero et al., 2007) a *H. intermedia* (Canestrelli et al. 2007a,b). Všechny studie byly založené na mitochondriální DNA a jedna ještě navíc porovnávala jaderný signál dle výsledků allozymové analýzy (Canestrelli et al. 2007a). Fylogeografické studie si zpravidla kladou za cíl odvozování evoluční historie populací daného druhu a případné taxonomické výstupy představují spíše doplňující výsledky. V tomto duchu jsou pojaty i všechny tři zmiňované práce, které sice odhalily hluboké divergence u obou jmenovaných druhů datované minimálně do období před 2 miliony let (*H. meridionalis*: Tunisko vs. Maroko + Evropa; *H. intermedia*: populace severně vs. jižně od Severních Apenin), ale neučinily k odlišení těchto linií žádné taxonomické kroky. Studie

[1] Popis tohoto druhu byl překvapivý již tím, že typová lokalita se nalézá v centru Jeruzaléma. Autoři popisu však uvádějí, že druh je možná již vyhynulý (Grach & Werner 2008). Navíc Stöck et al. (2008) upozornili na základě analýzy mtDNA, že tento taxon pravděpodobně představuje jen zavlečený dálnovýchodní druh *H. japonica*.

zkoumající u druhu *H. intermedia* také jaderné markery totiž poukázala na vzájemnou neshodu mezi některými allozymovými lokusy a částečnou neshodu vůči signálu mitochondriální DNA (Canestrelli et al. 2007a). Tento výsledek zřejmě svědčí pro případ hemiplasie, retence ancestrálního polymorfizmu kvůli nekompletnímu dělení linií, tzv. *incomplete lineage sorting* (Avise & Robinson 2008, Degnan & Rosenberg, 2009). Zdá se tedy, že tyto „vnitrodruhové" divergence ukazují zatím spíše na probíhající speciaci. Na otázku, zda by jednotlivé linie mohly reprezentovat samostatné taxony, se však nedá jednoznačně odpovědět, neboť se zde jedná spíše o záležitost názoru na koncepci druhu. Studie obou druhů se navíc potýkaly s metodickým nedostatkem, neboť neobsahovaly ostatní západopalearktické druhy pro srovnání, tudíž autoři nemohli zodpovědně přistoupit k rozřešení taxonomické otázky z důvodu nejistoty, zda některá ze separátních linií nemůže ve skutečnosti představovat jiný známý taxon, např. geograficky sousedící. V rámci široce rozšířených linií pak autoři detekovali u *H. meridionalis* tři (Recuero et al. 2007) a u *H. intermedia* dvě (Canestrelli et al. 2007b) podskupiny, které uvedli do souvislosti s glaciálními refugii studovaných druhů. Fylogeografický vzor italského druhu poukázal na zajímavou příbuznost sicilských a kalabrijských populací (čili přes mořskou úžinu Messinského průlivu), což autoři vysvětlují spojením obou populací v době poklesu mořské hladiny v průběhu glaciálů a tím umožnění toku genů v rámci tohoto jižního refugia. Další refugium pak předpokládají ve střední Itálii v rozšířených nížinách opět v době poklesu mořské hladiny. Naproti tomu fylogeografický vzor široce rozšířené linie *H. meridionalis* je odlišný a zdá se být silně ovlivněn lidským faktorem – transportem (Recuero et al. 2007). Původně byla tato linie zřejmě rozšířená jen v oblasti severozápadní Afriky, kde dnes vytváří tři geograficky separované podskupiny – severní Maroko, Střední Atlas a jižní Maroko. Zajímavé ale je, že populace z jihozápadu Iberského poloostrova není blíže příbuzná se severomarockou podskupinou, ale s jihomarockou. Izolovaná populace ze severovýchodu Iberského poloostrova, jižní Francie až severozápadní Itálie je pak příbuzná severomarocké populaci, stejně tak populace z Kanárských ostrovů. Zdá se tedy nanejvýš pravděpodobné, že iberská populace vznikla umělým transferem z jihozápadního atlantického pobřeží Maroka, zatímco francouzská populace ze severního mediteránního marockého pobřeží. Až následně zřejmě došlo ke kolonizaci Kanárských ostrovů, pravděpodobně transferem z východní části areálu evropské populace (Recuero et al. 2007).

Výše uvedené příklady ukazují na zásadní význam použití molekulárních metod v systematice rosniček. Pro komplexní pochopení jejich příbuzenských vztahů a tím potažmo i systematiky je při tom ale zapotřebí komplexní studium všech potenciálně příbuzných

taxonů a jejich populací. To učinil Stöck et al. (2008) pro populace západopalearktických rosniček (jejich výsledky jsou reprodukovány na obr. 3). Na základě studia mitochondriálních a jaderných genů dospěl k fylogenetickému stromu ukazujícímu na tři hlavní klady: *H. meridionalis*, *H. savignyi* a v krátkém čase radiující klad taxonů kolem druhu *H. arborea*. Autoři potvrdili přítomnost separátních kladů v rámci *H. meridionalis* a *H. intermedia*, a navíc detekovali hlubokou divergenci také u *H. savignyi* i *H. arborea*. Vzhledem k dostupnosti poddruhových či starších synonymních jmen pak rovnou přistoupili k taxonomické revizi druhého druhu, který rozdělili na *H. arborea*, *H. molleri* a *H. orientalis*. Otázka, zda je toto rozdělení oprávněné, však může být předmětem hlubší polemiky. Autoři totiž neměli k dispozici potřebné množství vzorků, obzvlášť pak z kontaktních oblastí jednotlivých linií/druhů, aby mohli zodpovědně zhodnotit šíři případného toku genů přes kontaktní zóny. Divergence mezi *H. molleri* a *H. orientalis* navíc nedosahuje velké hloubky. Navzdory sesterské příbuznosti těchto taxonů je velmi zajímavý jejich distantní geografický původ (*H. molleri*: Iberský poloostrov; *H. orientalis*: Pontická oblast), který však autoři blíže nekomentují.

Zatím nepublikované výsledky vlastních studií fylogenetických vztahů všech taxonů západopalearktických rosniček plynně navazujících na tuto dizertační práci jsou prezentovány na obr. 4. Ve srovnání s výsledky práce Stöck et al. (2008) lze hlavní rozdíl pozorovat v poměrně dobře podpořeném sesterském vztahu taxonů *H. arborea* a *H. orientalis-molleri*, jejich nepříliš velké hloubce divergence a velice nízké podpoře taxonu *H. orientalis* ve vztahu k *H. molleri*. To vše může poukazovat na možná zbytečné povyšování těchto tří evolučních linií na druhovou úroveň. Tato hypotéza je také předběžně podpořena neshodou mezi mitochondriálním a jaderným signálem v kontaktní zóně taxonů *H. arborea* a *H. orientalis*. Detailní studium této problematiky je předmětem pokračujícího intenzivního výzkumu (V. Gvoždík et al., in prep.).

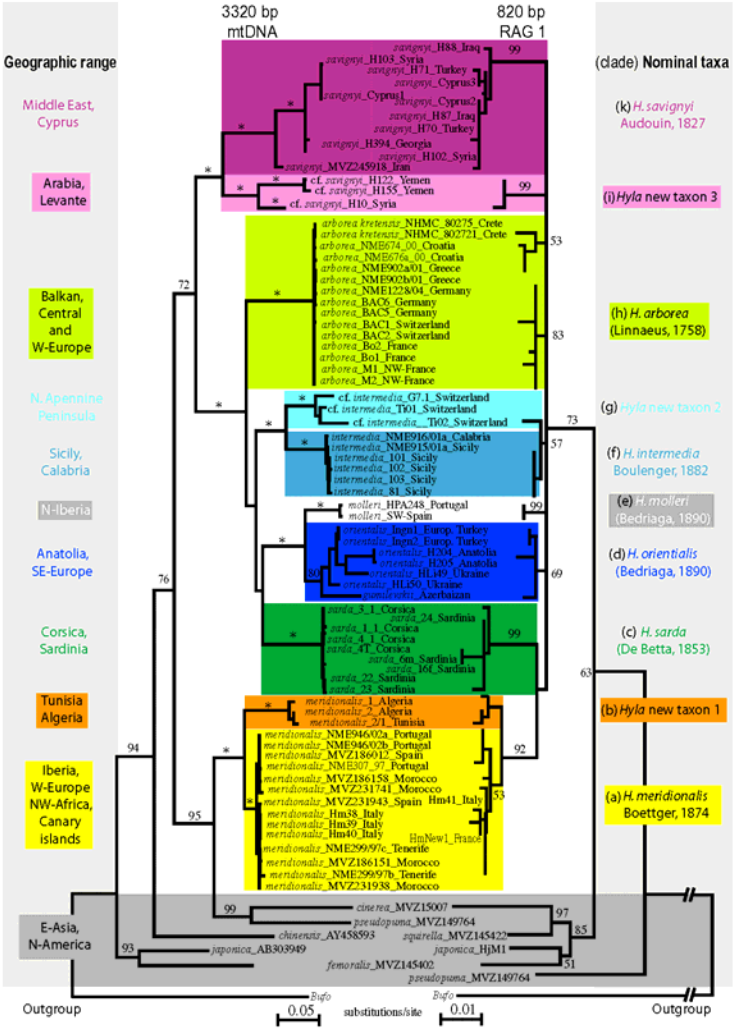
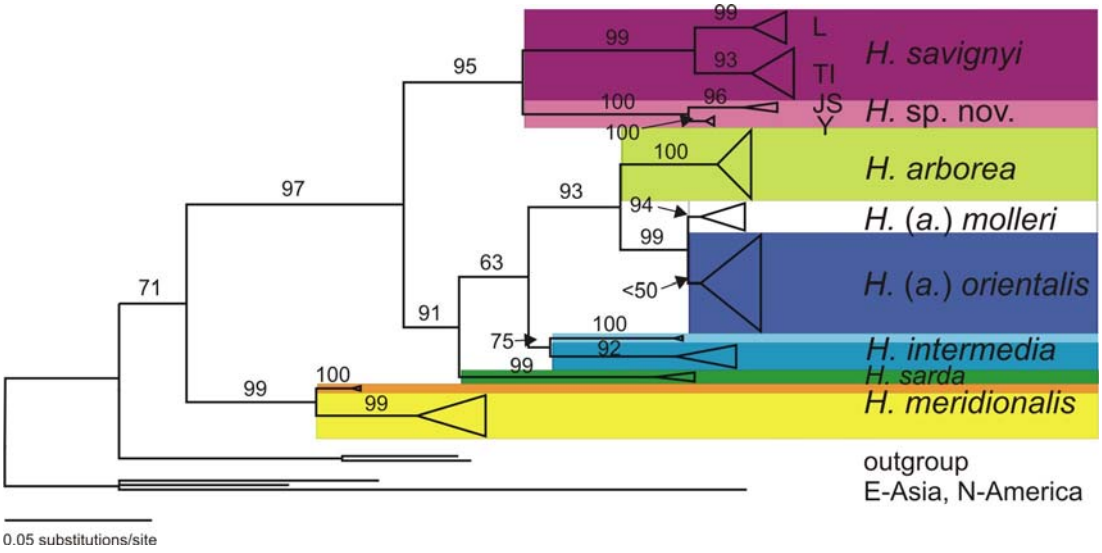


Fig. 2. Phylogenetic trees (Maximum-Likelihood phylograms) obtained with the program PhyML version 2.4.5 (Guindon and Gascuel 2003) with bootstrap support values obtained from 1000 resampled data sets (*refers to a Bootstrap support of 100%; major nodes below 50% remained unlabeled, below 30% were collapsed). Note that color codes of clades correspond to those of localities in Fig. 1.

Obr. 3. Fylogenetické stromy zobrazující příbuznost západopalearktických rosniček dle práce Stöck et al. (2008).



Obr. 4. Fylogenetický strom západopalearktických rosniček odvozený z mtDNA metodou maximum likelihood, čísla u větví jsou bootstrapové hodnoty. Jednotlivé taxony a linie jsou pro lepší orientaci podbarveny v podobném stylu jako v práci Stöck et al. (2008). Zkratky linií blízkovýchodních taxonů (*H. savignyi*, *H.* sp. nov.) odpovídají zkratkám použitých ve IV. kapitole výsledků této dizertační práce. Vlastní zatím nepublikované výsledky (V. Gvoždík et al., in prep).

Fenotypový versus molekulární přístup k systematice západopalearktických rosniček

Otázkou systematického postavení různých populací potažmo druhů západopalearktických rosniček se v posledních desetiletích zabývalo také několik autorů za pomocí tradičních morfologických metod. Jedna z prvních byla morfologická studie Těrěntěva (1960) zaměřená na testování validity poddruhu *H. arborea schelkownikowi* jeho srovnáním s nominotypickým poddruhem. Ve světle současného poznání (Stöck et al. 2008) však víme, že Těrěntěv nedělal srovnání s *H.* (*a.*) *arborea* nýbrž s *H. intermedia* a především s *H. orientalis*. Nicméně dospěl k závěru, že studované populace vykazují v západo-východním transektu klinální variabilitu v biometrických znacích a tudíž validita kavkazského taxonu je neopodstatněná. Zúžíme-li tento poznatek o klinální variabilitě jenom na studované populace *H. orientalis* (které navíc početně výrazně převyšovaly), zdá se, že to poukazuje na možný efekt vlivu graduálně se měnících klimatických podmínek na utváření tělesných proporcí rosniček. Podobné zjištění bylo dokumentováno také u *H. intermedia*, kdy odlišnosti ve tvaru těla pozitivně korelovaly s geografickými vzdálenostmi populací (Rosso et al. 2004), což ovšem zjevně popírá morfometrické rozdíly mezi severní a jižní evoluční linií tohoto druhu (cf. Canestrelli et al. 2007a,b), pro které Stöck et al. (2008) doporučují úroveň poddruhů. Morfologické porovnání tureckých populací druhů *H. arborea* (aktuálně *H. orientalis*) a *H. savignyi* (Zaloğlu 1972, Kaya 2001) neukázalo signifikantní rozdíly s výjimkou kresby v inguinální oblasti (*H. arborea*: přítomnost kličky; *H. savignyi*: absence). Jen populace *H. arborea* ze severovýchodu Turecka byly částečně morfometricky odlišné (Kaya 2001). To by mohlo poukazovat na přítomnost a odlišnost zpochybňovaného kavkazského taxonu *H. a. schelkownikowi* a/nebo opět na vliv vnějších podmínek tolik specifických pro tento pontický region Turecka (Sindaco et al. 2000). Ani ve světle současného poznání se k tomu nelze zodpovědně vyjádřit, neboť kavkazské populace *H. a. schelkownikowi* zatím nebyly geneticky hodnoceny. Nicméně dá se očekávat, že budou náležet k taxonu *H. orientalis*, což ovšem platí také pro západoturecké rosničky (Frost 2009). Máme zde tedy zjevný, i když původními autory nepodchycený, případ, kdy vnitrodruhová morfologická variabilita předčí mezidruhovou.

Jiný úhel pohledu se pokusily vnést bioakustické studie, které se rovněž snažily řešit otázku rozdílů mezi druhy *H. arborea* a *H. savignyi* v kontextu geografie jejich rozšíření v Turecku a ekologických vztahů (Kaya & Simmons 1999, Schneider 2001). Tyto práce naopak výrazné odlišnosti obou druhů potvrdily (v souladu s dnešními genetickými výsledky), nicméně nedoložily žádný případ syntopie či sympatrie. Z bioakustických příspěvků k systematice západopalearktických rosniček je zajímavá ještě jiná studie (Schneider 2000),

která poukazuje na totožnost hlasových projevů západotureckých rosniček a rosniček z Německa. Práce tedy upozorňuje na nejen druhovou nýbrž i poddruhovou identitu těchto populací rosniček. Dle současných genetických dat (Stöck et al. 2008, V. Gvoždík et al., in prep.) však víme, že se jedná o odlišné evoluční linie, možná i druhy. Schneider (1974, 2004b) a Scheider & Sinsch (2007) dále také kritizovali na základě akustických výzkumů a ve výsledku popřeli validitu poddruhů *H. a. kretensis* a *H. a. molleri* a Egiasarian & Schneider (1990) pak také *H. a. schelkownikowi*, vše ve srovnání s německými populacemi (nominotypickými) *H. arborea*. Přitom dle genetických výsledků se dá s těmito tvrzeními souhlasit jen v případě krétského taxonu *H. a. kretensis*, který spadá do shodné linie s německými populacemi, zatímco zbylé dva taxony patří do separátní linie/linií (Stöck et al. 2008, V. Gvoždík et al., in prep.). Z dalších bioakustických systematicky zaměřených prací lze jmenovat práci Castellano et al. (2002), která zjistila signifikantní rozdíly v oznamovacích hlasech mezi druhy *H. sarda*, *H. intermedia* a *H. arborea* (z Moldávie, tudíž aktuálně *H. orientalis*). Nicméně po detailním prostudovaní jejich práce je nutno poznamenat, že zjištěné rozdíly jsou velmi jemné (zvláště pak ve dvojici *H. intermedia – H. orientalis*), detekovatelné jen kvalitní akustickou a statistickou analýzou. Podobně drobné rozdíly mezi *H. intermedia* a *H. a. arborea* zjistil také Schneider (2004a). Z toho vyvstává otázka, zda jsou rosničky opravdu tak senzitivní vůči drobným rozdílům ve struktuře jejich oznamovacího hlasu, který navíc podléhá vlivu fyziologického stavu jedince (teplota), velikosti jedince a individuální variabilitě v hlasovém projevu (Schneider 1974, 2004b, Friedl 2006). Na druhou stranu je třeba se také zamyslet, zda mají alopatrické druhy zapotřebí odlišnosti v oznamovacích hlasech? V případě významných geografických bariér zjevně ne (např. případ ostrovní *H. sarda*). Tato potřeba však může vzejít až v zóně sekundárních kontaktů těchto druhů, čehož se dá částečně docílit procesem nazývaným vytěsnění znaků (tzv. *character displacement*; Brown & Wilson 1956), jak bylo již dokumentováno i na příkladu hlasů rosniček ze Severní Ameriky (Höbel & Gerhardt 2003, Moriarty Lemmon 2009). Vzájemné druhové rozpoznání je totiž nezbytné pro dlouhodobou koexistenci a reprodukční izolaci dvou alopatricky vzniklých sesterských druhů po jejich opětovném kontaktu a hlasové projevy jsou u žab významnou reprodukčně izolační bariérou (Schneider et al. 1984).

Z výše uvedeného je patrné, že použití molekulárně-genetických metod v kombinaci s fenotypovým přístupem umožňuje získat ucelenou představu o funkci a významu některých akustických a morfologických znaků. Kombinaci těchto přístupů lze tedy doporučit jako nejobjektivnější přístup ke studiu systematiky a evoluční historie nejen rosničkovitých žab.

Neotropické rosničky rodu Osteocephalus ve světle molekulární systematiky

Neotropická oblast je kolébkou diverzity celé čeledi Hylidae (Wiens et al. 2006). Jak již bylo řečeno výše, molekulární systematické přístupy posledních let se odrazily také na rodové systematice rosniček i na kompozici druhů v jednotlivých rodech (předně Faivovich et al. 2005). Rosničky rodu *Osteocephalus* v současnosti čítají kolem 20 druhů, přičemž asi polovina jich byla popsána v posledních 20 letech (cf. Frost 2009). Když k tomu připočítáme ještě 8 druhů (7 popsaných v 90. letech) sesterského rodu *Tepuihyla*, někdy považovaných za zástupce rodu *Osteocephalus*, máme před sebou typický příklad podhodnoceného a přehlíženého druhového bohatství. Rod *Osteocephalus* se proto jeví jako ideální modelová skupina pro bližší systematické studium. Uvážíme-li, že všechny nové druhy byly v posledních letech odhaleny a popsány jen na základě morfologie, je nasnadě, že při vyšetření variability DNA fylogeografickými metodami či přístupem DNA barcodingu (Vences et al. 2005) bude nalezena ještě řada dosud nepoznaných nebo nerozpoznaných druhů (cf. Fouquet et al. 2007). Tento genetický přístup je sice velikou výzvou, ale zároveň si musíme uvědomit, že ho lze provádět jen souběžně s tradičním taxonomickým přístupem, neboť určení řady taxonů není vždy nejjednodušší záležitostí, často možnou jen ve spolupráci se specializovanými taxonomy. Že tento komplexnější metodický postup není bohužel vždy dodržován, je dokumentováno také na příkladu rodu *Osteocephalus* v V. kapitole výsledků této dizertační práce.

Cíle práce

Jak bylo výše demonstrováno, systematika rosniček západního Palearktu prošla v minulých desetiletích bouřlivým vývojem, a to hlavně za přispění bioakustických a genetických přístupů. Přesto se dalo tušit, že řada poznatků zůstala stále zahalena rouškou tajemství, zvláště pak v případě některých opomíjených regionů.

Cílem práce bylo za pomocí kombinace fenotypových (morfologie, bioakustika) a molekulárně-genetických přístupů zhodnotit systematiku rosniček ve východní Evropě a na Blízkém východě, tedy v oblasti poněkud vědecky přehlížené a zároveň dobře reprezentované v herpetologických sbírkách Národního muzea a katedry zoologie PřF UK. Zvláštní pozornost pak byla od počátku věnována izolovaným populacím druhu *H. savignyi* z jihozápadu Arabského poloostrova a Kypru.

Dalším cílem pak bylo stejnými přístupy zhodnotit systematiku amazonských rosniček rodu *Osteocephalus* a za pomocí dostupných sekvencí DNA také odvodit fylogenetické vztahy uvnitř rodu.

Stručná anotace dílčích výstupů

I. práce se zabývá otázkou variability ve zbarvení, resp. kresbě mezi kyperskými a přilehlými pevninskými (Turecko, Sýrie, Libanon) populacemi druhu *H. savignyi*. Zjištěný frekvenční rozdíl v přítomnosti a typu dorzálního skvrnitého vzoru je diskutován s ohledem na možný taxonomický význam tohoto znaku.

II. práce řeší otázku geografické morfologické variability u parapatrických druhů *H. arborea* (a dle současné taxonomie také *H. orientalis*) a *H. savignyi* v ekologickém kontextu potenciálního vlivu klimatických podmínek na utváření tělesných proporcí. Původním cílem projektu bylo morfologické systematické zhodnocení rosniček dané oblasti, ale jak se postupně ukázalo, morfologický přístup nebyl pro tento cíl vhodný. Naopak byl vhodně využit k interpretacím evolučně-ekologického charakteru, jak je dokumentováno na případu morfologicky podobných, ale akusticky odlišných a geneticky nepříbuzných mediteránních populacích druhů *H. arborea* (a *H. orientalis*) a *H. savignyi*.

III. práce využívá akustický přístup k odlišení dvou druhů rosniček na území Íránu, odkud byl doposud hlášen jen jediný druh, *H. savignyi*.

IV. práce je komplexním vyústěním fylogeografické studie rosniček Blízkého východu za pomocí sekvencí mitochondriální i jaderné DNA. Díky tomuto molekulárně-genetickému přístupu byl objeven nový druh rosničky z oblasti Jemenu až jižní Levanty, následně identifikovaný také dle rozdílů v akustických znacích (oznamovacích hlasech samců v období rozmnožování) a v menší míře také dle rozdílů v morfologii. Tento nový druh bude v oficiálním článku formálně popsán (platnost popisu v dizertační práci je popřena dle pravidel ICZN 2009). Vedle tohoto výsledku se práce také stručně zabývá vnitrodruhovou taxonomií *H. orientalis*, resp. validitou taxonů *H. arborea schelkownikowi* a *H. arborea gumilevskii*, kterou popírá. Dalším výstupem práce jsou demografické analýzy jednotlivých populací celkem tří druhů (*H. savignyi*, *H. orientalis* a nový druh), které umožnily detailnější vhled do evoluční historie těchto druhů.

V. práce je popisem nového druhu amazonské rosničky rodu *Osteocephalus* na základě kombinace morfologických, ekologických a genetických přístupů. Práce také předkládá předběžné výsledky poznání fylogenetických vztahů uvnitř rodu, které stručně komentuje s ohledem na využívané reprodukční strategie různých druhů.

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VÝSLEDKY

Kapitola I.

Variation of *Hyla savignyi*: A color pattern of Cypriote and mainland populations.

Gvoždík V. & Moravec J., 2005.

Herpetologia Petropolitana. Ananjeva N. & Tsinenko O. (Eds.)
Russian Journal of Herpetology 12 (Suppl.): 32–34.

Hyla savignyi, Gazimağusa, Kypr (Cyprus)

VARIATION OF *Hyla savignyi*: A COLOR PATTERN OF CYPRIOTE AND MAINLAND POPULATIONS

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Keywords: Hylidae, *Hyla savignyi*, variation, color pattern, Cyprus.

INTRODUCTION

Audouin (1809) distinguished *Hyla savignyi* from *Hyla arborea* (Linnaeus, 1758) on the basis of its different color pattern (absence of upward loop on the lateral dark stripe). Later, Boulenger (1882) mentioned the former taxon as a variety of *H. arborea* whereas Nikolsky (1918) gave it a subspecific rank. More recently, Schneider and Nevo (1972) referred to differences in the mating calls of the both taxa and proposed to elevate the former one to the specific level. This approach was followed by number of other authors and today the name *H. savignyi* is widely used for the tree frog populations distributed in southern and eastern Turkey, Transcaucasia, western Iran, Iraq, Levant, north-eastern part of Sinai, Cyprus, and south-western part of Arabian Peninsula. Nevertheless, except of the description of the general coloration of *H. savignyi*, till now there are no available data dealing more thoroughly with the color pattern of this species and its possible geographical variation.

Examining the morphological variation of *H. savignyi* we noticed remarkably frequent occurrence of spotted to striped dorsal color pattern in Cypriote population. The aim of this brief report is (i) to draw attention to this phenomenon, (ii) to describe the patterned form of dorsal coloration of *H. savignyi*, and (iii) to quantify the geographic distribution of this form.

MATERIAL AND METHODS

The material consisted of 599 museum specimens of *H. savignyi* from its whole distribution area. Additional data were collected directly in the field in Cyprus, Turkey, Syria, Lebanon, Israel and Jordan. The comparison of the color pattern of the Cypriote population with the population from adjacent mainland (Mediterranean zone of Turkey, Syria, and Lebanon) was based on 421 specimens (Cyprus: 240, mainland: 181; see Fig. 1 for localities).

Variation in the dorsal color pattern consists in presence, shape and arrangement of dark dorsal spots, which, according to our observation on living specimens, can undergo on the background color independent color changes. We defined two basic groups of color pattern for needs of our study: (i) pattern-less and (ii) patterned. The latter was subdivided into two subgroups: (ii-a) spotted, individuals bearing irregularly distributed spots; (ii-b) striped, individuals with more or less complete longitudinal stripes formed by oblong spots. Pattern of dark permanent little dots, which occurred in *H. arborea* too, was omitted within our investigation of the dorsal color pattern.

RESULTS AND DISCUSSION

The examination of the studied material revealed that the frequency of the patterned specimens is higher in the Cypriote population than in the populations from the other parts of the range of *H. savignyi*. The comparison proper of the samples from Cyprus and the adjacent mainland approved a significantly higher occurrence of patterned specimens in the island population (45.4%, 109/240 vs. 23.2%, 42/181, $\chi^2 = 22.13$, $df = 1$, $p < 0.0001$; Fig. 2). There was also a significantly higher occurrence of striped

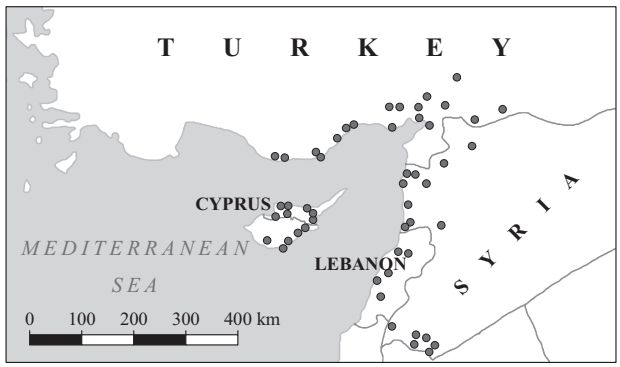

Fig. 1. Schematic map of localities of the material examined.

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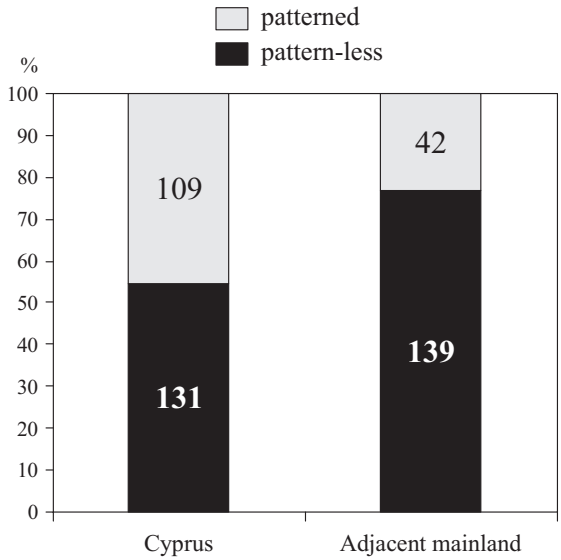

Fig. 2. Comparison of ratios of patterned specimens.

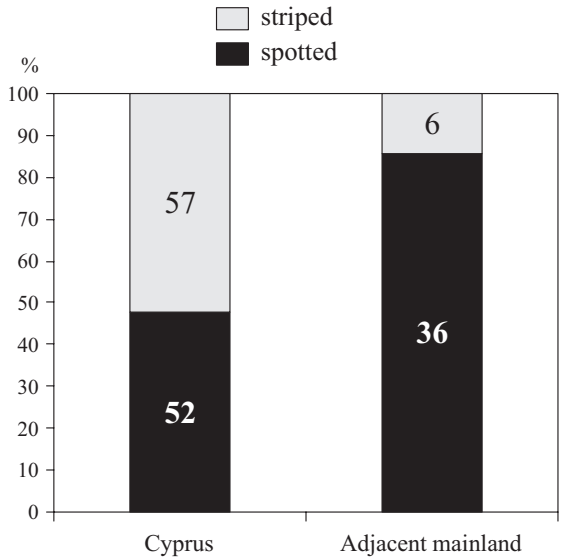

Fig. 3. Comparison of ratios of striped specimens within patterned specimens.

individuals within the patterned part of the Cypriote population (52.3%, 57/109 vs. 14.3%, 6/42, $\chi^2 = 18.01$, $df = 1$, $p < 0.0001$; Fig. 3).

The dorsal color pattern can be more or less obvious in dependence of the current physiological conditions. According to our observations from the field and captivity, the intensity of the dorsal color pattern changes in dependence on daily cycle and on activity of frog. The color pattern is more visible at nights, when the spots/stripes turn from green (different tone from background color) to dark brown or black. Nevertheless, it is obvious during daytime too. Interesting finding is that already Boulenger (1898) mentioned the striped or spotted pattern in *H. savignyi* (at that time as *H. arborea* var. *savignyi*) on page 251: "Some specimens (Cyprus) have four stripes or series of spots in addition to the lateral." He also supplied this record by figure of Cypriote specimen on plate XV (see Fig. 4).

Our findings indicate that Cypriote population of *H. savignyi* has undergone a certain degree of differentiation from the mainland populations. These findings are also supported by our other morphological and bioacoustic data. The most obvious difference is in body size. Tree frogs of Cypriote population are significantly smaller than tree frogs from adjacent mainland (Gvoždík et al., in preparation). Already Schmidtler (1984) gave notice of this phenomenon. He considered Cypriote tree frogs as a "dwarf form" of supposedly subspecific status. On the other hand Böhme and Wiedl (1994) discussed weak insular endemism of the herpetofauna of Cyprus. One of the possibilities how to answer this problem is the insufficient knowledge of amphibians and reptiles biodiversity in the region of eastern Mediterranean. The study pointing to specific status of Cypriote water frogs could be used as an example (Plötner et al., 2001). Tarkhnishvili and Gokhelashvili (1999) wrote about tree frogs from Cyprus that they "are morphologically similar to *H. savignyi*, although nowadays they are assumed to represent different species." This information is very interesting but certainly incorrect, be-

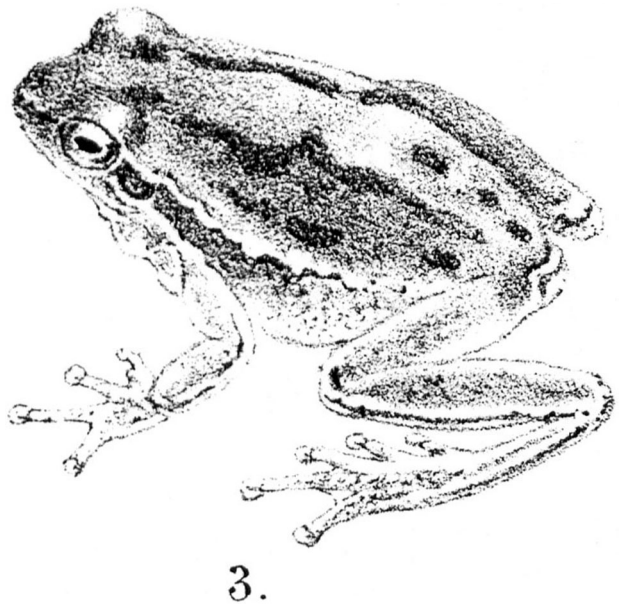

Fig. 4. Illustration of a Cypriote specimen of *H. savignyi* published by Boulenger (1898: Plate XV, Figure 3).

cause no particular investigations of variation of *H. savignyi* have been accomplished neither from Cyprus nor from other parts of the distribution range so far.

Therefore, taxonomic status of Cypriote tree frogs needs further verification on the basis of particular morphological, bioacoustic and genetic studies.

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Kapitola II.

Geographic morphological variation in parapatric Western Palearctic tree frogs, *Hyla arborea* and *Hyla savignyi*: are related species similarly affected by climatic conditions?

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Biological Journal of the Linnean Society 95: 539–556.

Hyla arborea, Gialova, Peloponés, Řecko (Greece)

Biological Journal of the Linnean Society, 2008, **95**, 539–556. With 5 figures

Geographic morphological variation in parapatric Western Palearctic tree frogs, *Hyla arborea* and *Hyla savignyi*: are related species similarly affected by climatic conditions?

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Tree frogs *Hyla arborea* and *Hyla savignyi* are similar, closely-related species distributed in Europe and the Middle East. We investigated geographic variation in body shape within and between these species, and tested its relationships to macroclimatic conditions. We used morphometric distances (based on size corrected external measurements) to construct phenetic trees (unweighted pair-group method of arithmetical averages, Neighbour-joining), and to test correlations between morphology, geography, and climate by the partial Mantel test. Regardless of their specific affiliation, the parapatric populations of both species from the eastern Mediterranean, where they occupy comparable habitats, are closer to each other in morphospace than to conspecific populations from distal regions. This local interspecific similarity is probably driven by the common response to environment, expressed here as macroclimatic conditions. In support, the geographically close but ecologically vicariant populations of both species from the Caucasus region differ quite substantially in body shape. We suggest that climate-provoked phenotypic variation in closely-related parapatric species should be taken into account as a potential complication to character displacement in morphology. Contrariwise, morphological diversification between related species or their populations could be enhanced by habitat shifts resulting in occupation of different environmental space.

ADDITIONAL KEYWORDS: amphibians – body shape – character displacement – climatic response – morphological convergence – morphometrics – zoogeography.

INTRODUCTION

Although controversy still exists with respect to the extent of individual geographic modes of speciation, it is commonly accepted that most species originated in allopatry (Coyne & Orr, 2004). As a result, ranges of the most of newly-formed species are not in contact, and recently observed parapatric or sympatric occurrence of closely-related species is ordinarily secondary. Related species inherit most of their traits from their common ancestor. Consequently, they are usually very similar in ecology as well as in phenotype. After the contact of their ranges, we can thus

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expect fierce competition for resources between these reproductively isolated, but ecologically largely equivalent entities. The most likely stable evolutionary outcomes of such competition is either exclusion of one of the competitors from the overlaying part of ranges leading to parapatry, in a long view, potentially even to total extinction of the inferior competitor, or the differentiation of ecological niches enabling long-term coexistence in sympatry. The differentiation of niches is often accompanied by the selection for divergent phenotypes that further minimize interactions between competitors. This process, termed character displacement (Brown & Wilson, 1956), generates adaptive shifts in phenotypes of one or both competing species reflecting resource division (Schluter, 2000). Because character displacement is thought to be an important mechanism for promoting adaptive diversification and consequently biodiversity (Schluter, 2000; Coyne & Orr, 2004), a number of studies has focused on identifying the conditions that encourage character displacement (Losos, 2000). However, many closely-related species have not differentiated their niches and live in parapatry (tree frogs *Hyla arborea* and *Hyla savignyi* in southern Anatolia being an example; Schneider, 2001). It seems that a process exists making phenotypic diversification between members of some species pair difficult (i.e. a process that prevents character displacement).

A candidate for such process could be climate-provoked phenotypic variation. Climatic conditions as an important agent influencing animal morphology have been documented in many ectothermic and endothermic animals (Rosso, Castellano & Giacoma, 2004; Schäuble, 2004; Davis, 2005; Kutrup, Bulbul & Yilmaz, 2006). Convergent emergence of ecomorphs during iterative climatic cycles suggests a strong potential of climatic conditions to form similar phenotypes even among distantly-related animals (Martin & Meehan, 2005). We should thus expect convergent evolution driven by common macroclimatic conditions still more likely in related species that share most of their genetic background. Closely-related species might also react to common environment by uniform plastic response because it is highly probable that they inherited the norm of reaction from the ancestor. To sum up, we can thus assume that shared environmental conditions might induce phenotypic resemblance of closely-related species in the common or near parts of their ranges, which could complicate species differentiation necessary for character displacement.

In the present study, we focus on the morphological variation in two parapatric species of tree frogs, *H. arborea* (Linnaeus, 1758) and *H. savignyi* Audouin [1827] '1809'. According to the immunological research, *H. arborea* and *H. savignyi* are closely-related species, which diverged probably by the end of the Miocene, approximately 5–6 Mya (Maxson & Wilson, 1975; Maxson, 1978; Riehl, Lell & Maxson, 1995). Fossil records of *H. arborea* and *H. savignyi* are known from the Pleistocene and Holocene, and the first species is likely recorded also from the Pliocene (Sanchiz, 1998). The known range of *H. arborea* extends from Iberian Peninsula in the west to Asia Minor and the Caucasus region in the east (Glandt, 2004). *Hyla savignyi* is widely-distributed in southern and eastern Turkey, eastern Transcaucasia, northern and western Iran, Iraq, Levant, and the north-eastern part of Sinai. Two isolated populations live in Cyprus and south-western Arabian Peninsula (Glandt, 2004). The ranges of *H. arborea* and *H. savignyi* are known to be in contact in two areas: in southern Anatolia, where both species live in parapatry (Kaya, 2001; Schneider, 2001, 2004), and in the Caucasus region, where parapatry was also documented in Armenia (Egiasarian & Schneider, 1990, 1991), but where a local sympatric occurrence was anticipated in other studies (Alekperov, 1978; Kuzmin, 1999; Tarkhnishvili & Gokhelashvili, 1999). However, sympatry was recently rejected by Litvinchuk *et al.* (2006).

Hyla savignyi was originally distinguished from *H. arborea* on the basis of colour pattern, and for a long time, it was understood as a subspecies of *H. arborea* (Duellman, 1977). Nevertheless, bioacoustic studies revealed substantial differences in male advertisement calls between *H. arborea* and *H. savignyi* (Schneider & Nevo, 1972; Schneider, 1974; Kaya & Simmons, 1999). Within the species, male advertisement calls tend to be stable across ranges (Schneider, 2004). Male advertisement calls are important components of courtship behaviour in anurans in general and in the Palearctic tree frogs in particular (Brzoska, Schneider & Nevo, 1982; Schneider *et al.*, 1984), and usually form interspecific premating reproductive barrier. Later authors revealed cytological and osteological traits further supporting the specific status of *H. savignyi* (Anderson, 1991; Kaya, 1997). By contrast, the differentiation between *H. arborea* and *H. savignyi* in external morphology appears to be minimal across populations in southern Anatolia (Zaloğlu, 1972; Kaya, 2001), although their advertisement calls considerably differ also in that region (Kaya & Simmons, 1999; Schneider, 2001). However, the studies focussing on morphology did not include material from other parts of species ranges, and do not allow morphological variation and differentiation to be compared among distant versus near populations of both species. Recently, a new species of tree frog, *Hyla heinzsteinitzi* Grach, Plesser & Werner (2007), was described from the range of *H. savignyi*. The new species is

known only from a restricted area in the surroundings of Jerusalem, Israel, and occurs in sympatry and apparently syntopy with *H. savignyi*. It was distinguished from *H. savignyi* by differences in head shape, coloration and advertisement call (Grach *et al.*, 2007).

The aim of the present study was to document variation in external morphology across the whole range of *H. savignyi* and eastern part of the range of *H. arborea*. We were interested in the degree of morphological differentiation between both species and, specifically, in morphological (dis)similarity in the regions near the contacts of their ranges (southern Anatolia and Caucasus). Furthermore, we tested whether climatic conditions influence the morphological variation across populations of both species, and whether similar environment leads in both species to comparable morphotypes, which would support the above-described scenario of climate-driven prevention of character displacement in morphology in closely-related species of organisms.

MATERIAL AND METHODS

Material

The material examined consisted of 308 preserved museum adult specimens of *H. savignyi* from the whole range of its distribution (195 males, 89 females, and 24 adults of undetermined sex), 238 preserved voucher adults of *H. arborea* from the central and eastern parts of its range (173 adult males, 50 adult females, and 15 adults of undetermined sex), and 20 preserved museum adult males of *Hyla meridionalis* from Canary Islands/Tenerife (for details, see Appendix). The Mediterranean species *H. meridionalis* was recently shown to be basal within the Western Palearctic clade (Smith, Stephens & Wiens, 2005), and we used it as the outgroup. With the exception of the Iberic subspecies *Hyla arborea molleri* Bedriaga, 1890 and recently described subspecies *Hyla arborea gumilevskii* Litvinchuk, Borkin, Rosanov, Skorinov, 2006, our material covers all other traditionally recognized subspecific taxa of *H. arborea* (Kuzmin, 1999; Valakos *et al.*, 2008), which have been distinguished on the basis of external morphology: *Hyla arborea arborea*, *Hyla arborea schelkownikowi* Chernov, 1926 (including 23 topotypes) and *Hyla arborea kretensis* Ahl, 1931 (including 34 syntypes). Although, some studies do not approve the validity of these subspecies (Schneider, 2004; Frost, 2007). The recently described *H. heinzsteinitzi* was not included in our study since the formal description was published after our analyses. Nevertheless, no tree frogs from the restricted range of the new species were included into our dataset; thus, any taxonomic confusion should not occur.

The material was divided into 13 operational taxonomic units (OTUs) defined according to the species identity and biogeographical division (for the map, see Fig. 1). The individuals of *H. savignyi* form seven OTUs: (1) Arabian Peninsula, (2) Iranian Highlands and Kurdistan, (3) Mesopotamia, (4) Levant, (5) Cyprus, (6) southern Anatolia, and (7) Transcaucasia. *H. arborea* was assorted into six OTUs: (8) Caucasus and adjacent regions, (9) western Anatolia and adjacent islands, (10) Crete, (11) Balkan Peninsula and adjacent islands, (12) Danube Delta, and (13) Central Europe. Six individuals from six localities outside the ranges of defined OTUs were used only in the interspecific comparisons. An extraordinary OTU (14) was established for the outgroup, *H. meridionalis* from Tenerife Island (the type locality).

Sex and sexual maturity were determined according to the presence of vocal sac in males and enlarged abdomen (presence of eggs) in females. The snout–urostyle length of the smallest adult males and females (excluding several miniature individuals considered extreme) was taken as an arbitrary limit of sexual maturity for individuals in nonreproductive phase of life (29.0 mm for males and 31.8 mm for females). The sex of individuals collected during nonreproductive phase was determined by dissection (which was possible only for the material deposited in the National Museum, Prague).

Specific determination: colour pattern

The colour pattern of the dark side strip (*linea marginalis*) and the inguinal region was examined in all individuals of *H. arborea* and *H. savignyi*. *Hyla meridionalis* does not possess the dark strip along the body. The coloration of the inguinal region has been suggested to be the key morphological trait discriminating *H. arborea* and *H. savignyi* (Baran & Atatür, 1998), with the exception of the subspecies *H. arborea gumilevskii* from south-eastern Azerbaijan and presumably northern Iran with reduced inguinal loop (Litvinchuk *et al.*, 2006). The specific determination of the material from the region of parapatry was confirmed by molecular evidence (mitochondrial and nuclear DNA sequences; V. Gvoždík, J. Moravec, P. Kotlík *et al.*, unpubl. data). Three types of inguinal colour pattern were distinguished: (1) *linea marginalis* continuously builds a regular inguinal loop, (2) spot(s) instead of the loop, or a thin loop is separated from *linea marginalis*, and (3) loop or spot(s) are entirely absent. Photographs of representatives of the three groups are provided in the Supporting Information (Fig. S1). In six specimens, the condition of the skin colour did not allow an evaluation of the inguinal colour pattern.

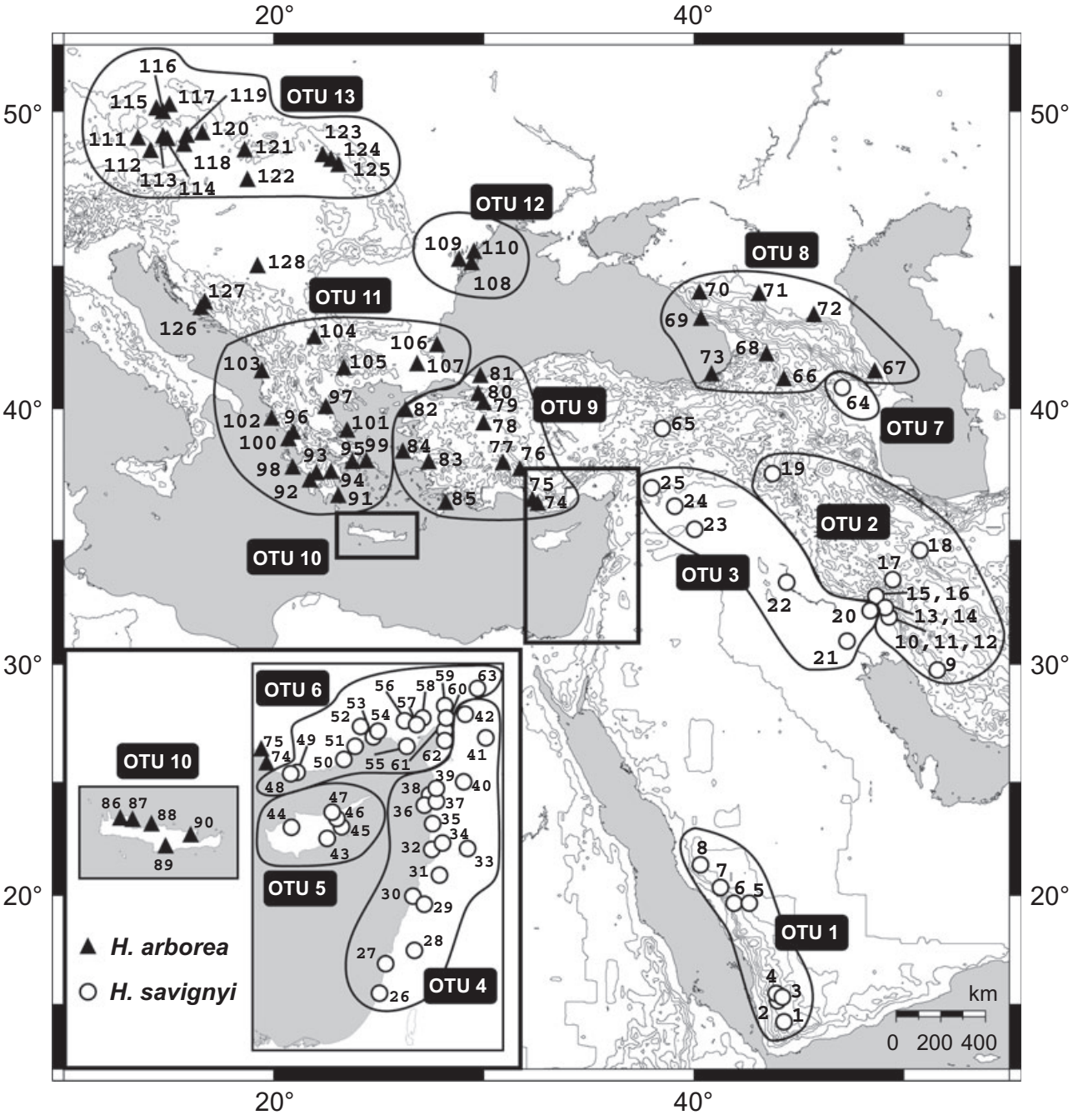

Figure 1. Map of localities. For further details, see Appendix.

MORPHOMETRICS

Seventeen external measurements were taken with the callipers in each adult specimen by V.G. to the nearest 0.1 mm in the standardized manner (Fig. 2): SUL (snout–urostyle length: from the tip of snout to the posterior margin of urostyle); FmL* (femur length: from the middle of cloacal gap to the external margin of knee joint, when thighs and shins are in perpendicular position to body axis); TbL* (tibia length: from the external margin of knee joint to the external margin of heel articulation); TrL* (tarsus length: from the external margin of heel articulation to the proximal edge of inner metatarsal tubercle); HW (head width: the largest head width); HLt⁺ (head length: from the tip of head to the posterior margin of

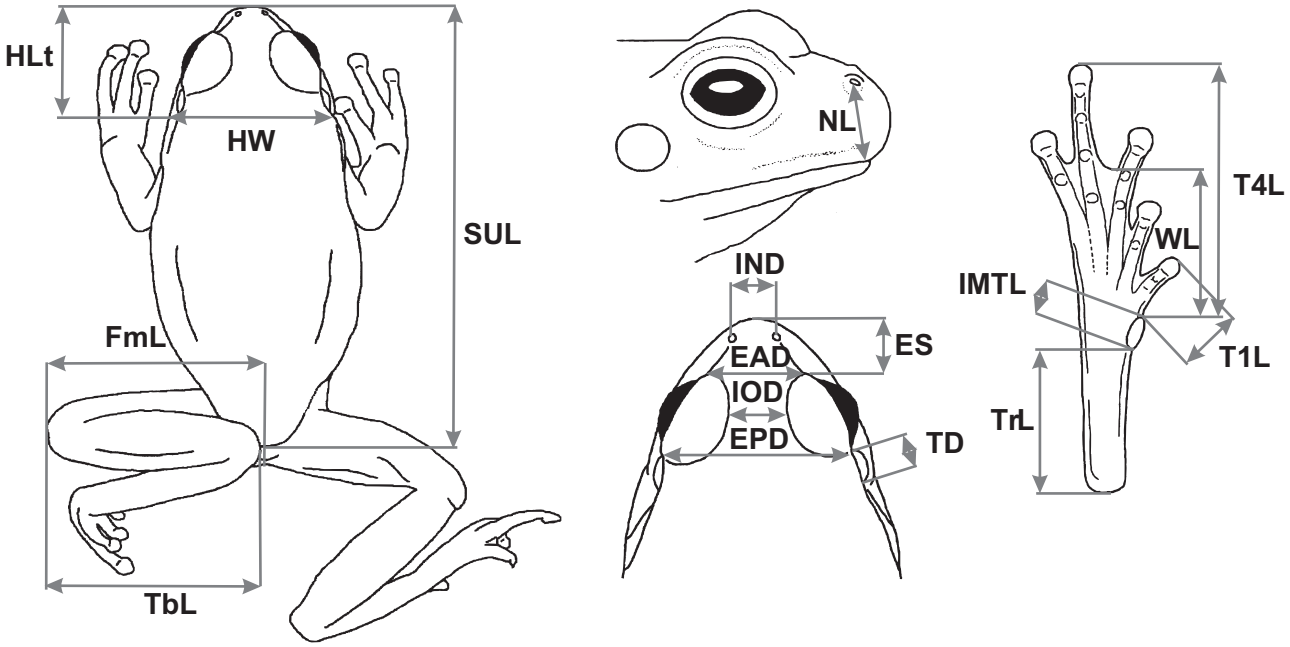

Figure 2. Morphological measurements. SUL, snout–urostyle length: from the tip of snout to the posterior margin of urostyle; FmL, femur length: from the middle of cloacal gap to the external margin of knee joint, when thighs and shins are in perpendicular position to body axis; TbL, tibia length: from the external margin of knee joint to the external margin of heel articulation; TrL, tarsus length: from the external margin of heel articulation to the proximal edge of inner metatarsal tubercle; HW, head width: the largest head width; HLt, head length: from the tip of head to the posterior margin of tympanum; ES, eye-snout distance: from the tip of head to the anterior corner of eye; TD, horizontal tympanum diameter; EAD, distance between the anterior corners of eyes; IOD, interorbital distance: the shortest distance between upper eye lids; EPD, distance between the posterior corners of eyes; IND, internarial distance: the distance between the midpoints of nostrils; NL, nostril-upper lip distance: the distance from the midpoint of nostril to the margin of the upper lip; IMTL, inner metatarsal tubercle length: the length of the base of tubercle; T1L, first toe length: from the distal edge of the inner metatarsal tubercle to the tip of the first toe; T4L, fourth toe length: see T1L; and WL, webbing length: from the distal margin of the inner metatarsal tubercle to the margin of the webbing in the middle between the third and the fourth toe.

tympanum); ES^{+} (eye-snout distance: from the tip of head to the anterior corner of eye); TD (horizontal tympanum diameter); EAD (distance between the anterior corners of eyes); IOD (interorbital distance: the shortest distance between upper eye lids); EPD (distance between the posterior corners of eyes); IND (internarial distance: the distance between the midpoints of nostrils); NL (nostril-upper lip distance: the distance from the midpoint of nostril to the margin of the upper lip); IMTL* (inner metatarsal tubercle length: the length of the base of tubercle); T1L* (first toe length: from the distal edge of the inner metatarsal tubercle to the tip of the first toe); T4L* (fourth toe length: see T1L); and WL* (webbing length: from the distal margin of the inner metatarsal tubercle to the margin of the webbing in the middle between the third and the fourth toe). The means of left and right sides were computed for bilateral traits to reduce effects of fluctuating asymmetry and measurement error (asterisks). The measurements denoted by cross ($^{+}$) were measured in parallel to body axis using calliper modified according to Goren & Werner (1993). One or more measurements were missing in 21 individuals due to their damage. These missing values (representing less than 0.3% of all measurements) were replaced with the means of values predicted from regressions of traits involved against SUL, FmL, TbL, and HLt in individuals from a given OTU. Predicted values obtained using different independent variables were subsequently averaged to produce a single estimate of missing values (for an analogous method, see Merilä, 1997).

Analysis of the pattern of morphometric variation

The original measurements were natural log-transformed before the further analyses. Every comparative analysis has to take into account the growth pattern of studied organisms. Tree frogs typically

mature at small sizes relative to their asymptotic size, and continue growing extensively after maturity (Moravec, 1990; Friedl & Klump, 1997; Kyriakopoulou-Sklavounou & Grumiro, 2002). Ignoring this fact during morphometric comparisons, the discovered variation could reflect differences in age composition among samples rather then factual differences among populations. Therefore, we decided to focus on quantitative morphological variation in body shape after statistical control for body size. The traditionally used method for computing size-adjusted data (residuals of linear regressions) was recently subjected to severe criticism (Smith, 1999; García-Berthou, 2001) and, due to the structure of our data (nonparallel regression lines of many variables against SUL among groups), it would be obviously misleading in our case. Therefore, to remove the effects of size we used the geometric means method of Mosimann (1970), which does not distort group configuration (Butler & Losos, 2002; Losos & Miles, 2002). We defined the individual index of body size as the arithmetic mean of all 17 log-transformed variables (equivalent to the geometric mean of original variables if computed before log-transformation). Each individual was then size-adjusted by taking the difference of each log-variable with this body size index. To obtain linearly independent shape variables for successive multivariate analyses, we arbitrarily omitted one variable (size-adjusted ES). Further on we refer to size-adjusted variable as a variable/S (e.g. size-adjusted femur length as FmL/S).

Principal component analysis (PCA) on 16 size-adjusted measurements of all individuals was used to estimate the pattern of correlation and covariation among variables. The number of interpretable PCA axes was determined using broken-stick model as recommended by Jackson (1993). Retained individuals' scores on the significant principal components were then treated by general linear models (GLM) with sex and OTU as factors to test sexual and geographical differences in our dataset within the ingroup species, *H. arborea* and *H. savignyi*. Next, we performed two canonical discriminant function analyses (DFA): the first DFA with all individuals including the outgroup, *H. meridionalis*, for investigation of general pattern, and the second DFA restricted to the ingroup species, *H. arborea* and *H. savignyi*, to obtain morphological distance matrix for further testing of geographic variation causality. The differences among all groups were tested for reliability by establishing the percentages of individuals correctly classified by classification functions. Further, we calculated squared Mahalanobis distances (D^2) as a measure of morphometric distances among groups of different geographic origin. Morphological phenograms were constructed from the distances between all OTUs (i.e. including *H. meridionalis*) by unweighted pair-group method of arithmetical averages (UPGMA) cluster analysis and the Neighbour-joining method.

The program STATISTICA, version 6.0 (StatSoft) was used for all morphometric calculations. Phenetic trees were constructed by PHYLIP, version 3.65 (Felsenstein, 2005) and depicted in TREEVIEW, version 1.6.6 (Page, 2001).

Analysis of morphometric/geographic/climatic causality

The causes of geographic variation between and within the species were tested by the partial Mantel test of matrix association (Smouse, Long & Sokal, 1986). We used the morphometric matrix (i.e. the matrix of squared Mahalanobis distances between particular *H. arborea* and *H. savignyi* OTUs) as a dependent matrix. The independent matrices were the matrix of geographic distances and the climatic matrix. Mantel tests were run on 10 000 randomizations. Geographic distances between approximate centres of the ranges of OTUs were taken from the Lambert azimuthal projection map. The macroclimatic data [i.e. 12 average monthly temperatures (°C) and 12 monthly precipitations (mm month^{-1}) for each single locality] were obtained from the International Water Management Institute Climate Atlas Web Query service (IWMI, 2006). These values show the annual climate cycle experienced by a population through a year. The climatic matrix was constructed as the matrix of Euclidean distances between-OTUs based on the weighted means of all 12 average monthly temperatures and all 12 monthly precipitations for particular OTU. The means for each climatic trait were weighted with the number of individuals from each locality to control for biases caused by uneven representation of individual localities within OTUs (for the weighted means of all climatic traits, see Supporting Information, Table S1). The program IBD, version 1.52 (Bohonak, 2002) was employed for the Mantel tests.

RESULTS

Colour pattern

Most specimens of *H. savignyi* ($N = 207$; 68.1%), lacked any dark inguinal loop or spot(s). The dark spot separated from *linea marginalis* was present in 95 specimens (31.2%), and the loop of irregular shape on the lateral strip was developed in only two specimens (0.7%) from areas in the middle of the range of *H. savignyi*. Interestingly, the populations of *H. savignyi* from Iranian Highlands and Kurdistan possess high rate of absence of both spot(s) and loop ($N = 39$; 97.5%). On the other hand, a loop was present in

Table 1. Factor loadings of size-adjusted external measurements for the first two principal components

	PC 1	PC 2
SUL/S	–0.42	0.54
FmL/S	–0.84*	0.17
TbL/S	–0.82*	0.31
TrL/S	–0.77*	0.14
HW/S	–0.04	0.04
HLt/S	0.09	0.75*
TD/S	0.22	0.14
EAD/S	0.44	0.23
IOD/S	0.04	0.23
EPD/S	0.41	0.17
IND/S	0.64	–0.24
NL/S	0.71	–0.09
IMTL/S	0.32	–0.52*
T1L/S	–0.44	–0.48*
T4L/S	–0.59	–0.56*
WL/S	–0.46	–0.66*

*Characters most strongly correlated with respective principal component. For abbreviations, see Fig. 2.

220 (93.2%) of the examined adult specimens of *H. arborea*. The colour pattern in the form of a separated loop was observed in 13 individuals (5.5%), and the absence of any shaped pattern was noticed in three (1.3%) specimens of *H. arborea* only. All 34 examined individuals of *H. arborea* from western Anatolia (OTU 9) possess well-developed loop of a regular shape. For a graphic expression of inguinal colour pattern distribution, see the Supporting Information (Fig. S2).

Geographic variation of body shape

The broken-stick model revealed that only the first two principal axes summarizing 27.1%, respectively 15.6% of the total variance in body shape are statistically significant. PC1 catches mostly variation in measurements concerning hind limbs (FmL/S, TbL/S, TrL/S), whereas PC2 reflects variation in head length (HLt/S) and in foot characters (IMTL/S, T1L/S, T4L/S, WL/S); factor loadings summarized in Table 1. The studied species differ significantly in factor scores of PC1 as well as PC2 [analysis of variance (ANOVA), $P < 0.0001$], although post-hoc tests revealed that only *H. arborea* and *H. savignyi* differ significantly in both principal components (Tukey HSD tests for unequal N, $P < 0.0001$). *Hyla meridionalis* does not differ significantly from neither *H. arborea*, nor *H. savignyi* in PC1 factor scores, and differs only from *H. arborea* in PC2 factor scores (Tukey HSD for unequal N test, $P < 0.05$). Nevertheless, *H. arborea* and *H. savignyi* show an extensive overlap in the morphometric space, and *H. meridionalis* is imbedded directly between the two other species (Fig. 3A, B, C). For descriptive statistics of morphological characters, see the Supporting Information (Table S2).

GLM ANOVA of PC1, respectively PC2 scores, performed separately for each ingroup species, revealed substantial geographic intraspecific variation in body shape (factor OTU: *H. savignyi*: PC1: $F = 27.271$, $P < 0.001$; PC2: $F = 13.081$, $P = 0.003$; *H. arborea* PC1: $F = 8.313$, $P = 0.018$; PC2: $F = 15.538$, $P = 0.005$). Compared with OTU, the variation explained by the sex factor is trivial and statistically nonsignificant after Bonferroni correction for multiple tests: *H. savignyi*: PC1: $F = 5.822$, $P = 0.027$; PC2: $F = 0.467$, $P = 0.497$; *H. arborea*: PC1: $F = 2.428$, $P = 0.155$; PC2: $F = 0.063$, $P = 0.808$; as well as the sex × OTU interactions, nonsignificant, $P > 0.072$ in all cases. Therefore, we pooled data on both sexes and added data on 39 adults of unknown sex in our further analyses.

DFA further confirmed the differences in body shape among individual OTUs (Wilks' $\lambda = 0.028$, $F = 10.99$, $P < 0.0001$; Fig. 4A). However, the success of classification functions in classification of individuals with respect to their origin was moderate (62.8% correctly classified individuals on average; range = 25.0–90.9%; Table 2); for discriminant coefficients of classification functions, see the Supporting Information (Table S3). Classification into incorrect species was relatively low for all OTUs of *H. savignyi* (10.1% specific misclassifications on average, range = 3.4–17.1%; 8.0% into *H. arborea*, 2.1% into *H. meridionalis*), and three OTUs of *H. arborea* [only 2.3% specific misclassifications within the OTU 8 (Caucasus), OTU 12 (Danube Delta), and OTU 13 (Central Europe), on average; range = 0–4.3%; all misclassified as *H. savignyi*]. On the other hand, a relatively high number of misclassification into different species occurred within the southern OTUs of *H. arborea*: OTU 9 (western Anatolia), OTU 10 (Crete), and OTU 11 (Balkan); 24.6% erroneous on average; range = 17.6–35.3%; 21.2% into *H. savignyi*, 3.4% into *H. meridionalis*. The individuals of these populations were often misclassified into the Mediterranean and Mesopotamian populations of *H. savignyi*. *H. meridionalis* was incorrectly classified in 45% (30% as *H. savignyi*, 15% as *H. arborea*).

Initially, the phenetic trees based on the squared Mahalanobis distances between OTUs calculated by alternative computational methods (UPGMA and NJ) appear to be rather different (Fig. 4B, C). However, the main information included in both of them does not differ. Both trees demonstrate low morphometric distances among the eastern Mediterranean populations of *H. arborea* and the Mediterranean and Mesopotamic populations of *H. savignyi*. On the other hand, OTUs with the largest geographic distances from the Anatolian zone of parapatry, and/or with the

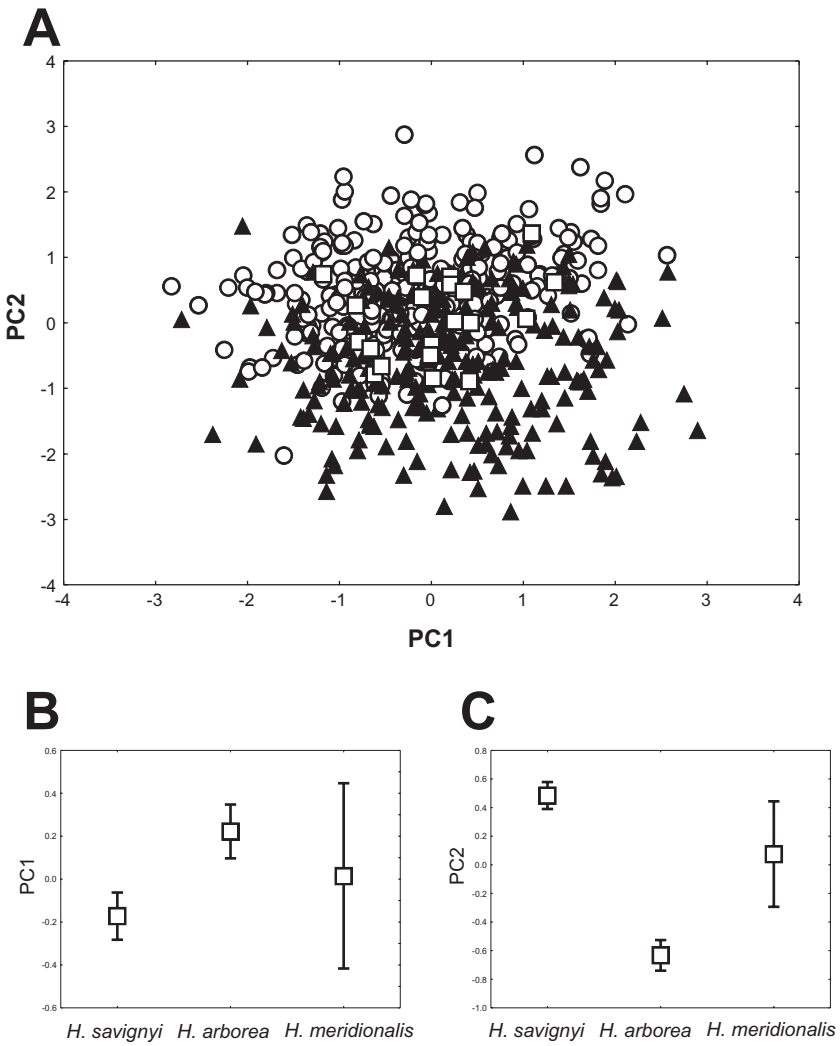

Figure 3. Factor scores of the first two principal components shown as (A) scatterplot for all cases (triangles, *Hyla arborea*; circles, *Hyla savignyi*; squares, *Hyla meridionalis*) and mean values with 95% confidence intervals of (B) PC1 and (C) PC2 for each species separately. The large confidence intervals of *H. meridionalis* mirror smaller sample size in comparison to *H. arborea* and *H. savignyi*.

most outermost climatic conditions (Central European *H. a. arborea*, Caucasian *H. a. schelkownikowi*, Danube Delta populations of *H. arborea*, and Arabian, Iranian and Transcaucasian populations of *H. savignyi*) are relatively dissimilar to each other as well as to the cluster of the Mediterranean and Mesopotamic tree frogs.

Effect of geography and climate

The Mantel tests performed on distances among populations of *H. arborea* and *H. savignyi* revealed significant correlation between body shape and geography ($r = 0.635$, $P < 0.0002$), as well as between body shape and climate defined by temperature and precipitation ($r = 0.373$, $P = 0.0037$) (Fig. 5A, B). Although the correlation between body shape and geography is stronger, several outliers contradicting this correlation are present (Caucasus versus Transcaucasia, Caucasus versus Iran, and Caucasus versus Mesopotamia). These interspecific outliers reflect deep morphometric differences across relatively short geographic distances. On the other hand, the geographically close Anatolian *H. arborea* and *H. savignyi* populations are close also in morphospace.

Because geographical and climatical matrices are significantly correlated (Mantel test: $r = 0.266$, $P = 0.0242$), the partial Mantel tests were used to analyse their independent association with body

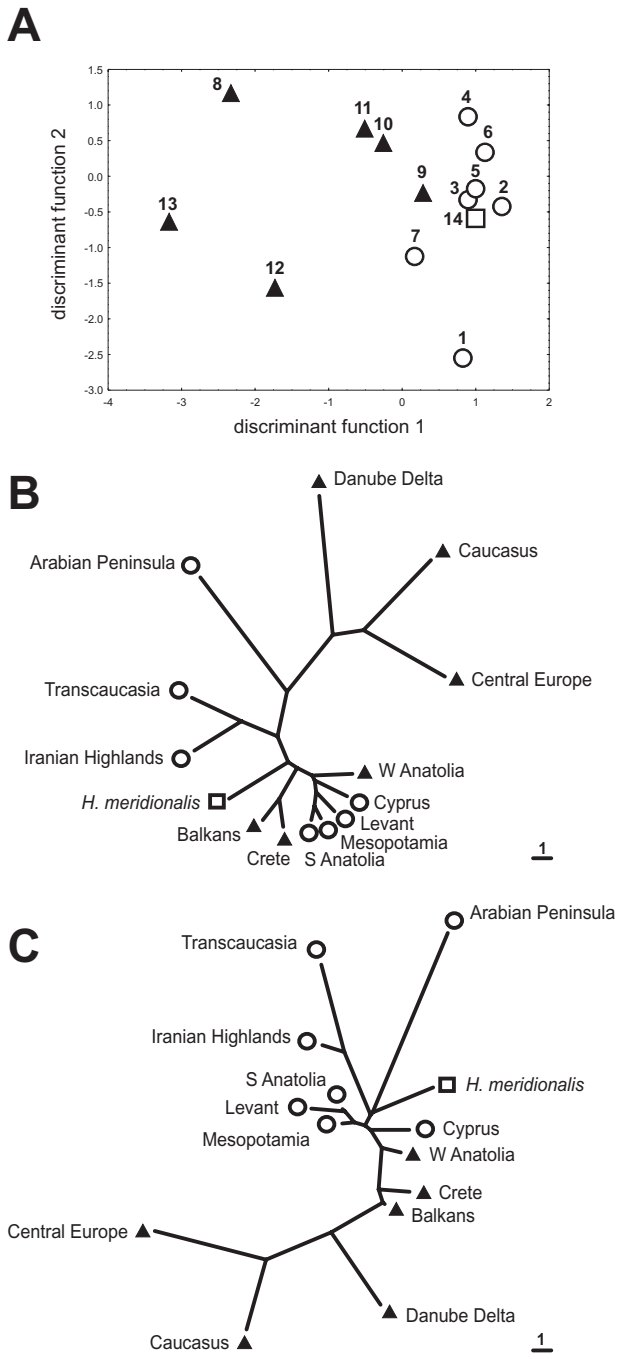

Figure 4. Results of the discriminant analysis presented as (A) median values of the canonical scores of the first two discriminant functions for each single operational taxonomic unit (OTU) (for OTU identification, see text) and unrooted phenetic trees based on morphometric distances among OTUs using (B) unweighted pair-group method of arithmetical averages cluster analysis, and (C) the Neighbour-joining method. Triangles, *Hyla arborea*; circles, *Hyla savignyi*; squares, *Hyla meridionalis*.

shape. After statistical control of the covariation with geography, climate still showed significant association with body shape ($r = 0.274$, $P = 0.0227$).

DISCUSSION

In closely-related species, we can expect considerable phenotypic similarity caused by their shared ancestry. After speciation, the reproductive barriers prevent interbreeding among populations between species, while individual populations within species are essentially free to interbreed. Consequently, after a longer period of independent evolution, each species should possess its own species-specific morphotype. None or only small interspecific overlap in many morphological traits usually allows us to find key traits useful for determination of even closely-related species. The colour pattern of the inguinal region (either the absence or presence, respectively, of the shape of the dark anterodorsally-oriented loop) has been considered to be the main morphological difference between *H. arborea* and *H. savignyi* since the description of *H. savignyi* (Audouin, [1827] '1809'). Our material more or less confirms the coloration as the key morphological differential diagnostics. However, some individuals of both species do not follow the coloration typical for their species, which warns against determination of tree frogs solely on the base of coloration. This view is also supported by recent description of a subspecies of *H. arborea* with reduced inguinal loop (Litvinchuk *et al.*, 2006). Therefore, we strongly recommend the use of molecular markers or bioacoustics for the verification of the determination of these two species of tree frogs.

We found a statistically significant interspecific differentiation between *H. arborea* and *H. savignyi* in measurements concerning hind limbs and head length, which corresponds to a general historical approach to taxonomy of the Palearctic tree frogs (Terentjev & Chernov, 1949). However, these two species do not form well-separated clusters in the morphospace delimited by external measurements (Fig. 3A). Multivariate analyses revealed that individuals from the populations of the Mediterranean and adjacent regions (the Balkans, Crete, Anatolia, Levant, Cyprus, Mesopotamia) are very similar in body shape regardless of specific affiliation. This means that the individuals of *H. arborea* and *H. savignyi* from these areas are mutually more similar in body shape to each other, irrespective of the sharp difference in their advertisement calls (Schneider, 2001, 2004), than to conspecific individuals (with identical advertisement calls; Schneider, 2000, 2004) from more distal populations.

Significant portion of variation in body shape among particular OTUs can be explained by two basic

Table 2. A posteriori classification of the discriminant function analysis. Percentage of correctly classified individuals for respective OTU in bold.

			Predicted group													
			Hyla savignyi							*Hyla arborea*						*Hyla meridionalis*
		OTU	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Actual group	*Hyla savignyi*	1	26	0	0	0	0	1	1	0	0	0	0	0	1	0
		$N = 29$	**89.8%**					3.4%	3.4%						3.4%	
		2	0	25	0	3	3	1	2	0	0	5	1	0	0	1
		$N = 41$		**61.0%**		7.3%	7.3%	2.4%	4.9%			12.2%	2.4%			2.4%
		3	1	1	5	1	2	7	0	0	2	0	0	0	0	1
		$N = 20$	5.0%	5.0%	**25.0%**	5.0%	10.0%	35.0%			10.0%					5.0%
		4	0	6	0	51	4	6	1	0	0	3	5	0	0	2
		$N = 78$		7.7%		**65.4%**	5.1%	7.7%	1.3%			3.8%	6.4%			2.6%
		5	0	1	1	8	47	3	0	0	0	2	2	0	0	1
		$N = 65$		1.5%	1.5%	12.3%	**72.3%**	4.6%				3.1%	3.1%			1.5%
		6	1	3	0	13	11	25	4	0	1	0	1	0	0	2
		$N = 61$	1.6%	4.9%		21.3%	18.0%	**41.0%**	6.6%		1.6%		1.6%			3.3%
		7	0	3	0	0	0	1	7	0	0	0	0	1	0	0
		$N = 12$		25.0%				8.3%	**58.3%**					8.3%		
	Hyla arborea	8	1	0	0	0	0	0	0	28	1	3	3	0	3	0
		$N = 39$	2.6%							**71.8%**	2.6%	7.7%	7.7%		7.7%	
		9	0	1	1	3	5	1	0	4	15	1	2	0	0	1
		$N = 34$		2.9%	2.9%	8.8%	14.7%	2.9%		11.8%	**44.1%**	2.9%	5.9%			2.9%
		10	0	1	0	3	3	0	1	1	2	29	4	1	1	2
		$N = 48$		2.1%		6.3%	6.3%		2.1%	2.1%	4.2%	**60.4%**	8.3%	2.1%	2.1%	4.2%
		11	0	1	0	3	1	0	0	3	3	8	12	2	0	1
		$N = 34$		2.9%		8.8%	2.9%			8.8%	8.8%	23.5%	**35.3%**	5.9%		2.9%
		12	1	0	0	0	0	0	0	1	0	0	0	20	1	0
		$N = 23$	4.3%							4.3%				**87.0%**	4.3%	
		13	0	0	0	0	0	0	0	4	0	1	0	0	50	0
		$N = 55$								7.3%		1.8%			**90.9%**	
	Hyla meridionalis	14	0	3	0	0	0	3	0	1	2	0	0	0	0	11
		$N = 20$		15.0%				15.0%		5.0%	10.0%					**55.0%**

OTU, operational taxonomic unit.

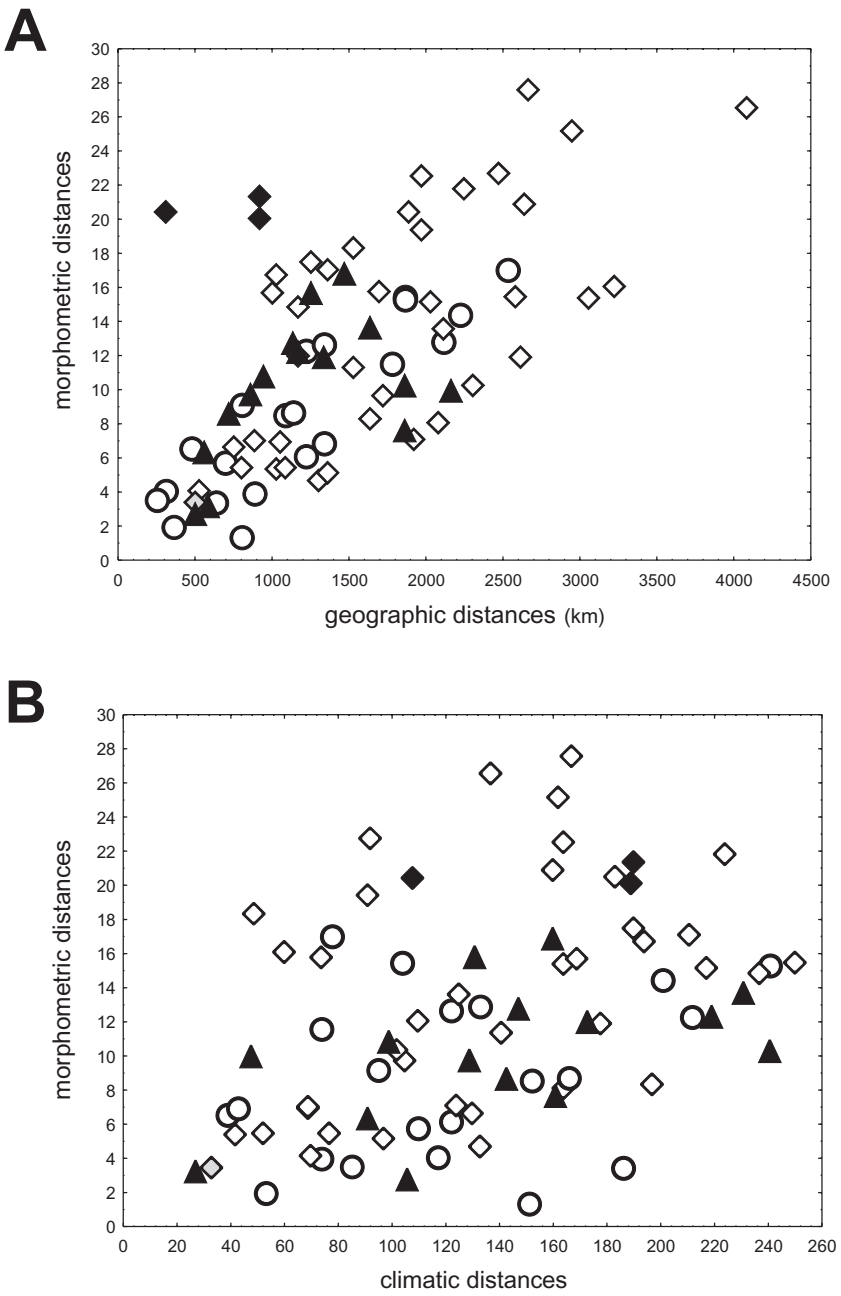

Figure 5. Morphometric distances among operational taxonomic units (OTUs) plotted against (A) geographic and (B) climatic distances. Triangles, distances within *Hyla arborea*; circles, distances within *Hyla savignyi*; rhomboids, distances between OTUs of *H. arborea* versus *H. savignyi* (black rhomboid, Caucasian *H. arborea* versus Transcaucasian, Iranian and Mesopotamian *H. savignyi*; grey rhomboid, Anatolian *H. arborea* versus Anatolian *H. savignyi*).

climatic factors (temperature and precipitation) even after statistical control of the covariation between climate and geography. It suggests that climatic conditions were an important factor in the formation of morphological variation. It appears that they lead to dissimilar morphotypes in populations living in areas with outermost climatic conditions, but to similar body shape in populations of both species occupying areas with comparable climate.

Most remarkably for the present study, *H. arborea* and *H. savignyi* are more or less undistinguishable in body shape in southern Anatolia, but substantially differ in the Caucasus regions, despite their ranges are in contact in both areas. The Anatolian

populations thus appear to support the scenario of climate-driven geographic morphological variation, whereas the Caucasian populations appear to ostensibly contradict it. This apparent discrepancy could be explained by the very diverse patterns of coexistence of tree frogs in these two zones of parapatry. *H. arborea* and *H. savignyi* inhabit similar habitats on the southern coast of Anatolia (Schneider, 2001; V. Gvoždík, unpubl. data), and they hence face comparable environmental conditions there. On the other hand, the high morphological distance between the populations of *H. savignyi* from Transcaucasia and *H. arborea* from the Caucasus area (but short geographic distance, approximately 300 km in average; Fig. 5A) could reflect substantial ecological vicariance of both species in this second zone of parapatry. In accordance with what we can infer from their ecophysiological differences (*H. savignyi* possess on average 1 °C higher tissue thermal resistance; Egiasarian & Andronnikov, 1986), in the Caucasus, *H. arborea* inhabits relatively humid and cold localities generally placed in the higher elevations, whereas *H. savignyi* prefers drier and warmer places (Alekperov, 1978; Egiasarian & Schneider, 1990, 1991; Kuzmin, 1999; Tarkhnishvili & Gokhelashvili, 1999). We can conclude that the two species of tree frogs occupy fairly different environmental space in the Caucasian area, and their morphological divergence thus supports, rather than denies, the hypothesis of climate-driven morphological variation.

The results obtained by Kaya (2001) on morphometric differences between the populations of *H. arborea* from the northeastern part of Turkey and the other Anatolian populations of both *H. arborea* and *H. savignyi* fit the hypothesis as well. Environmental conditions of north-eastern Turkey (Ponto-Caspian zoogeographic area) are different from those in southern Anatolia (Sindaco *et al.*, 2000). Tree frogs from the north-eastern part of Turkey used to be assigned as the Caucasian subspecies *H. a. schelkownikowi* (Kuzmin, 1999). This taxon is extensively distant from the western Anatolian populations of *H. arborea* in body shape also in our analyses.

Thus, only the morphometric sovereignty of Kurdistano-Iranian (OTU 2) and Transcaucasian (OTU 7) populations of *H. savignyi* and their mutual similarity somewhat contradict the climate-provoked phenotypic variation in the tree frogs. The Iranian tree frogs occupy mostly highlands, the Transcaucasian populations are rather a lowland form. But we shall notice that, in contrast to the OTU 7, the OTU 2 has been composed relatively unnaturally. OTU 2 covers the extensive area with both high and low elevations. Almost half of the individuals are from the southern foot of the Zagros Mountains in Khuzestan Province from low elevations of approximately 300 m a.s.l. (Fig. 1; see also Appendix).

We ascribed the morphological similarity of southern Anatolian parapatric populations of *H. arborea* and *H. savignyi* to an equivalent response of both species to a shared environment. Alternatively, the similarity between species near the contact of their ranges could be explained by interspecific hybridization and subsequent introgression of morphotype-mediated genes (Grant & Grant, 2002). Nevertheless, this explanation is not likely to be applicable to *H. arborea* and *H. savignyi*. These species appear to be isolated by a strong reproductive barrier as a result of substantial differences in the species-specific advertisement call and, to our knowledge, no natural hybridization between them has ever been demonstrated despite several studies performed on tree frogs in this area. Molecular data, both mitochondrial and nuclear markers, further support the non-existence or maximal rarity of hybridization between *H. arborea* and *H. savignyi* near the Anatolian zone of parapatry. All the Anatolian specimens of either species determined by the characteristic colour pattern are well-nested within their respective species-specific molecular clade (V. Gvoždík J. Moravec, P. Kotlik, unpubl. data). Also, the hybridization hypothesis, as opposed to equivalent response to environmental conditions, cannot explain the morphological similarity of tree frogs of both species in the isolated populations from the Mediterranean area (Crete, Cyprus), or in the other populations with similar climate distant from the actual zone of parapatry (the Mediterranean coast of Levant).

Although the correlation between body shape and climatic variables among OTUs of *H. arborea* and *H. savignyi* indicates an important role of climatic conditions in the incitement of morphological variation in these taxa, many interpretations of our results suffer from the obvious problems of the correlational framework. Future experimental work (e.g. common environment or reciprocal transplant experiments in conjunction with quantitative genetics) may identify the evolutionary and ecological processes responsible for the observed matching between climate and morphology in examined tree frogs. Such experimental approaches could decide whether similar morphotypes in Mediterranean populations of *H. arborea* and *H. savignyi* living in more or less the same environmental conditions are caused by convergent evolution or shared phenotypically plastic response to environmental conditions. Nevertheless, the case of two species of tree frogs described in the present study suggests that climate-provoked morphological variation in closely-related parapatric species should be taken into account as a potential process complicating character displacement in morphology. On the other

hand, morphological diversification between closely-related species or their populations could be enhanced by habitat shifts resulting in the occupation of different environmental space as in the Caucasian and Transcaucasian tree frogs. Recent knowledge (Streelman & Danley, 2003) indicates that habitat shifts regularly precede morphological diversification during evolutionary events encompassing character displacements in morphology and adaptive radiations. Further studies should investigate how frequently the climate-driven variation can serve as an obstacle to morphological differentiation between close relatives and, conversely, how often the shifts in habitat are connected to changes in environmental space, which could potentially directly drive morphological differentiation and, consequently, facilitate coexistence after secondary contact.

Only after knowing the extent of the contribution of phenotypic plasticity versus genetic evolution to the variation in body shape among particular OTUs, and after estimation of the rate of morphological evolution driven by climatic conditions, will we be able to judge, whether our phenetic trees contain also certain information on the biogeography of the populations within the studied species. Because the morphology in anurans is particularly well-known to be highly sensitive to environmental conditions (Emerson, 1986; Emerson, Travis & Blouin, 1988; Blouin & Loeb, 1991; Castellano & Giacoma, 1998; Blaustein *et al.*, 1999; Castellano, Giacoma & Dujsebayeva, 2000; Rosso *et al.*, 2004; Schäuble, 2004; Kutrup *et al.*, 2006; Lougheed *et al.*, 2006; Lind & Johansson, 2007), the biogeographic signal in morphometric data in tree frogs can be completely concealed by the plastic response to environmental conditions or rapid climate-provoked evolutionary changes. Several lines of indirect evidence suggest that it is indeed the case. First, as we have documented, some populations of the Middle Eastern and south-eastern European tree frogs belonging to different species have probably been genetically isolated for a few millions years, but are almost uniform in body shape. *Hyla savignyi* has been specified as an Irano-Turanian zoogeographical element with later Mediterranean penetration (Bodenheimer, 1944), whereas *H. arborea* occurs across almost whole Europe from west to east, with Anatolian populations on the eastern distributional margin. Accordingly, the populations of both species in southern Anatolia are probably younger than the populations in the middle of specific ranges, but possess a very similar body shape. Their body shape is also similar to the Canarian population of *H. meridionalis*, which is bioacoustically and genetically more distant (Schneider, 1974, 2004; Smith *et al.*, 2005). Next, the colonization of the Arabian Peninsula by *H. savignyi* is probably more recent than 5 Mya because, until this period Arabia was connected to Eastern Africa (Braithwaite, 1987) where tree frogs or their fossils are completely absent (Duellman, 1977; Sanchiz, 1998). The isolation of the Arabian population restricted to the Asir Mountains in south-western Arabia started approximately only 5000–6000 years ago, when a period of aridization began in the Middle East (Klütsch *et al.*, 2004). A rather recent isolation is supported by low immunological divergence between the Saudi Arabian and Israeli tree frogs and no immunological distinction between Yemeni and Israeli populations of *H. savignyi* (Riehl *et al.*, 1995). The substantial morphological distinction of the Arabian population thus presumably mirrors the extraordinary environmental conditions of this climatically extreme part of the species area.

The independence of genetic and morphological variation is relatively well known in frogs. In some cases, morphology is 'conserved' whereas genetic evolution goes forward (Borkin *et al.*, 2004; Camargo, de Sá & Heyer, 2006). For example, in the Far East, *Hyla japonica* Günther, [1859] '1858' and *Hyla suweonensis* Kuramoto, 1980 are morphologically very similar but genetically distant (Kuramoto, 1980; Lee *et al.*, 1999). In other cases, a significant geographic morphological variation exists but does not match genetic relationships among populations. For example, Nevo & Yang (1979) demonstrated the independence of genetic and morphological variation in Israeli populations of *H. savignyi* and, likewise, Kyriakopoulou-Sklavounou, Karakousis & Alexiou (1992) and Kyriakopoulou-Sklavounou (2000) demonstrated the same in Greek populations of *H. arborea*. Thus, within the species complex of the tree frogs, it is impossible to put definitive taxonomic implications and biogeographic scenarios based solely on the external morphology. An investigation of variation of bioacoustic parameters and mainly molecular markers using phylogeographic methods will be necessary to uncover taxonomy and phylogenetic relationships of the tree frogs' population systems.

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APPENDIX

Material examined

Hyla savignyi: **OTU 1 – ARABIAN PENINSULA: Yemen:** 1. 130 km S of Sana'a (14°13′N; 44°16′E) – 1 ex. (ZFMK 32272); 2. 31 km from Sana'a in direction to Hodeida (15°11′N; 43°59′E) – 4 ex. (ZFMK 42847-42849, 42852); 3. Sana'a (15°21′N; 44°12′E) – 4 ex. (ZMH A4130-A4131, ZFMK 37039-37040); 4. Shibam (15°31′N; 43°54′E) – 1 ex. (ZFMK 43108); **Saudi Arabia:** 5. Wadi Mahra 1 (19°38′N; 42°38′E) – 2 ex. (CAS 145328, 145347); 6. Wadi Mahra 2 (19°38′N; 41°54′E) – 3 ex. (CAS 145320-145322); 7. Barahard (20°21′N; 41°15′E) – 12 ex. (CAS 136516-136527); 8. Wadi Amagk (21°21′N; 40°18′E) – 2 ex. (CAS 139732-139733). **OTU 2 – IRANIAN HIGHLANDS AND KURDISTAN: Iran:** 9. Shapur (29°46′N; 51°34′E) – 2 ex. (ZMUC R1426, R1428); 10. Masjid-i-Suleiman (31°57′N; 49°16′E) – 3 ex. (CAS 86286, 86355-86356); 11. 15 km N of Masjid-i-Suleiman (32°04′N; 49°09′E) – 5 ex. (CAS 86296-86300); 12. 17 km N of the road Lali – Masjid-i-Suleiman (32°02′N; 49°13′E) – 1 ex. (CAS 86312); 13. Zeloi (32°13′N; 49°04′E) – 7 ex. (CAS 86253-86255, 86257-86260); 14. Lali (32°20′N; 49°06′E) – 2 ex. (CAS 86304-86305); 15. Dum – Dum (32°55′N; 48°46′E) – 2 ex. (ZMUC R1424-R1425); 16. Shabazan (32°47′N; 48°39′E) – 3 ex. (ZMUC R1429-R1431); 17. Äzna (33°27′N; 49°27′E) – 1 ex. (ZMUC R1432); 18. Ghom (34°36′N; 50°45′E) – 4 ex. (NMP P6V 34538/1-4); 'Persia' – 3 ex. (ZMB 4284, 63378-63379); **Turkey:** 19. Hakkari (37°34′N; 43°44′E) – 8 ex. (NMP P6V 70790/1-8). **OTU 3 – MESOPOTAMIA: Iran:** 20. 10 km E of the cross of the roads Andimeshk – Ahvaz – Shush, in the direction to the Ab-e Dez River (32°12′N; 48°23′E) – 1 ex. (CAS 141117); **Iraq:** 21. Euphrate River between Garma and Khor Hamor, Basra (30°57′N; 47°17′E) – 1 ex. (ZMB 43457); 22. Baghdad (33°20′N; 44°24′E) – 5 ex. (CAS 159967, NMP P6V 71117/1-4); 'Central Mesopotamia' – 1 ex. (ZMB 31601); **Syria:** 23. Ayyash, Deir ez Zur (35°26′N; 40°02′E) – 3 ex. (NMP P6V 71365/1-3); 24. 2 km S of Al Ghazi, Raqqa (36°20′N; 39°05′E) – 8 ex. (NMP P6V 71364/1-8); **Turkey:** 25. Birecik (37°02′N; 37°59′E) – 1 ex. (ZFMK 14053). **OTU 4 – LEVANT: Israel:** 26. Sarona, Jaffa (32°04′N; 34°47′E) – 1 ex. (ZMB 32186); 27. Tantura (= Dor), Ramot Hasharim (32°37′N; 34°55′E) – 1 ex. (ZMH A04126); 28. Tabgha, Tiberias (32°52′N; 35°33′E) – 1 ex. (ZMB 31743); **Lebanon:** 29. Ammik (33°43′N; 35°46′E) – 1 ex. (CAS 159047); 30. Beirut (33°52′N; 35°31′E) – 1 ex. (ZMH A04127); 31. Bsharri (34°15′N; 36°06′E) – 1 ex. (NMP P6V 70517); 'Kadisha Valley' – 1 ex. (ZFMK 60939); 'Lebanon' – 3 ex. (ZMB 3134-3136); **Syria:** 32. Al Hamidiyah (34°43′N; 35°56′E) – 22 ex. (NHMC 80.2.23.31-80.2.23.52); 33. Homs (34°44′N; 36°43′E) – 3 ex. (MZLU L955/3063-(1-3)); 34. 5 km S of Safita, Tartus District (34°50′N; 36°10′E) – 1 ex. (NMP P6V 71367); 35. 1,5 km S of Baniyas (35°11′N; 35°57′E) – 1 ex. (NMP P6V 34733); 36. Lattakia (35°31′N; 35°46′E) – 3 ex. (NHMC 80.2.23.6-80.2.23.8); 37. Al Haffah, 40 km E of Lattakia (35°35′N; 36°02′E) – 1 ex. (NHMC 80.2.23.9); 38. Maquam Assayedh, 20 km N of Lattakia (35°42′N; 35°53′E) – 21 ex. (NHMC 80.2.23.10-80.2.23.30); 39. NW of Rabi'ah, Lattakia (35°49′N; 36°02′E) – 3 ex. (NMP P6V 70713/1-3); 40. Idlib (35°56′N; 36°38′E) – 4

ex. (NMP P6V 34732/1-4); **Turkey:** 41. Kilis (36°43′N; 37°07′E) – 2 ex. (NMP P6V 70775/1-2); 42. Sendschirli (= Zencirli) (37°08′N; 36°40′E) – 7 ex. (ZMB 11034, 14464, 22487, 63385-63388). **OTU 5 – CYPRUS:** 43. Larnaca (34°55′N; 33°38′E) – 2 ex. (MTKD D18594, D18597); 44. Lefka (35°07′N; 32°51′E) – 2 ex. (MHNG 1393.81-82); 45. Famagusta (35°08′N; 33°57′E) – 27 ex. (NMP P6V 71570/1-16, 72539/1-2, ZFMK 14399-14407); 46. Sınırüstü (35°16′N; 33°51′E) – 4 ex. (NMP P6V 71571/1-4); 47. Yalı (35°24′N; 33°45′E) – 14 ex. (NMP P6V 71572/1-7, 71572/9-15); 'Cyprus' – 16 ex. (ZMB 11698, 18142, 63389-63392, 63394-63396, 63398-63402, ZMH A04132-A04133). **OTU 6 – SOUTHERN ANATOLIA: Turkey:** 48. Anamur (36°05′N; 32°50′E) – 20 ex. (NMP P6V 71573/1-3, 71574/1-9, 72537/1-8); 49. Bozyazı (36°06′N; 32°58′E) – 1 ex. (NMP P6V 72538); 50. Kurtuluş (36°20′N; 34°00′E) – 1 ex. (NMP P6V 71575/1); 51. Lamaskalesi (36°34′N; 34°15′E) – 9 ex. (NMP P6V 70795/1-9); 52. Fundukbunar (= Findikpinari), Taurus Mts. (36°55′N; 34°22′E) – 1 ex. (ZMB 32227); 53. Mersin (36°44′N; 34°39′E) – 2 ex. (ZMB 21265, MTKD D25225); 54. Kazanlı (36°50′N; 34°45′E) – 3 ex. (NMP P6V 71576/1-3); 55. Karataş (36°34′N; 35°23′E) – 6 ex. (NMP P6V 70772/1-3, 70772/6, 70774/1-2); 56. Adana (37°01′N; 35°20′E) – 4 ex. (NMP P6V 70776/1-4); 57. between Adana and Ceyhan (36°57′N; 35°36′E) – 1 ex. (ZFMK 7703); 58. Yilanlikale (37°04′N; 35°44′E) – 1 ex. (ZFMK 48062); 59. Karatepe (37°17′N; 36°13′E) – 1 ex. (ZMH A03038); 60. Osmaniye (37°04′N; 36°15′E) – 2 ex. (NMW 18536:3-4); 61. Dörtyol (36°51′N; 36°13′E) – 2 ex. (CAS 105299-105300); 62. Sariseki (36°40′N; 36°13′E) – 5 ex. (CAS 105307-105308, 105317-105319); 63. Kahramanmaraş (37°35′N; 36°56′E) – 2 ex. (NMW 18537:14, 18537:33). **OTU 7 – TRANSCAUCASIA: Azerbaijan:** 64. Mingaçevir (40°46′N; 47°03′E) – 12 ex. (NMP P6V 70773/1-6, 70777/1-2, 70778/1-2, 70792/1-2). **Not assigned to any OTU: Turkey:** 65. Kemaliye (39°16′N; 38°29′E) – 1 ex. (ZMH A03021); 'Turkey' – 1 ex. (MTKD D33909).

***Hyla arborea*: OTU 8 – CAUCASUS REGION (*H. arborea schelkownikowi*): Armenia:** 66. Kuybyshev, Stepanavan (**topotypes**) (41°01′N; 44°17′E) – 23 ex. (MTKD D12058-D12067, D12070, D12073-D12074, D12267-D12269, D12272, NMW 24779:1-3, ZFMK 18721-18723); **Azerbaijan:** 67. Nukadi, Kara-Çaj River (41°19′N; 48°35′E) – 3 ex. (ZFMK 39104); **Georgia:** 68. Akhaldaba (41°55′N; 43°29′E) – 2 ex. (ZFMK 70385-70386); 69. Pizunda (43°09′N; 40°21′E) – 1 ex. (ZFMK 38378); **Russia:** 70. Malenkij Sakhray River, Republic of Adygeya (44°02′N; 40°18′E) – 2 ex. (ZMB 57374, 57379); 71. Pjatigorsk (44°03′N; 43°04′E) – 1 ex. (ZMB 18960); 72. Groznyj (43°19′N; 45°42′E) – 3 ex. (NMW 14868:1-3); **Turkey:** 73. Pazar (41°11′N; 40°53′E) – 4 ex. (CAS 105577-105580). **OTU 9 – WESTERN ANATOLIA AND ADJACENT ISLANDS: Turkey:** 74. Gazipaşa (36°16′N; 32°19′E) – 4 ex. (NMP P6V 72536/1-4); 75. Syedra, 15 km SE of Alanya (36°29′N; 32°07′E) – 2 ex. (NMP P6V 72535/1-2); 76. Beyşehir (37°41′N; 31°44′E) – 2 ex. (NMP P6V 33320/1, 33320/3); 77. Mahmatlar (37°56′N; 30°56′E) – 1 ex. (NMP P6V 70793); 78. Kütahya (39°25′N; 29°59′E) – 1 ex. (MHNG 908.34); 79. Bileçik (40°09′N; 29°59′E) – 2 ex. (NMP P6V 70789/1-2); 80. Nicaea Lake, Iznik (40°26′N; 29°43′E) – 2 ex. (ZMH A03029, A03030); 81. 10 km SW of Ağva (41°06′N; 29°48′E) – 1 ex. (ZFMK 56746); 82. Troy, Bursa (39°56′N; 26°16′E) – 1 ex. (ZFMK 16331); 83. Selçuk – Ephesos (37°57′N; 27°22′E) – 8 ex. (NMP P6V 71098/1-4, 72534/1-3, ZFMK 56677); **Greece:** 84. Chios Is. (38°22′N; 26°08′E) – 8 ex. (NMP P6V 70794/1-8); 85. Maritsa, Rhodes Is. (36°26′N; 28°13′E) – 2 ex. (NHMC 80.2.7.2, 80.2.7.3). **OTU 10 – CRETE (*H. arborea kretensis*, syntypes in bold):** 86. Chania (35°31′N; 24°01′E) – 7 ex. (**NMW 18413:1-4**, **ZMB 31575**, **63407**, NHMC 80.2.7.4); 87. Almiros (35°27′N; 24°12′E) – 1 ex. (NMP P6V 34173); 88. Skopelos, Panormos (35°25′N; 24°42′E) – 1 ex. (ZFMK 61476); 89. Kapetaniana, Asterousia Mts. (34°58′N; 25°02′E) – 1 ex. (NMW 5833:5); 90. Lasithi Plateau (35°11′N; 25°36′E) – 11 ex. (NHMC 80.2.7.5-80.2.7.8, 80.2.7.12, ZFMK 18697-18700, NMP P6V 70780/2, **NMW 18413:5**); 'Crete' – 27 ex. (**ZMB 31569**, **63408-63430**, **63432-63434**). **OTU 11 – BALKANS: Greece:** 91. 8 km S of Monemvassia, Peloponnese (36°41′N; 23°03′E) – 1 ex. (ZFMK 40803); 92. 7 km E of Kalon Neron, Peloponnese (37°17′N; 21°42′E) – 3 ex. (ZMB 49823-49824, MTKD D33150); 93. Gortys, Peloponnese (37°33′N; 22°03′E) – 1 ex. (NMW 29159:5); 94. Argos, Peloponnese (37°38′N; 22°44′E) – 2 ex. (NMP P6V 70796/1-2); 95. Athens (37°59′N; 23°44′E) – 1 ex. (ZMUC R14500); 96. between Arta and Salaora (39°05′N; 20°55′E) – 1 ex. (MHNG 1010.65); 97. Platamon (40°02′N; 22°28′E) – 3 ex. (ZFMK 37703, ZMB 49825-49826); 98. SE of Zakynthos Is. (37°47′N; 20°54′E) – 1 ex. (ZFMK 27186); 99. Karystos, Evia Is. (38°01′N; 24°25′E) – 1 ex. (ZMB 13511); 100. Levkas, Levkas Is. (38°50′N; 20°42′E) – 3 ex. (MHNG 1186.53-55); 101. Skiathos Is. (39°10′N; 23°29′E) – 1 ex. (ZFMK 24082); 102. Corfu Is. (39°37′N; 19°53′E) – 2 ex. (ZFMK 24081, ZMB 32332); **Albania:** 103. Durres (41°19′N; 19°27′E) – 1 ex. (MTKD D3131); **Serbia:** 104. Vranje (42°33′N; 21°54′E) – 1 ex. (ZMUC R14544); **Bulgaria:** 105. Dolno Spachevo (41°25′N; 23°23′E) – 1 ex. (NMP P6V 34163); 106. Primorsko (42°16′N; 27°46′E) – 3 ex. (NMP P6V 7669, 70771/1-2); **Turkey:** 107. Havsa, Edirne (41°33′N; 26°49′E) – 9 ex. (NMP P6V 72533/1-9). **OTU 12 – DANUBE DELTA: Romania:** 108. Tuldscha (= Dulcea)

(45°10′N; 28°48′E) – 1 ex. (NMW 5805:13); 109. Periplava (45°24′N; 29°32′E) – 1 ex. (NMP P6V 70783/1); 110. Caraorman (45°05′N; 29°24′E) – 21 ex. (NMP P6V 70784/1-20, 70781/4). **OTU 13 – CENTRAL EUROPE: Czech Republic:** 111. Kašperské Hory (49°09′N; 13°34′E) – 1 ex. (NMP P6V 70813); 112. Černá v Pošumaví (48°44′N; 14°07′E) – 1 ex. (NMP P6V 70820); 113. Veselí nad Lužnicí (49°11′N; 14°42′E) – 19 ex. (NMP P6V 70785/1-2, 70806, 70808, 70812/1-15); 114. Stráž nad Nežárkou (49°04′N; 14°54′E) – 2 ex. (NMP P6V 70804/1-2); 115. Praha (50°04′N; 14°24′E) – 2 ex. (NMP P6V 70807/1-2); 116. Říčany (49°59′N; 14°39′E) – 8 ex. (NMP P6V 33703/1-8); 117. Nymburk (50°11′N; 15°02′E) – 1 ex. (NMP P6V 33001); 118. Bítov (48°57′N; 15°43′E) – 2 ex. (NMP P6V 70786/1-2); 119. Třebíč (49°13′N; 15°53′E) – 2 ex. (NMP P6V 70816/1-2); 120. Ořešín, Brno (49°17′N; 16°36′E) – 1 ex. (NMP P6V 71523); **Slovakia:** 121. Prievidza (48°46′N; 18°38′E) – 1 ex. (NMP P6V 70814); 122. Kováčovské kopce (47°50′N; 18°44′E) – 1 ex. (NMP P6V 32457); **Ukraine:** 123. Uzhgorod (48°37′N; 22°18′E) – 5 ex. (NMP P6V 70805/1-5); 124. Mukachevo (48°27′N; 22°43′E) – 7 ex. (NMP P6V 70811/1-4, 6-8); 125. Irshava (48°19′N; 23°03′E) – 2 ex. (NMP P6V 70810/1-2). **Not assigned to any OTU: Croatia:** 126. Salona (43°33′N; 16°30′E) – 1 ex. (ZMB 22673); 127. Sinj (43°42′N; 16°38′E) – 1 ex. (NMP P6V 70823); 'Croatia' – 1 ex. (ZMB 23729); **Serbia:** 128. Morovic (45°00′N; 19°13′E) – 1 ex. (ZMUC R14545).

***Hyla meridionalis*: OTU 14 – CANARY ISLANDS:** Tenerife Is. (28°22′N; 16°43′W) – 20 ex. (NMW 5822, 5825:1-2, 5852:1-2, 5857:3, 5858:2, 5861:2, 5869:2, 5871:1-4, 5873:2, 5874:1-2, 5877:1-2, 5880:1-2).

Museum abbreviations: CAS, California Academy of Sciences, San Francisco, CA, USA; MHNG, Muséum d'Histoire Naturelle Genève, Switzerland; MTKD, Museum für Tierkunde Dresden, Germany; MZLU, Museum of Zoology Lund, Sweden; NHMC, Natural History Museum of Crete, Irakleio, Greece; NMP, National Museum Prague, Czech Republic; NMW, Naturhistorisches Museum Wien, Austria; ZFMK, Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; ZMB, Museum für Naturkunde Berlin, Germany; ZMH, Zoologisches Institut und Zoologisches Museum der Universität Hamburg, Germany; ZMUC, Zoological Museum, Copenhagen, Denmark.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representatives of three categories according to the types of inguinal colour pattern.
Figure S2. Distribution of three categories of inguinal colour patterns in *Hyla savignyi* and *Hyla arborea*.
Table S1. Average monthly temperature and monthly precipitation values for each single operational taxonomic unit of *Hyla savignyi* and *Hyla arborea*.
Table S2. Descriptive statistics of characters examined.
Table S3. Discriminant coefficients of classification functions.

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SUPPLEMENTARY MATERIAL

Gvoždík V, Moravec J, Kratochvíl L. Geographic morphological variation in parapatric Western Palearctic tree frogs, *Hyla arborea* and *Hyla savignyi*: Are related species similarly affected by climatic conditions? *Biological Journal of the Linnean Society*.

Figure S1. Representatives of three categories according to the types of inguinal colour pattern.

Figure S2. Distribution of three categories of inguinal colour patterns in *H. savignyi* and *H. arborea*.

Table S1. Average monthly temperature and monthly precipitation values for each single OTU of *H. savignyi* and *H. arborea*.

Table S2. Descriptive statistics of characters examined.

Table S3. Discriminant coefficients of classification functions.

Figure S1. Representatives of three categories according to the types of inguinal colour pattern: (1) *linea marginalis* continuously builds a regular inguinal loop, (2) spot(s) instead of the loop, or a thin loop is separated from *linea marginalis*, (3) loop or spot(s) are entirely absent. Photo: Václav Gvoždík

(1) *H. arborea*, Selçuk, western Anatolia, OTU 9
(2) *H. savignyi*, Anamur, southern Anatolia, OTU 6
(3) *H. savignyi*, Anamur, southern Anatolia, OTU 6

Figure S2. Distribution of three categories of inguinal colour patterns in *H. savignyi* and *H. arborea*. See text for more details.

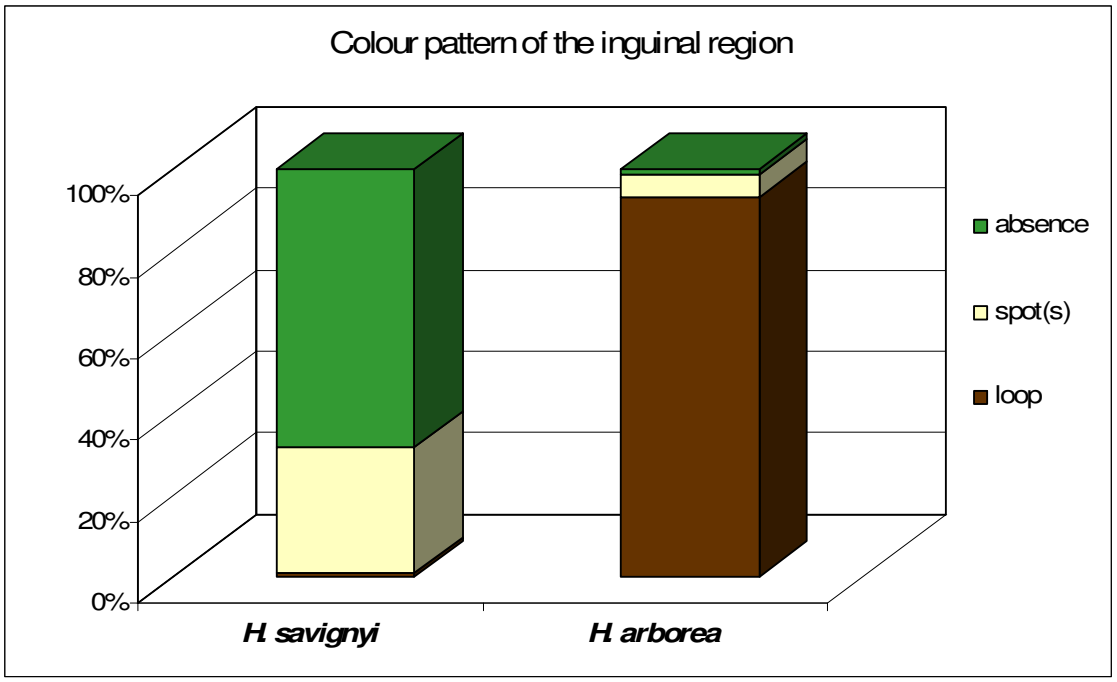

Table S1. Average monthly temperature and monthly precipitation values for each single OTU of *H. savignyi* and *H. arborea*, calculated as weighted means for each single OTU in respect to the number of individuals from each locality. These values were used for calculation of a climatic distance matrix.

		Hyla savignyi							*Hyla arborea*					
	OTU	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
temperature (°C)	January	14.5	5.2	8.1	9.2	11.3	8.6	3.1	-6.2	6.6	8.5	5.6	-0.1	-2.6
	February	16.1	7.1	10.1	10.3	11.6	9.5	4.4	-5.0	7.3	8.6	6.7	0.9	-0.6
	March	18.7	11.4	14.0	12.8	13.5	12.2	8.4	0.0	9.5	9.8	8.9	4.3	3.5
	April	20.2	16.8	19.3	16.4	17.0	15.9	15.0	7.0	13.3	12.8	12.5	10.3	8.5
	May	22.9	22.6	25.0	20.0	20.8	19.7	19.8	11.6	17.3	16.4	17.3	16.0	13.4
	June	25.2	27.4	29.7	23.4	24.8	23.7	24.0	15.4	21.4	20.4	21.3	20.2	16.5
	July	25.8	30.4	32.5	26.0	27.4	26.6	27.3	19.1	23.9	22.5	23.7	22.1	18.0
	August	25.4	29.7	31.8	26.4	27.4	26.8	26.1	18.8	23.7	22.4	23.5	21.8	17.5
	September	23.9	25.9	28.1	24.7	25.2	24.3	22.3	14.7	20.8	20.1	20.5	18.1	13.9
	October	19.8	19.9	22.0	20.7	21.5	19.9	15.8	8.3	16.3	16.6	15.7	12.8	8.8
	November	16.9	13.1	14.8	15.4	16.9	14.7	10.3	2.7	12.0	13.3	11.2	7.4	3.4
	December	14.8	7.4	9.6	11.0	13.0	10.4	5.4	-3.0	8.5	10.3	7.4	2.7	-0.7
precipitation (mm month^{-1})	January	3.4	64.6	36.5	137.8	67.7	120.4	16.6	39.9	109.2	166.5	81.2	19.5	33.0
	February	3.2	49.8	30.2	97.7	52.0	97.1	22.2	39.4	85.9	116.2	68.6	20.1	29.7
	March	16.6	48.9	30.0	83.9	37.9	72.3	26.2	47.6	66.6	94.0	59.0	15.9	33.7
	April	24.7	42.2	19.1	41.8	15.3	42.3	37.5	64.3	39.8	35.8	45.0	20.8	41.5
	May	19.0	18.0	6.9	17.5	8.5	26.6	51.5	93.5	25.8	14.4	35.7	28.0	65.8
	June	5.6	3.2	0.3	1.7	0.9	8.7	45.0	93.8	12.2	1.7	23.9	33.5	80.7
	July	19.2	0.9	0.0	0.2	0.0	2.5	21.8	63.7	5.4	0.1	13.2	26.6	70.9
	August	21.8	0.4	0.0	0.4	0.0	2.2	20.8	52.6	4.9	0.0	13.6	24.0	69.0
	September	10.2	0.7	0.1	2.4	0.6	6.1	18.5	50.1	10.5	4.5	25.8	25.3	43.8
	October	3.0	13.0	5.9	42.4	16.3	45.9	46.1	58.5	39.6	72.7	63.4	15.7	30.8
	November	7.3	27.3	17.0	75.6	35.7	74.9	24.6	49.2	71.9	80.2	91.7	22.1	40.4
	December	3.5	58.2	35.7	135.6	79.1	131.1	18.7	44.8	122.3	137.0	101.2	26.2	38.5

Table S2. Descriptive statistics of characters examined.

Loop = qualitative character, see text or Fig. S1 for code explanation; other characters are measurements in mm; mean ± SD / min. - max.; asterisk denotes bilateral characters measured on both sides and averaged.

	H y l a s a v i g n y i													
OTU	**1 - Arabian Peninsula**			**2 - Iran and Kurdistan**		**3 - Mesopotamia**		**4 - Levant**		**5 - Cyprus**		**6 - southern Anatolia**		**7 - Transcaucasia**	
sex	males (n = 3)	females (n = 9)	undetermined (n = 17)	males (n = 30)	females (n= 10)	males (n = 16)	females (n = 4)	males (n = 49)	females (n = 25)	males (n = 47)	females (n = 17)	males (n = 47)	females (n = 13)	males (n = 3)	females (n = 9)
Loop	2.3 2-3	2.7 2-3	2.4 2-3	3 2-3	3 3	2.7 2-3	2.8 2-3	2.6 2-3	2.4 1-3	2.7 2-3	2.8 2-3	2.6 1-3	2.5 2-3	3 3	3 3
SUL	36.2±1.4 34.6-37.4	41.5±2.4 37.6-44.0	37.6±2.4 34.8-42.3	36.8±3.0 32.2-45.4	40.7±3.0 37.0-45.8	37.9±3.8 33.1-44.5	41.8±5.1 36.6-48.6	37.9±2.5 33.3-43.4	41.7±3.3 33.7-48.3	33.5±2.0 29.0-37.3	36.5±2.4 32.5-39.8	37.4±2.8 29.0-42.4	40.1±3.2 34.7-45.7	37.2±3.6 33.1-39.6	40.3±2.9 34.0-43.2
FmL*	16.5±0.7 15.8-17.2	19.9±1.1 18.1-21.3	17.8±1.3 15.8-20.5	17.7±1.8 15.2-22.4	19.0±2.4 16.2-24.5	18.4±2.0 15.6-22.1	20.2±2.5 17.2-23.2	18.7±1.4 15.2-21.9	20.5±1.8 16.6-24.0	16.1±1.1 13.6-17.9	17.9±1.5 15.4-19.9	18.4±1.5 14.3-21.5	19.4±1.8 15.9-22.7	18.1±1.7 16.4-19.7	19.6±1.7 16.2-21.6
TbL*	16.5±0.7 16.0-17.3	20.2±1.1 18.2-21.2	18.3±1.7 15.7-21.7	17.8±1.8 15.5-22.4	19.2±2.3 16.5-24.6	18.8±2.0 15.9-22.3	20.8±2.0 18.4-23.2	18.9±1.3 15.1-22.2	20.7±1.8 17,6-23.9	16.3±1.1 13.7-18.5	18.1±1.4 15.8-20.1	18.7±1.5 14.6-21.9	19.6±1.8 16.3-23.0	18.2±1.4 16.6-19.4	19.8±1.5 16.7-21.5
WL*	8.6±0.4 8.1-8.9	10.0±0.5 8.9-10.6	9.1±0.8 7.8-10.4	9.0±0.9 7.7-11.4	9.8±1.0 8.5-11.6	9.4±1.1 8.0-11.6	10.5±0.8 9.9-11.7	9.6±0.8 7.5-11.8	10.6±1.0 8.3-12.5	8.1±0.6 6.8-9.5	8.8±0.8 7.5-9.9	9.3±0.9 7.3-11.3	10.1±0.9 8.7-11.9	9.6±1.0 8.5-10.4	10.0±0.6 8.8-10.6
T4L*	14.5±0.6 13.8-15.0	17.2±0.9 15.4-18.0	15.6±1.2 14.1-18.0	15.0±1.6 12.8-19.0	16.3±1.9 13.9-20.5	16.3±1.9 13.6-20.0	17.8±1.3 16.9-19.8	16.4±1.4 13.3-19.6	18.2±1.6 14.6-21.6	14.2±1.0 12.5-16.3	15.6±1.2 13.2-17.5	16.0±1.4 12.3-18.3	17.2±1.7 14.9-20.7	15.6±2.0 13.3-16.8	16.7±1.3 14.4-19.1
T1L*	3.9±0.1 3.8-4.0	4.8±0.2 4.5-5.3	4.5±0.4 4.0-5.3	4.0±0.5 3.2-5.4	4.4±0.6 3.6-5.6	4.5±0.5 3.7-5.3	4.8±0.2 4.7-5.1	4.4±0.4 3.7-5.7	4.8±0.5 3.8-5.6	3.8±0.3 2.8-4.5	4.1±0.4 3.6-4.8	4.3±0.4 3.1-5.4	4.7±0.4 4.2-5.4	4.4±0.4 4.0-4.7	4.7±0.4 4.0-5.3
IMTL*	1.8±0.2 1.7-2.0	2.0±0.3 1.7-2.7	1.8±0.1 1.5-2.0	1.8±0.2 1.5-2.5	2.0±0.2 1.6-2.4	1.9±0.3 1.5-2.5	2.1±0.2 1.9-2.4	1.8±0.2 1.5-2.3	1.9±0.2 1.4-2.3	1.6±0.1 1.3-2.0	1.8±0.2 1.6-2.2	1.8±0.2 1.4-2.4	1.9±0.2 1.5-2.3	1.8±0.2 1.6-2.0	2.0±0.3 1.7-2.6
TrL*	9.3±0.5 9.0-9.9	10.9±0.6 10.3-11.9	10.1±0.8 8.8-11.6	9.5±1.1 8.0-12.0	10.3±1.2 8.8-12.8	10.1±1.0 8.5-11.8	10.8±1.4 9.5-12.7	10.4±0.8 8.3-13.2	11.3±0.9 9.8-13.6	8.7±0.6 7.4-9.8	9.7±0.8 8.5-11.1	10.0±0.8 7.9-11.6	10.8±1.0 9.0-12.5	9.8±0.5 9.3-10.3	10.8±0.7 9.6-11.8
HW	12.9±0.4 12.4-13.2	15.1±0.9 13.7-16.2	13.4±0.9 12.2-15.3	12.7±1.4 10.9-16.3	13.6±1.3 12.0-16.1	12.9±1.4 11.3-15.4	14.3±1.7 12.5-16.5	13.0±1.0 11.0-15.4	14.2±1.1 11.0-15.9	11.4±0.7 9.7-12.7	12.5±1.1 10.7-14.5	12.6±1.1 9.5-15.1	13.6±1.1 11.7-15.4	13.2±1.1 12.0-14.0	14.1±1.1 11.7-15.1
HLt	10.6±0.5 10.2-11.2	12.2±0.6 11.1-12.9	11.0±0.7 9.4-12.1	10.4±0.8 9.1-12.7	11.3±0.9 10.0-12.8	10.6±1.2 8.9-12.4	11.8±1.5 10.3-13.9	10.5±0.7 9.2-12.3	11.4±0.9 9.2-13.7	9.5±0.5 8.3-10.8	10.2±0.7 9.1-11.5	10.3±0.9 7.5-12.1	11.2±0.9 9.5-13.1	10.7±1.4 9.1-11.6	11.3±0.7 10.0-12.4
ES	4.3±0.4 3.9-4.6	5.0±0.4 4.1-5.5	4.6±0.5 3.9-5.5	4.4±0.5 3.6-5.5	4.6±0.5 3.9-5.5	4.5±0.6 3.4-5.6	5.0±0.9 4.4-6.3	4.5±0.3 3.6-5.5	4.8±0.5 3.8-5.7	4.1±0.3 3.5-4.6	4.1±0.3 3.6-4.5	4.3±0.4 2.8-5.1	4.7±0.6 3.7-5.7	4.4±0.6 3.7-4.9	4.6±0.3 4.2-5.1
NL	3.2±0.2 3.0-3.3	3.7±0.3 3.3-4.1	3.2±0.3 2.6-3.7	2.9±0.2 2.6-3.5	3.2±0.3 2.8-3.7	3.0±0.3 2.6-3.5	3.2±0.3 2.8-3.6	2.9±0.3 2.4-3.4	3.2±0.3 2.6-3.9	2.7±0.2 2.3-3.0	3.0±0.2 2.4-3.4	2.9±0.2 2.3-3.4	3.1±0.3 2.7-3.7	3.0±0.4 2.5-3.3	3.2±0.3 2.8-3.5
IND	3.2±0.2 3.0-3.3	3.3±0.2 3.0-3.6	3.1±0.2 2.7-3.4	3.0±0.3 2.6-3.8	3.3±0.3 2.8-3.8	3.1±0.3 2.7-3.7	3.2±0.5 2.7-3.8	2.9±0.3 2.5-3.7	3.2±0.3 2.7-3.8	2.7±0.2 2.3-3.0	3.0±0.2 2.5-3.4	3.0±0.2 2.3-3.7	3.1±0.3 2.4-3.5	3.3±0.2 3.1-3.4	3.6±0.3 3.0-4.0
EAD	6.3±0.3 6.0-6.6	7.5±0.5 6.8-8.3	6.5±0.4 5.7-7.2	6.8±0.7 5.7-8.9	7.3±0.8 6.6-9.0	6.8±0.7 5.6-8.1	7.4±0.7 6.7-8.4	6.8±0.6 5.7-7.8	7.4±0.6 6.2-8.6	6.2±0.4 5.4-7.0	6.7±0.5 5.9-7.4	6.7±0.5 5.2-7.5	7.2±0.5 6.6-8.3	7.2±0.5 6.7-7.6	7.5±0.9 6.2-8.8
IOD	3.7±0.3 3.5-4.0	3.7±0.3 3.3-4.0	3.5±0.3 2.9-4.0	3.5±0.4 2.9-4.8	3.8±0.5 3.4-5.0	3.4±0.3 2.9-4.2	3.7±0.2 3.4-3.9	3.5±0.3 2.9-4.5	3.8±0.4 3.1-4.6	3.0±0.3 2.4-3.6	3.5±0.5 2.8-4.8	3.3±0.3 2.5-4.2	3.5±0.4 3.1-4.2	3.6±0.0 3.6-3.6	4.2±0.4 3.4-4.7
EPD	11.2±0.6 10.5-11.6	12.5±0.6 11.5-13.0	11.0±0.6 10.0-12.0	10.6±0.8 9.5-13.0	11.3±1.1 9.3-13.1	10.8±1.0 9.4-12.6	11.8±1.0 10.6-13.1	10.8±0.8 9.2-12.7	11.7±1.0 9.9-14.0	9.8±0.6 8.6-11.0	10.6±0.8 9.6-12.1	10.6±0.8 8.8-12.5	11.4±1.0 9.8-13.3	11.2±1.3 9.8-12.2	11.3±1.1 9.8-13.0
TD	2.4±0.3 2.1-2.6	2.8±0.1 2.6-3.0	2.4±0.2 2.0-2.9	2.3±0.2 1.9-2.9	2.4±0.2 2.2-2.8	2.5±0.4 1.8-3.2	2.7±0.5 2.1-3.4	2.3±0.2 1.8-3.0	2.6±0.3 2.0-3.0	2.0±0.2 1.6-2.3	2.2±0.2 1.8-2.6	2.3±0.2 1.9-3.2	2.6±0.3 2.3-3.3	2.4±0.2 2.1-2.5	2.7±0.2 2.3-3.1

	H y l a a r b o r e a													***Hyla meridionalis***
OTU	**8 - Caucasus**			**9 - western Anatolia**		**10 - Crete**		**11 - Balkans**		**12 - Danube Delta**		**13 - Central Europe**		**14 - Tenerife Is.**
sex	males (n = 31)	females (n = 2)	undetermined (n = 6)	males (n = 25)	females (n = 7)	males (n = 33)	females (n = 13)	males (n = 18)	females (n = 13)	males (n = 21)	females (n = 2)	males (n = 41)	females (n = 13)	males (n = 20)
Loop	1.1 1-2	1 1	1 1	1 1	1 1	1.1 1-2	1.2 1-3	1.1 1-2	1.2 1-3	1.1 1-2	2 1-3	1 1-2	1.1 1-2	- -
SUL	38.6±2.2 33.0-41.9	35.8±2.8 33.8-37.7	39.5±1.1 38.3-41.5	37.0±2.4 32.6-42.4	36.5±2.2 33.5-39.0	35.2±2.5 29.9-39.6	37.8±4.2 32.1-45.8	35.7±3.2 29.1-42.0	37.5±4.0 29.3-42.2	29.2±2.8 26.0-38.5	32.4±5.2 28.7-36.0	36.9±3.0 29.2-43.6	37.7±2.7 33.4-41.2	36.1±2.2 31.1-40.5
FmL*	19.5±1.3 16.7-22.0	17.6±0.8 17.0-18.2	20.2±0.7 19.6-21.2	18.5±1.7 15.8-22.1	18.0±1.2 15.9-19.3	17.1±1.4 13.7-19.4	18.2±2.3 14.9-21.8	17.7±1.7 14.4-20.5	19.2±1.9 15.2-22.1	14.2±1.4 12.4-18.9	16.2±1.9 14.8-17.6	17.8±1.5 14.8-20.6	18.5±1.7 15.5-21.2	17.2±1.3 15.2-19.7
TbL*	19.1±1.2 16.8-22.4	17.6±0.2 17.5-17.8	19.4±0.9 18.5-20.6	18.7±1.8 15.8-23.2	18.2±1.1 16.1-19.1	17.3±1.4 14.4-20.9	18.3±2.1 14.9-21.6	17.9±1.9 14.3-21.4	19.2±1.8 15.3-22.2	14.2±1.4 12.4-18.8	15.9±1.6 14.8-17.1	17.5±1.5 14.8-20.6	18.2±1.5 15.6-20.1	17.9±1.3 15.7-20.4
WL*	10.3±0.8 8.8-12.8	9.5±0.3 9.3-9.7	10.6±0.5 9.9-11.0	9.3±1.1 7.8-12.2	9.0±0.8 8.0-10.2	9.1±0.9 7.8-11.1	9.7±1.5 7,6-12.3	9.4±1.2 7.3-11.6	10.0±1.1 7.7-11.8	7.3±0.9 6.4-10.3	8.6±1.4 7.6-9.6	10.1±0.9 8.0-11.8	10.4±0.9 8.9-12.1	9.1±0.7 7.3-10.1
T4L*	17.5±1.2 15.0-20.4	15.8±0.7 15.3-16.3	17.7±0.6 17.1-18.5	16.1±1.6 13.4-19.8	15.5±1.1 13.9-17.0	15.3±1.4 12.4-17.7	16.3±2.4 13.5-21.0	15.6±1.7 12.2-18.5	16.8±1.6 13.3-19.0	12.8±1.4 11.0-17.5	14.6±1.8 13.3-15.9	16.8±1.4 13.4-20.0	17.4±1.4 15.1-19.8	15.5±1.2 13.1-17.8
T1L*	4.7±0.3 3.9-5.3	3.8±0.1 3.8-3.9	4.9±0.2 4.5-5.2	4.4±0.4 3.6-5.3	4.3±0.3 3.8-4.7	3.9±0.4 3.2-4.8	4.2±0.7 3.5-5.3	4.2±0.5 3.3-5.2	4.5±0.5 3.4-5.2	3.4±0.4 2.7-4.6	3.8±0.6 3.4-4.3	4.5±0.4 3.6-5.3	4.8±0.4 4.1-5.4	4.1±0.4 3.2-4.8
IMTL*	2.1±0.2 1.5-2.4	2.0±0.1 2.0-2.1	2.1±0.1 1.9-2.3	1.9±0.3 1.5-2.6	1.8±0.3 1.5-2.2	1.8±0.2 1.4-2.0	1.8±0.3 1.5-2.4	1.8±0.2 1.5-2.1	2.0±0.2 1.7-2.5	1.5±0.2 1.4-2.3	1.8±0.3 1.6-2.0	2.0±0.2 1.7-2.5	2.2±0.2 1.9-2.5	1.9±0.1 1.7-2.1
TrL*	10.8±0.7 9.3-12.2	10.1±0.4 9.8-10.3	11.1±0.6 10.5-12.0	10.2±0.9 8.8-12.2	10.0±0.6 9.2-10.7	9.4±0.7 8.1-10.7	9.9±1.2 7.9-11.8	9.7±1.0 7.9-11.0	10.5±1.0 8.3-11.6	7.9±0.8 6.8-10.8	9.2±1.2 8.4-10.1	9.8±0.9 8.0-11.4	10.2±0.9 8.8-11.8	9.8±0.7 8.6-11.5
HW	14.0±0.9 11.8-15.6	12.6±0.6 12.1-13.0	14.2±0.6 13.3-15.1	12.9±1.1 10.5-15.0	12.5±0.8 11.2-13.5	12.4±0.9 10.2-14.0	13.0±1.6 11.0-15.7	12.5±1.1 10.9-14.9	13.2±1.5 10.3-15.3	10.4±1.1 9.1-14.3	11.3±1.6 10.1-12.4	13.4±0.9 11.3-15.0	13.4±1.1 11.8-14.8	12.2±0.6 10.8-13.4
HLt	10.3±0.6 9.3-11.6	9.9±0.4 9.6-10.2	10.1±0.6 9.4-11.0	10.0±0.7 8.5-11.2	10.0±0.6 9.3-10.7	9.7±0.7 8.4-11.0	10.0±0.9 8.5-11.5	9.8±1.0 8.0-11.5	10.2±1.0 8.3-11.3	8.4±0.8 7.2-11.0	8.7±0.8 8.1-9.2	10.1±0.6 9.0-11.8	10.3±0.8 9.2-11.9	10.2±0.5 9.0-11.2
ES	4.4±0.3 4.0-5.1	4.1±0.3 3.9-4.3	4.5±0.4 4.1-5.1	4.4±0.4 3.5-5.1	4.4±0.5 3.7-5.3	4.2±0.3 3.5-5.0	4.0±0.4 3.4-4.7	4.2±0.4 3.3-5.0	4.2±0.2 3.6-4.5	3.8±0.3 3.3-4.4	3.4±0.2 3.2-3.5	4.6±0.3 4.0-5.4	4.3±0.5 3.7-5.5	4.3±0.2 3.9-4.6
NL	3.0±0.2 2.6-3.6	3.1±0.0 3.1-3.1	3.0±0.2 2.7-3.4	3.1±0.3 2.7-3.6	2.9±0.4 2.4-3.3	2.9±0.2 2.3-3.3	3.0±0.3 2.7-3.6	3.0±0.2 2.7-3.6	3.1±0.3 2.6-3.5	2.7±0.2 2.4-3.4	2.9±0.1 2.8-3.0	3.3±0.2 2.8-3.8	3.3±0.2 3.1-3.8	3.0±0.1 2.7-3.2
IND	3.3±0.2 2.6-3.7	3.1±0.1 3.0-3.1	3.4±0.2 3.1-3.7	3.1±0.3 2.7-3.6	2.9±0.4 2.4-3.5	2.8±0.2 2.3-3.2	2.9±0.4 2.5-3.8	2.9±0.2 2.6-3.3	3.1±0.2 2.6-3.4	2.7±0.2 2.5-3.5	2.9±0.1 2.8-3.0	3.4±0.2 2.8-4.1	3.3±0.2 3.1-3.9	3.0±0.1 2.6-3.2
EAD	7.0±0.4 6.2-7.8	6.8±0.5 6.4-7.1	7.2±0.3 6.8-7.5	6.8±0.5 5.7-7.7	6.6±0.5 5.8-7.1	6.5±0.4 6.0-7.4	6.7±0.6 6.0-7.6	6.7±0.5 5.8-7.9	6.7±0.5 5.7-7.4	5.8±0.6 5.1-8.0	6.6±0.0 6.6-6.6	7.1±0.5 5.9-8.0	7.1±0.4 6.6-7.8	6.4±0.3 6.0-7.0
IOD	3.8±0.3 3.1-4.6	3.7±0.1 3.6-3.8	3.8±0.1 3.8-4.0	3.3±0.3 2.9-4.1	3.2±0.3 3.0-3.7	3.3±0.4 2.6-4.4	3.6±0.6 2.8-4.6	3.4±0.5 2.7-4.4	3.6±0.4 2.6-3.9	2.8±0.3 2.4-3.9	3.6±0.2 3.4-3.7	3.5±0.4 2.8-4.4	3.3±0.2 3.0-3.7	3.3±0.3 2.9-3.8
EPD	11.6±0.7 10.3-12.9	10.8±0.4 10.5-11.0	10.8±0.6 10.1-11.4	10.9±0.8 9.3-12.3	10.6±0.7 9.5-11.2	10.5±0.7 9.1-11.7	11.0±1.0 9.7-13.0	10.7±0.9 9.1-12.3	11.2±1.0 9.0-12.5	9.2±0.8 8.3-12.0	10.0±0.9 9.3-10.6	11.2±0.8 9.5-13.0	11.5±0.9 10.0-13.0	10.3±0.5 9.1-11.1
TD	2.3±0.2 2.0-2.9	2.2±0.4 1.9-2.5	2.3±0.1 2.2-2.3	2.3±0.2 1.9-2.7	2.2±0.3 1.8-2.6	2.1±0.2 1.8-2.5	2.2±0.3 1.8-2.8	2.2±0.3 1.7-2.8	2.3±0.2 1.9-2.7	1.9±0.2 1.7-2.7	2.1±0.1 2.0-2.1	2.4±0.2 1.9-2.9	2.5±0.2 2.1-3.0	2.2±0.2 1.9-2.7

Table S3. Discriminant coefficients of classification functions as summarized in the discriminant analysis. Highest and lowest values per character are given in bold. *H. m.* = *Hyla meridionalis*.

OTU	*Hyla savignyi*							*Hyla arborea*						*H. m.*
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	p = 0.052	p = 0.073	p = 0.036	p = 0.140	p = 0.116	p = 0.109	p = 0.021	p = 0.070	p = 0.061	p = 0.086	p = 0.061	p = 0.041	p = 0.098	p = 0.036
SUL/S	1930.1	**1976.3**	1952.8	1944.6	1958.9	1958.4	1950.4	1907.2	1934.1	1946.6	1915.2	**1886.2**	1899.6	1960.3
FmL/S	133.2	157.9	132.6	155.7	163.9	164.1	154.4	**218.8**	164.8	154.4	186.3	162.2	172.8	**91.7**
TbL/S	776.7	800.6	815.9	776.9	783.5	796.3	804.8	664	781.2	765.1	757.7	709.3	**658.3**	**830.7**
WL/S	-136.8	-88.2	-121.9	-112.3	**-142.6**	-108.5	-91.8	-92.8	-132	-84.1	-90.8	-140.3	**-75.5**	-103.2
T4L/S	778.1	**742.8**	797.1	814.9	**824.1**	798	754.6	814.8	788.5	802.6	792.8	817.7	820.3	795.6
T1L/S	**-176.8**	-212.8	-196.2	-209.3	-199.4	-200.1	-190.5	-208.1	-196.5	**-216.9**	-206.9	-214.7	-202.1	-209.9
IMTL/S	-99.5	-87.1	-93.5	-107.9	-93.4	-94.5	-85	-90.3	-95.6	-96.3	-92.5	**-108.5**	-95.2	**-78**
TrL/S	-453.9	**-502.9**	-496.4	-475.3	-484.9	-478.5	-481.3	**-442.3**	-462	-475.6	-462.3	-451.4	-459.3	-456.3
HW/S	**188.6**	142.6	127.4	135.2	132.4	128.5	154.7	170.3	141	165.9	144.2	136.1	159	**124.1**
HLt/S	**254.2**	239.5	229	224.2	237.1	231.7	237.2	**185.7**	199.4	207.2	207.9	187	186	247.7
NL/S	**-159.8**	-199.5	-197.1	-200.4	-187.4	-195.9	**-205.9**	-202.8	-183.8	-177.9	-177.5	-172.5	-182.5	-186.6
IND/S	78.6	92.9	79.9	70.3	83.4	91	**125.3**	90	87.3	**70.2**	76.9	87	88.8	94.8
EAD/S	**246.8**	289.1	278.8	288.6	291.3	290.9	**294.1**	270.8	275.9	290.2	279.2	273.9	282.3	269
IOD/S	19.8	23.4	11.9	15.7	19.1	10.7	**32**	22.4	9.1	23.4	20.8	10.7	**8.2**	17.7
EPD/S	**297.1**	**261.1**	271.7	270.4	282.2	273.6	264.8	293.2	287.1	288	293.9	295	282.7	272.6
TD/S	-44.4	-56.4	-44.9	-54.2	-59.4	-46.3	**-35.4**	**-65.9**	-57.3	-58.5	-55.1	-55.3	-51.5	-51.3
Constant	-2512.3	-2585.7	-2571.2	-2612.2	-2608.8	-2596	-2501.2	-2510	-2543.8	-2587.3	-2525.5	-2435.3	-2424.1	-2549.6

Kapitola III.

Second species of tree frog, *Hyla orientalis* (formely *H. arborea*), from Iran confirmed by acoustic data.

Gvoždík V.

Submitted manuscript.

Hyla orientalis, Motalla Sara-ye Lemir, Írán (Iran)
(= dříve/former *H. arborea gumilevskii*)

Second species of tree frog, *Hyla orientalis* (formely *H. arborea*), from Iran confirmed by acoustic data

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Abstract. Presence of the second tree frog species, *Hyla orientalis*, from Iran is confirmed based on advertisement call. According to available data, it seems that *H. orientalis* occupies the lowlands along the southern Caspian coast, and presumably also the eastern slopes of the Talesh Mts. and northern slopes of the Alborz Mts. The widely distributed *H. savignyi* is present west- and southward these mountain systems.

Keywords. *Hyla orientalis*; *Hyla arborea*; *Hyla savignyi*; distribution; Iran; bioacoustics.

For a long time, Iran had been expected to be occupied by the only tree frog species, *Hyla savignyi* Audouin, 1827 [“1809”] (Leviton et al., 1992; Baloutch and Kami, 1995). According to Cheatsazan et al. (2005), the species should be distributed in the northern and south-western part of the country. However, Litvinchuk et al. (2006) recently suggested that the northern part could be occupied by *Hyla arborea gumilevskii* Litvinchuk, Borkin, Rosanov, Skorinov, 2006, which they described from south-western Azerbaijan from the Talysh Mts. (cross-border mountains between Azerbaijan and Iran; usually spelled as the Talesh Mts. in Iran). The authors also listed 15 locality records from the literature and morphologically investigated 13 museum voucher individuals from Iran, which they assigned to *H. arborea gumilevskii*. However, Gvoždík et al. (2008) demonstrated that application of morphometric characters does not allow to distinguish properly between *H. arborea* and *H. savignyi*, and suggested the colour pattern of the inguinal region (i.e. inguinal loop present in *H. arborea*) to be more appropriate character for interspecific recognition. Nevertheless, *H. arborea gumilevskii* is characterized by a reduction or even absence of the inguinal loop (Litvinchuk et al., 2006), which makes it morphologically almost indistinguishable from *H. savignyi*. Thus, occurrence of *H. arborea* in Iran has been adopted only provisionally as a “presence uncertain” (Kaya et al., 2008; Schneider, 2009; Schneider and Grosse, 2009). Furthermore, recently, Stöck et al. (2008) split *H. arborea* into three species based on molecular data, and resurrected the name *Hyla orientalis* Bedriaga, 1890 for the eastern populations including *H. arborea gumilevskii*, which was thereby synonymized with *H. orientalis*. Herein, the occurrence of *H. orientalis* in Iran is confirmed based on species-specific acoustic data.

The recordings of tree frogs’ advertisement calls were obtained from four localities in Iran during short-term field survey in May-June 2005: (1) 12 km E of Qareh Ziya Eddin, 38.89° N, 45.02° E, 16.5 – 18.5 °C, n = 3; (2) 10 km W of Mianeh, 37.41° N, 47.72° E, 20.0 °C, n = 2; (3) Tonekabon, 36.81° N, 50.88° E, 21.0 °C, n = 2; (4) Motalla Sara-ye Lemir, 38.20° N, 48.87° E, 18.5 – 20.5 °C, n = 3 (Fig. 1A). Rice fields form tree frogs' habitats in all localities. The recordings were taken by Olympus DM-1 portable recorder with Sony ECM-MS907 electret condenser microphone, environmental temperature was measured by Viking

AB 06912 digital thermometer in the exact place occupied by the calling male. The calls were analyzed in BatSound – Sound Analysis 1.2 software (Pettersson Elektronik AB). Oscillograms and spectrograms were investigated, and call segment (pulse group sensu Schneider, 2004) length and number of pulses per segment were measured and counted, respectively. Averaged values were taken from five consecutive call segments from the middle of the call.

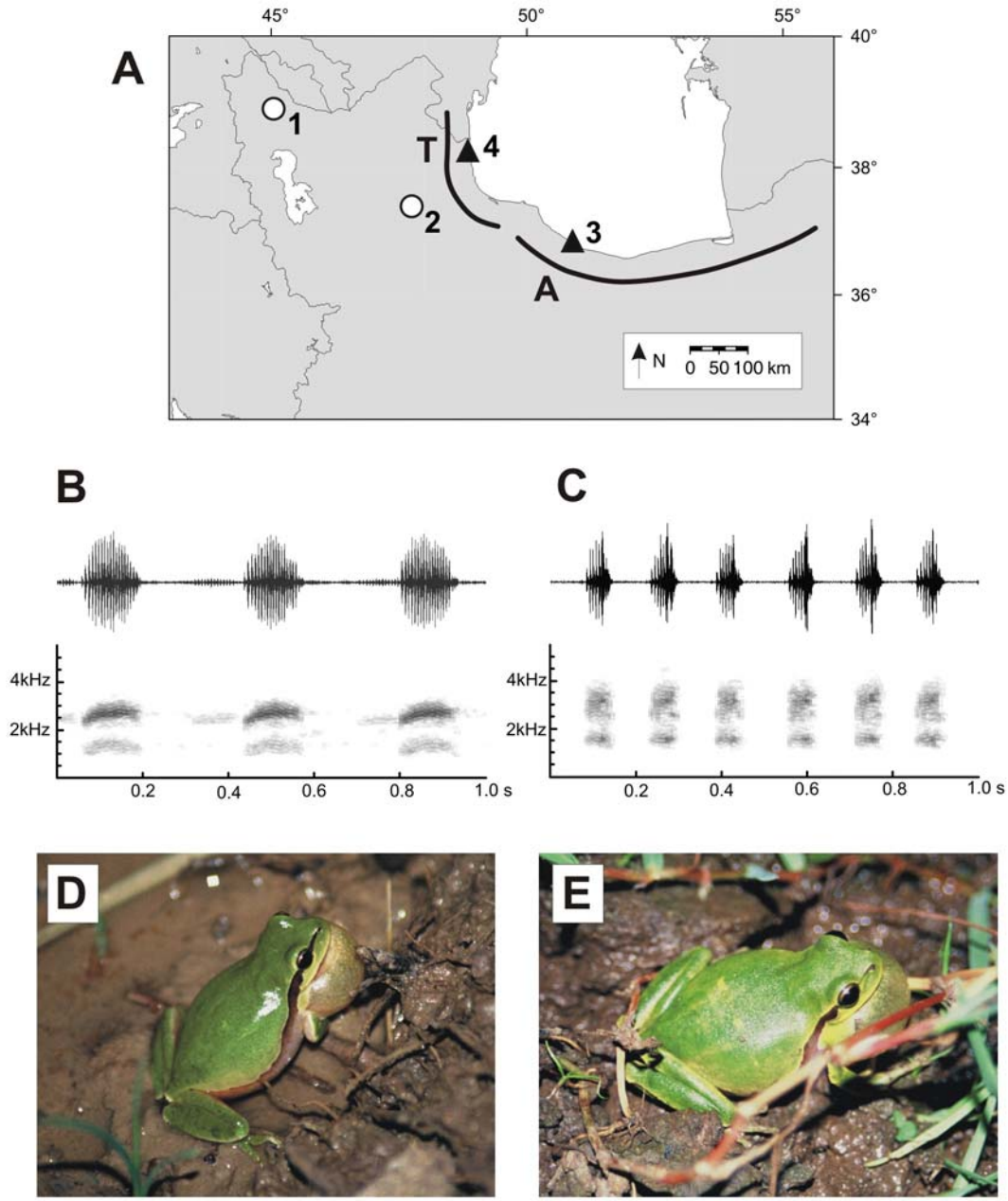

Fig. 1. (A) Map of north-western Iran showing localities where recordings of advertisement calls were taken, *H. savignyi* (circles), *H. orientalis* (triangles), Talesh Mts. (T) and Alborz Mts. (A). Numbers correspond to the list in the text. Advertisement calls of (B) *H. savignyi* (12 km E of Qareh Ziya Eddin, loc. 1, 16.5 °C) and (C) *H. orientalis* (Tonekabon, loc. 3, 21.0 °C) as represented by oscillograms and respective spectrograms of the call segments (pulse groups) at 1 s sections. An alternating male is evident in-between the call segments of *H. savignyi* in the background. Photographs of calling males of (D) *H. savignyi* (10 km W of Mianeh, loc. 2) and (E) *H. orientalis* (Motalla Sara-ye Lemir, loc. 4) demonstrate that the two species are morphologically very similar each other in Iran as the Caspian populations of the latter have a strongly reduced or absent inguinal loop.

Although the recordings were not made by a professional recorder, and the conditions were not ideal during some recording sessions due to a chorus of many calling frogs present, they allowed to inspect main call characteristics, which clearly separated the recorded advertisement calls into two different groups. The advertisement calls from the localities 1 and 2 (Kurdistan and south-west of the Talesh Mts.) had longer call segments and higher number of pulses (152 ms, 133 – 169 ms; 19.0 pulses, 18 – 20 pulses; means and ranges; Fig. 1B) than the advertisement calls recorded in the localities 3 and 4 on the southern Caspian coast (77 ms, 72 – 81 ms; 8.5 pulses, 8 – 9 pulses, Fig. 1C).

From the above results, it is evident that the recorded advertisement calls belong to two species. The calls from the inland clearly correspond to *H. savignyi*, while the calls from the Caspian coast are assignable to the *H. arborea*-like type (e.g. cf. Schneider, 2004). This finding is in concordance to the assumptions of Litvinchuk et al. (2006), who expected *H. arborea* to be present in northern Iran. However, according to the current taxonomy (Stöck et al., 2008), the Caspian population should be assigned to *H. orientalis*. This species has a circum-Pontic distribution including Asia Minor and the Caucasus (Stöck et al., 2008; Gvoždík et el., in prep.) and it is known that possesses the same (or very similar) advertisement call as *H. arborea*, since Schneider (2000, 2004) demonstrated that tree frogs from western Turkey and northern Armenia (both formerly *H. arborea*, now *H. orientalis*) possess very similar advertisement call to that of *H. arborea* from Germany. Thus, the obtained acoustic data show that the Iranian-Caspian populations belong to *H. orientalis* (Fig. 1E). This finding was recently confirmed also genetically (Gvoždík et al., in prep.). No *H. savignyi* calls were detected among the recordings from the Caspian region.

Based on the data obtained, it can be hypothesized that the Caspian coast in Iran is inhabited by *H. orientalis*, while *H. savignyi* (Fig. 1D) is distributed west- and southward the Talesh and Alborz Mts. It seems that the two species are mutually parapatric in Iran, however more data (acoustic or genetic) are needed to clarify the situation. Cheatsazan et al. (2005) and Kami (2005) published a north-eastward range extension of *H. savignyi* as they discovered a remote population in the Golestan Province. In the light of current findings, it is more likely that the Golestan population represents rather *H. orientalis*. Nevertheless, farther data coming from the Golestan tree frogs must be investigated to confirm this preliminary assumption.

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Kapitola IV.

Phylogeography of the Middle Eastern tree frogs (*Hyla*, Hylidae, Amphibia) as inferred from nuclear and mitochondrial DNA variation, with a description of a new species. *

Gvoždík V., Moravec J., Klütsch C. & Kotlík P.

Submitted manuscript.

***Hyla* sp. nov.**, Wadi Mujib, Jordánsko (Jordan)

* The proposed scientific name of the new species has been removed from the manuscript and will be published in the official paper.

Phylogeography of the Middle Eastern tree frogs (*Hyla*, Hylidae, Amphibia) as inferred from nuclear and mitochondrial DNA variation, with a description of a new species

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Abstract
Evolutionary relationships of the tree frogs from the Middle East and the demographic histories of their populations were studied using a combination of mitochondrial and nuclear genes. *Hyla savignyi* and neighboring populations of *H. orientalis* (former eastern populations of *H. arborea*) were the main focus taxa. Within *H. savignyi*, a deep phylogenetic divergence dated about 8.4 Ma was discovered. Southern populations from Yemen, Jordan, southern Syria and extreme north-eastern Israel are hereby described as a new species, *Hyla* sp. nov. Our study points to a biogeographic connection of the south-western Arabian Peninsula and southern Levant and to the importance of the Dead Sea Rift as a historical barrier geographically separating the new species from *H. savingyi*. Major genetic breaks revealed within species (*Hyla* sp. nov.: Yemen vs. Jordan-Syria; *H. savignyi* sensu stricto: Levant vs. Turkey-Iran) are probably connected to climate changes during the Plio-Pleistocene boundary, while the finer phylogeographic structuring probably resulted from the Quaternary climate oscillations. The Cypriote population of *H. savignyi* originated from southern Anatolia relatively recently. *H. orientalis* from the southern Black Sea region seems to be genetically quite uniform, although two phylogeographic units with western Turkish and Caucasus-Caspian affinities might be detected. *H. savignyi* and *H. orientalis* carry signals of population expansions dated to the middle to late Pleistocene, while populations of *Hyla* sp. nov. seem to have been rather constant in size.

Keywords: Hyla savignyi; *Hyla* sp. nov.; *Hyla orientalis*; *Hyla arborea*; DNA; 12S and 16S rRNA; Rhodopsin; Tyrosinase; Biogeography; Demography; Taxonomy

1. Introduction

The Middle East constitutes an important zoogeographic region on the crossroads of Palearctic, Oriental and Afrotropic ecozones. Some areas within this region, such as the Mediterranean, Caucasus Mountains or southern Caspian Sea coast, served during the Pleistocene climatic cycles as important refugia for predominantly European biota (e.g. Hewitt, 1999), while other areas, such as central Anatolia or Gulf of Persia provided refugia for strictly Middle Eastern species (e.g. Fritz et al., 2008). However, up to now, few phylogeographic vertebrate studies covering a substantial part of the Middle East have been published (e.g. Dubey et al., 2007a; Fritz et al., 2007, 2008; Kapli et al., 2008; Kyriazi et al., 2008; Macholán et al., 2007; Plötner et al., 2001; Prager et al., 1998; Stöck et al., 2006). Most of the available phylogeographic studies focused only on the situation in Anatolia and/or Transcaucasia (Bohlen et al., 2006; Dubey et al., 2007b; Furman et al., 2009; Gündüz et al., 2005, 2007; Hrbek et al., 2002, 2004; Tarkhnishvili et al., 2000, 2001; Veith et al., 2003, 2008; Weisrock et al., 2001). The studies have shown different phylogeographic patterns in different taxa, although, some general patterns have been found as well. The first group consists of taxa which are endemic to some smaller part of the Middle East (usually Anatolia), though possessing a high genetic diversity [e.g. fishes (Bohlen et al., 2006; Hrbek et al., 2002, 2004), salamanders (Tarkhnishvili et al., 2000; Veith et al., 2008; Weisrock et al., 2001), rodents (Gündüz et al., 2007)]. The second group includes widespread taxa, which form two or more lineages covering larger geographic areas [e.g. water and brown frogs (Plötner et al., 2001; Plötner, 2005; Tarkhnishvili et al., 2001; Veith et al., 2003), tortoises and terrapins (Fritz et al., 2007, 2008, 2009), lizards (Kapli et al., 2008; Kyriazi et al., 2008), shrews (Dubey et al., 2007a,b), mice (Macholán et al., 2007), bats (Furman et al., 2009)], or a single widespread lineage but with substantial genetic variation, as was documented in green toads (Stöck et al., 2006). These studies often related the deepest genetic divergences to the plate tectonics in the Miocene and/or associated geological events, such as the Messinian salinity crisis, and such divergences are considered interspecific, forming cryptic species complexes. Shallower, younger genetic structuring is then usually interpreted as a result of climatic oscillations (and associated aridification) during the Pliocene or the Quaternary. Furthermore, in some species present phylogeographic patterns may have also been influenced by human activity, such as continental or overseas transport, as inferred for commensal mice (Gündüz et al., 2005) or shrews (Dubey et al., 2007a).

Tree frogs of the genus *Hyla* are small, semiaquatic vertebrates, widely distributed throughout the Middle Eastern region. For their reproduction, tree frogs are dependent on open waters (e.g. pools, springs, artificial water reservoirs), with rather warm-climate preference. Their distribution in this relatively arid and topographically variable region is therefore limited by the availability of such habitats. Taking into account their relatively low mobility (apart from possible accidental transport by human; Recuero et al., 2007), high and cold mountain ridges of Anatolia, Kurdistan or Iranian Highlands, or deserts in central Iran, eastern Levant or most of Arabian Peninsula might form effective barriers to dispersal of tree frogs. These attributes make tree frogs an excellent model for a study of phylogeography and biogeography of the Middle East.

Systematics and taxonomy of tree frog species occurring in the Middle East, particularly Asia Minor, Transcaucasia, Iranian Plateau, Mesopotamia, Levant, Cyprus and Arabian Peninsula, have not been sufficiently resolved yet. Therefore, it remains unclear if there are three or four species of the genus *Hyla* present in the area.

(1) *Hyla savignyi* Audouin, 1827 [“1809”], occurs in southern and eastern Asia Minor, eastern Transcaucasia, northern and western Iran, Iraq, Levant, extreme north-eastern Sinai, and there are two isolated populations on Cyprus and south-western Arabian Peninsula (Schneider, 2009). *H. savignyi* was traditionally considered as a monotypic species. However, recent studies suggested that on the basis of preliminary molecular data this taxon might be

composed of two divergent evolutionary lineages, presumably species (Gvoždík et al., 2007a; Stöck et al., 2008).

(2) *Hyla orientalis* Bedriaga, 1890 ["1889"], is distributed parapatrically north- and westward of the range of *H. savignyi* in Asia Minor and the Caucasus. This species was previously known as *H. arborea* (Linnaeus, 1758). However, Stöck et al. (2008) suggested all "Middle Eastern" *H. arborea* to be considered as a separate species, for which they resurrected the name *H. orientalis*. Recently, *H. arborea gumilevskii* Litvinchuk, Borkin, Rosanov, Skorinov, 2006, was described from the Talysh Mts. (Azerbaijan), and considered as possibly occurring also in northern Iran (Litvinchuk et al., 2006), but it was subsequently synonymized with *H. orientalis* (Stöck et al., 2008). The Caucasus area has been believed to be inhabited by *H. arborea schelkownikowi* Chernov, 1926 (Kuzmin, 1999; Tarkhnishvili and Gokhelashvili, 1999), although validity of this taxon was disputed (Litvinchuk et al., 2006; Schneider, 2004; Terentjev, 1960).

(3) *Hyla heinzsteinitzi* Grach, Plesser, Werner, 2007, is a recently described endemic species known from three localities in Jerusalem and the adjacent Judean Hills in Israel and the Palestinian territories (Grach et al., 2007). Grach and Werner (2008) reported that the species might be extinct as no surviving population is currently known, while Stöck et al. (2008) suggested according to mitochondrial DNA of a sample from the type locality that this taxon might be based on introduced populations of *H. japonica* Günther, 1859 ["1858"].

In contrast to most previous studies, which were almost exclusively based on mitochondrial DNA and limited sampling sizes, we used here sequences of multiple mitochondrial (12S rRNA and 16S rRNA) and nuclear genes (rhodopsin and tyrosinase) as well as a dense sampling approach covering the distributions of tree frogs in the Middle East in order to infer the evolutionary relationships within this group (Supplementary data; Fig. S1, Table S1). Although our study builds on earlier work (Gvoždík et al., 2007a; Stöck et al., 2008), only with our new approach, based on the combination of genetic, bioacoustic, and morphological analyses, the dataset becomes adequate to provide a compelling description of phylogeography and evolutionary history of tree frogs in the Middle East. Our goals were as follows: (i) Firstly, to evaluate concordance in phylogeographic signals among different markers. (ii) To identify major intraspecific phylogeographic breaks within the studied taxa and estimate approximate dates of their origins. (iii) To test if *H. savignyi* s.l. and *H. orientalis* are reciprocally monophyletic phylogenetic lineages. (iv) To infer genetic relationships of the two isolated populations of *H. savignyi* s.l. from the Arabian Peninsula and Cyprus. (v) To evaluate taxonomic status of the previously reported two major clades of *H. savignyi* s.l. (vi) To test a validity of the Caucasian taxon *H. arborea schelkownikowi*. (vii) To infer demographic history of the main population subsets. (viii) Finally, we also revise mutual spatial distribution limits of *H. savignyi* and *H. orientalis*.

2. Material and methods

2.1. Samples for genetic study

Tissue samples of individual specimens (*H. savignyi* sensu lato (s.l.): n = 150, 66 localities; *H. orientalis*: n = 44, 19 localities) were obtained from museum voucher specimens (National Museum, Prague, Czech Rep.). In addition, toe clips, tadpole tail tips, and oral swabs were collected in the field (Fig. S1, Table S1). Additional sequences of three individuals of *H. savignyi* s.l. (yielding one additional locality) and an outgroup species *H. japonica* were taken from GenBank [Yemen: AY843665, AY844654, AY844107, Faivovich et al. (2005); Syria: EF566954, Moriarty Lemmon et al. (2007) & DQ055843, Smith et al. (2005); and Japan: AY843633, AY844615, AY844078, Faivovich et al. (2005)]. Another outgroup species, *H. meridionalis* Boettger, 1874, a specimen from Tenerife Island, the type locality, was sequenced by us. Outgroup position of selected outgroup taxa was shown by

Faivovich et al. (2005), Smith et al. (2005), Stöck et al. (2008) and Wiens et al. (2006). No samples of *H. heinzsteinitzi* were at our disposal for this study.

2.2. Molecular laboratory procedures

Total genomic DNA was extracted from tissue samples using different commercial kits following the manufacturers' protocols. We targeted two fragments of mitochondrial DNA (mtDNA), 12S rRNA and 16S rRNA (12S and 16S), and two nuclear genes (nDNA), rhodopsin, exon 1, and tyrosinase precursor, exon 1. The 12S fragment was amplified using primers 12Sa (5'-CTGGGATTAGATACCCCACTA-3') and 12Sbs (5'-TGAGGAGGGTGACGGGCGGT-3'), adapted from Kocher et al. (1989); the 16S segment by primers 16SL1 (5'-CGCCTGTTTAACAAAAACAT-3') and 16SH1 (5'-CCGGTCTGAACTCAGATCACGT-3'), adapted (16SL1) or taken (16SH1) from Palumbi et al. (1991). Nuclear genes for rhodopsin and tyrosinase were amplified using primers Rhod1A and Rhod1C, and Tyr1C and Tyr1G respectively (Bossuyt and Milinkovitch, 2000). Amplification of all fragments involved an initial cycle of denaturation at 94 °C for 15 min, and 35 subsequent cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Annealing temperature was 55, 59 and 57 °C for the mtDNA fragments, rhodopsin and tyrosinase, respectively. The resulting PCR products were directly cycle-sequenced with the same primers as those used for PCR. The sequence analysis was carried out on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) at the Macrogen sequencing service (Macrogen Inc., Seoul, Korea). Nucleotide sequences of each unique haplotype identified in this study have been deposited in the GenBank database under the accession numbers GQ916691–GQ916820 (for details see Supplementary data).

2.3. DNA sequence evaluation

Alignment of the mtDNA segments was made by ClustalW (Thompson et al., 1994) as implemented in BioEdit 7.0 (Hall, 1999) and checked by eye (aligned length of the 12S segment = 355 bp and the 16S segment = 544 bp). Alignment of nuclear genes was prepared in the same program by hand as the genes are protein-coding exons with no indels (rhodopsin segment = 276 bp; tyrosinase segment = 496 bp). In the nDNA, three individuals showed more than one heterozygote positions (one *H. savignyi* s.l. in rhodopsin; one *H. savignyi* s.l. and one *H. orientalis* in tyrosinase). In these cases, the coalescent-based Bayesian method implemented in Phase 2.1 (Stephens et al., 2001; Stephens and Scheet, 2005) was employed to infer haplotypes. The analysis was run separately for each species, with each analysis repeated several times (at least 5x) and with different seeds for the random number generator to check if the estimated gametic phase was consistent through the runs according to goodness-of-fit values. Each run was conducted under the parent-independent mutation model with a burn-in-period of 100 iterations, which was followed by 1000 iterations. All most likely haplotypes statistically inferred were used in phylogenetic analyses. No stop codons were detected in the haplotypes as checked by translation with the standard genetic code using BioEdit 7.0 (Hall, 1999). Haplotype networks for both nuclear markers were constructed using the statistical parsimony algorithm implemented in TCS 1.21 (Clement et al., 2000) under the 95% limit of parsimony.

Haplotype diversity (h), number of segregating sites (S), nucleotide diversity π (Nei and Li, 1979) and a population parameter θ_W (Watterson, 1975) for each locus, as well as the minimum number of recombination events in nuclear loci for different subsets of tree frog populations were estimated in DnaSP 5.00 (Librado and Rozas, 2009). One GenBank sequence (DQ055843), which covers only the 12S fragment, was not included in the genetic polymorphism evaluation. It was omitted also from calculation of genetic distances, based on individual haplotypes, which were computed in PAUP* 4.0b10 (Swofford, 2003) and averaged between groups in MEGA 4.0 (Kumar et al. 2008; Tamura et al., 2007).

2.4. Phylogenetic analyses

For the phylogenetic analyses, all sequences, mtDNA and phased nDNA, were sorted into distinct haplotype sets using Collapse1.2 (Posada, 2006). The best-fit model of sequence evolution was selected using jModelTest 0.1.1 (Posada, 2008). Because Posada and Buckley (2004) argued that the Akaike information criterion (AIC; Akaike, 1974) and the Bayesian information criterion (BIC; Schwarz, 1978) offer important advantages over the hierarchical likelihood-ratio tests, we checked results from both information criteria (Table 1).

Phylogenies were reconstructed using maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP) methods. The ML analyses were performed by the BEST approach implemented in PhyML version 3.0.1 (Guindon and Gascuel, 2003), which combines the NNI (nearest neighbour interchanges) and SPR (subtree pruning and regrafting; Hordijk and Gascuel, 2005) algorithms to maximize tree likelihood and using the best-fit model according to the AIC for each data set [TIM2+G, Posada (2003) for concatenated mitochondrial data; TrN+G, Tamura and Nei (1993) for rhodopsin; SYM+G, Zharkikh (1994) for tyrosinase] and 10 random starting BioNJ trees. We computed bootstrap values based on 1000 resampled data sets (Felsenstein, 1985), as well as the approximate likelihood-ratio test for branches (aLRT; Anisimova and Gascuel, 2006), to assess the branch supports.

Bayesian analyses were carried out with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood settings corresponded to the general time-reversible model with rate heterogeneity (GTR+G; Tavaré, 1986), which is the closest approximation of the models selected for each mitochondrial fragment (Table 1) available in MrBayes. Parameters were optimized for two partitions, 12S and 16S rRNA fragments separately. The analysis was performed with two runs and four chains for each run for six million generations, and sampling every 100th tree. First 10,000 trees (burn-in value) were discarded to be sure to avoid unstationary trees, however log-likelihood scores of sampled trees plotted against the generation time showed that stationarity was fully achieved already with the approx. 300th tree. A majority rule consensus tree was subsequently produced from the remaining trees after discarding the burn-in trees, and the posterior probabilities calculated as the frequency of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001). Each BI analysis was repeated several times with random starting trees and the results were compared and checked if assessed convergence.

PAUP* 4.0b10 (Swofford, 2003) was used for the MP analyses. All characters were equally weighted, gaps were treated as a fifth state, and a heuristic search was conducted with 100 random taxon stepwise addition replicates using tree bisection and reconnection (TBR) branch swapping. The topology was reconstructed as the 50% majority rule consensus of most parsimonious trees, and support values were assessed using 1000 bootstrap pseudoreplicates (Felsenstein, 1985).

2.5. Estimation of divergence times

Divergence dates among the main mitochondrial clades were estimated using a Bayesian coalescence approach, as implemented in BEAST 1.4.8 (Drummond et al., 2006; Drummond and Rambaut, 2007). Because we were principally interested in dating the divergence between major phylogenetic lineages, we only selected basal and the most common haplotypes from each major mitochondrial clade for this analysis. We employed the Yule tree prior, which assumes a constant speciation rate per lineage. The GTR+G model with Jeffreys priors for substitution rates with the TIM2+G values of the jModelTest output was applied with an uncorrelated lognormal relaxed molecular clock. The prior for the mean mutation rate was specified as a normal distribution, with a mean of 0.0028 and standard deviation of 0.0010. This normal distribution covered the relevant range from 0.1% to 0.5% substitutions per site per My, which is concordant with assumed speed of evolution of the studied mitochondrial fragment in amphibians (Lymberakis et al., 2007). The mean 0.0028

substitution/site/My follows suggested average 12S/16S mutation rate as was recently estimated directly for hylid frogs (Moriarty Lemmon et al., 2007). The search was started with an UPGMA tree. Two independent runs of 20 x 10^6 generations were conducted. The results were checked for convergence and stationarity of the different runs using Tracer 1.4.1 (downloadable from the BEAST website http://beast.bio.ed.ac.uk) and combined with the BEAST module LogCombiner 1.4.8, after discarding the burn-in 4 x 10^6 generations from each analysis. The final molecular clock tree was summarized in the BEAST module TreeAnnotator 1.4.8 using medians as node heights.

2.6. Historical demography

We used several different approaches to examine past population dynamics and signatures of refugial expansion of the individual lineages. We first considered the distribution of the number of pairwise nucleotide differences (mismatch distribution) within the lineages by contrasting observed distributions with those expected from models of population size change. We tested whether the data fitted the sudden demographic expansion model (Rogers & Harpending, 1992; Slatkin and Hudson, 1991) or the instantaneous range expansion model (Excoffier, 2004; Ray et al., 2003), using Arlequin 3.11 (Excoffier et al., 2006). A parametric bootstrapping approach (Schneider & Excoffier, 1999) with 1000 replicates was used to estimate the significance of the Harpending's raggedness index (*r*; Harpending, 1994) and to obtain the probability that the observed data conform to each model using the sum of square deviations (SSD) between the observed and expected mismatch distribution as a test statistic.

To determine the effect of population structure on mismatch distribution in a case where regional populations were not reciprocally monophyletic but carried unique sets of haplotypes (one mtDNA clade of *H. savignyi*), we applied the mismatch distributions also to these geographically delimited population subsets. The distribution of the number of pairwise nucleotide differences between these regional populations (intermatch distribution) was calculated using the program IWaVe (Sherry, 1994). Two lineages belonging to the same population expansion should show similar mismatch distributions and the main mode of their mismatch distribution should correspond to the mode of the intermatch distribution (Excoffier, 2004). On the contrary, the lack of fit between the mismatch and intermatch distributions is expected if their diversity has been shaped by expansions of different ancestral populations (Harpending et al., 1993; Excoffier, 2004).

Second, we calculated three tests of selective neutrality for each population subset. Fu's (1997) F_S, one of the most powerful neutrality test for detecting expansions on non-recombining genomic regions (Ramírez-Soriano et al., 2008), and Tajima's (1989) *D* tests were performed with 1000 simulated replicates in Arlequin 3.11 (Excoffier et al., 2006). The Ramos-Onsins and Rozas's (2002) R_2 statistics, which has more statistical power in small population sizes and is more resistant to possible recombinations (Ramírez-Soriano et al., 2008), was calculated and its significance tested with 1000 replicates in DnaSP 5.00 (Librado and Rozas, 2009). If recombinations were detected in nuclear markers, the tests' significance was assessed by coalescent simulations with 1000 replicates considering the recombination parameter R (Hudson, 1987) in the same program. The significant departure of the tests' null hypotheses indicates that the population has deviated from neutrality and/or could undergone expansion or decline (negative values of the Fu's F_S and Tajima's *D*).

Third, historical population demography of main phylogeographic lineages were investigated using a coalescence approach of Bayesian skyline plots (BSP; Drummond et al., 2005), as implemented in BEAST 1.4.8 (Drummond et al., 2006; Drummond and Rambaut, 2007). This technique calculates the effective population size (N_e) through time directly from sampled sequences and does not require a specific demographic model as a prior. The appropriate substitution model (GTR+G) and the mean mutation rate under a relaxed uncorrelated lognormal molecular clock were set as in the analysis of divergence dates (see

previous section). We applied 10 groups as time segmentation and the linear skyline model. Two independent runs of 30×10^6 iterations for each grouping scenario were performed. Times to most recent common ancestor (t_{MRCA}) of different population subsets were also assessed from the analyses. Convergence of chains, effective sample size (ESS), estimates and credible intervals for each parameter and demographic reconstructions, and burn-in values were examined in Tracer 1.4.1. When the convergence did not occur and/or ESS were low, additional longer analyses were run.

A sequence from GenBank (DQ055843) covering only the 12S fragment was not included in the demographical analyses.

2.7. Morphology

Specimens examined, the type series of the new species and the referred material, are listed within the description of the new species and in Supplementary data. Morphometric methodology is given in Gvoždík et al. (2008), measurement and museum abbreviations are listed in Supplementary data.

2.8. Acoustics

Advertisement call structure of all ingroup species, whose determination was confirmed genetically, was investigated. A portable Sony Walkman WM-GX550 cassette-recorder with a Sony ECM-MS907 electret condenser microphone was used. Calls were digitized using BatSound – Sound Analysis 1.2 software (Pettersson Elektronik AB) under a sampling rate of 22,050 Hz with a sample size of 16 bits in the mono mode and analyzed by the same software. Oscillograms, spectrograms and power spectra were inspected with the following settings: FFT size 256 samples and Hanning FFT window for spectrograms and power spectra, and FFT samples overlap 75% for spectrograms. Call segment (pulse group sensu Schneider, 2004) length, and dominant frequency were measured, and number of pulses per segment were counted. Values of the parameters were averaged from five consecutive call segments from the middle of the call.

3. Results

3.1. Mitochondrial DNA variation, phylogeny and characterization of the main groups identified

Considerable genetic variation within ingroup taxa was found in both the 12S and 16S rRNA genes. The 355 bp long 12S fragment was with 30 unique haplotypes from 197 ingroup sequences less variable (43 variable characters, of which 28 parsimony informative) than the 542 bp long 16S fragment, which revealed 57 different haplotypes from 196 ingroup individuals (78 variable characters, of which 54 parsimony informative). Both mitochondrial fragments concatenated, including outgroup and considering indels (899 bp aligned length), yielded 78 haplotypes; 154 characters were variable, of which 99 were parsimony informative (without outgroup: 897 bp, 76 haplotypes, 121 variable, of which 85 parsimony informative). The best-fit substitution models for each fragment and the concatenated mitochondrial data set as predicted by both AIC and BIC are in Table 1. ML analysis of the concatenated mtDNA data set with the TIM2+$G_{0.054}$ model resulted in the most likely tree with log likelihood (ln*L*) = -2643.78 (Fig. 1). All independent BI runs resulted in essentially identical topologies and likelihood estimates (mean ln*L* = -2841.39). MP analysis produced 630 most-parsimonious trees with a length of 244 steps (consistency index, CI = 0.746; retention index, RI = 0.964), which all had exactly identical topologies with respect to the main clades.

H. savignyi s.l. with 54 haplotypes and *H. orientalis* with 22 haplotypes were mutually monophyletic in all analyses (ML, BI, MP), however a further deep divergence was found within *H. savignyi* s.l. (Figs. 1, 2a). The mean divergence between the two well-supported lineages with parapatric distributions in the Levant was 9.6% ML genetic distances (4.5%

uncorrected pairwise distances in concatenated mtDNA, and 4.3% in the 16S rRNA gene solely), which suggests a specific level of the two clades (cf. Fouquet et al., 2007; Vieites et al., 2009). Thus, the northern clade would correspond to *H. savignyi* sensu stricto (hereafter as only *H. savignyi*) because it included specimens from the putative type locality (see Discussion), while the southern clade would represent a new species, hereafter provisionally referred to as *Hyla* sp. nov.

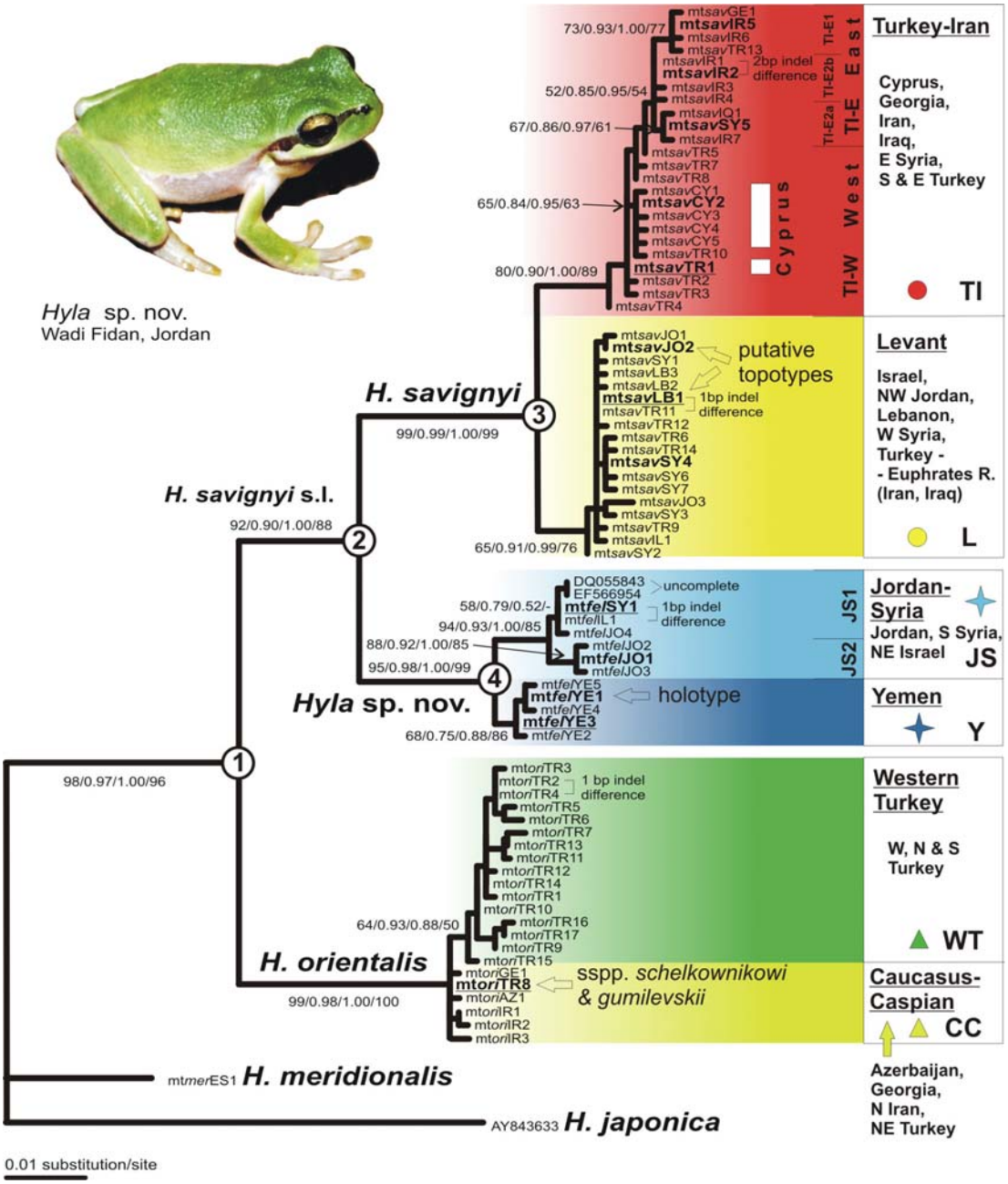

Fig. 1. Maximum likelihood phylogeny (TIM2+G) of the Middle Eastern tree frogs (*Hyla*) using mitochondrial data. For nodal support, maximum likelihood (ML) bootstrap (1000 pseudoreplicates), approximate likelihood-ratio test for branches for ML, Bayesian posterior probabilities (BPP < 0.50 not shown) and maximum parsimony bootstrap (1000 pseudoreplicates) are given. Group names and geographic distributions are shown on the right, for further explanation see text. Haplotypes in bold were found in ≥ 5 individuals. Numbers in nodes indicate split events dated by the Bayesian coalescent approach, with underscored haplotypes indicating those included in the analysis.

3.1.1. Hyla savignyi

H. savignyi forms two distinct clades of intermediate (ML, MP) or high (BI) statistical support and with generally parapatric distributions. One clade composed of 18 haplotypes, hereafter called the Levant lineage (L), comprises individuals from the Levant region (Israel, north-western Jordan, Lebanon, western Syria) including from the type locality, and northward along the Euphrates River as far as at least 39° 36' N (Kemah, Turkey). The northern Euphrates population interrupts the geographical distribution of the second clade, hereafter referred to as the Turkish-Iranian lineage (TI; 24 haplotypes), which comprises tree frogs from Cyprus and southern Mediterranean Turkey (Western TI, TI-W; 13 haplotypes), and eastern Turkey, Transcaucasia, Iran, Iraq and eastern Syria (Eastern TI, TI-E; 11 haplotypes). TI-E population forms a monophyletic subclade in the ML phylogram, although with rather low statistical support, while TI-W is a paraphylum in ML (Fig. 1). Two individuals from the Levant lineage were found also in central Iraq (Bagdad) and south-western Iran (Choqa Zanbil). Two localities in Turkey from the contact zone of the two lineages (Ovaçiftliği, Payas) and one locality outside the Levant (Bagdad, Iraq) contained individuals from both lineages. In samples from the southern Caspian coast (Iran), we found only mitochondrial haplotypes belonging to *H. orientalis*, not to *H. savignyi* as have been supposed till the recent time (Cheatsazan et al., 2005).

3.1.2. Hyla sp. nov.

Hyla sp. nov. is distributed from Yemen northward as far as southern Syria. There is a known disjunction in tree frogs' occurrence between the Asir Mts. in Saudi Arabia and Jordan due to a desert habitat present there (Balletto et al., 1985; Klütsch et al., 2004). We found two main clades, one in Yemen (5 haplotypes discovered; hereafter called the Yemeni lineage, Y), and the other in the southern Levant – western Jordan, southern Syria and extreme north-eastern Israel (7 haplotypes; Jordanian-Syrian lineage, JS). Only at one place (Karkom, Israel) mt haplotypes of both species, *H. savignyi* (n = 5; k = 3) and *Hyla* sp. nov. (n = 3; k = 2), were mixed, which suggests a hybrid origin of this population.

3.1.3. Hyla orientalis

Populations of *H. orientalis* from the Anatolian and Caucasian region form a compact cluster, which can be subdivided into two haplogroups: Caucasian, which extends along to the southern Caspian coast as far as to northern Iran (6 haplotypes; hereafter referred to as the Caucasus-Caspian haplogroup, CC), and which seems to be basal and paraphyletic in respect to the Anatolian population (16 haplotypes; hereafter as the Western Turkish haplogroup, WT) forming a monophylum, however, with only intermediate statistical support. One locality (Hopa, north-eastern Turkey) was found to represent a geographical contact between the two groups as it carried haplotypes from both haplogroups (n = 4 vs. n = 1 of the WT).

3.2. Nuclear DNA sequence diversity

Sequences of nDNA (coding sequences of the rhodopsin and tyrosinase genes) were obtained for selected samples from each species and major mitochondrial haplogroups (Fig. 1). Nuclear DNA sequences can be sometimes difficult to analyze due to heterozygous positions if present more than once in a sequence. We found three individuals, which showed more than one heterozygous positions (one in rhodopsin, two in tyrosinase). For rhodopsin (Karkom, Israel) the phase was determined with the probability of 1.00 in each of all five Phase runs and the results indicated a hybrid origin of this individual (*H. savignyi* and *Hyla* sp. nov.). For the tyrosinase heterozygotes, the phase was identified in one *H. savignyi* individual (Choqa Zanbil, Iran) with the probability of only 0.66 (average for multiple runs) for one of the two heterozygous sites, and in one *H. orientalis* individual (Marmaris, Turkey) with the probability of 0.84 for one of the three heterozygous sites. Despite lower

	AIC	BIC
12S rRNA	TIM2 + G	TrNef + G
16S rRNA	GTR + G	TIM2ef + G
12 + 16S rRNA	TIM2 + G	TIM2ef + G
rhodopsin	TrN + G	TrNef + G
tyrosinase	SYM + G	K80 + G

Table 1. Results of substitution model selection using the Akaike (AIC) and Bayesian (BIC) information criteria for different data subsets.

Table 2. Combination of haplotypes of three different markers as found in four hybrid individuals (*H. savignyi – Hyla* sp. nov.) from Karkom, Israel (loc. 67).

Sample ID	mt DNA	Rhodopsin A	Rhodopsin B	Tyrosinase A	Tyrosinase B
H299	mt***sav***IL1	R***fel***1	R***fel***1	T***sav***2	T***sav***3
H300	mt***fel***SY1	R***fel***1	R***sav***1	T***fel***6	T***fel***6
H302	mt***fel***SY1	R***fel***1	R***fel***1	T***fel***3	T***fel***5
H304	mt***fel***IL1	R***sav***1	R***sav***1	T***sav***2	T***fel***7

Table 3. Results of the Bayesian coalescent based estimation of divergence dates and the time to the most recent common ancestor (t_{MRCA}) of extant haplotypes of different subset populations. The numbers in the left column correspond to the splits in mtDNA phylogeny (Fig. 1). Median values in bold and 95% HPD in brackets. See text for population abbreviations.

	Species / Populations	Divergence time estimate (Ma)
1	***orientalis*** vs. ***savignyi*****-sp. nov.**	**11.100** (4.854–22.961)
2	***savignyi*** vs. **sp. nov.**	**8.408** (3.208–18.231)
3	***savignyi*** **L** vs. **TI**	**2.753** (0.018–7.479)
4	**sp. nov. Y** vs. **JS**	**2.018** (0.035–5.911)
		t_{MRCA}
	H. savignyi	
	L	**0.410** (0.106–1.296)
	TI	**0.817** (0.207–2.325)
	TI-W	**0.616** (0.129–1.938)
	TI-W-Cy	**0.266** (0.065–0.828)
	TI-E	**0.516** (0.107–1.639)
	TI-E1	**0.212** (0.055–0.612)
	TI-E2	**0.392** (0.086–1.278)
	TI-E2a	**0.176** (0.042–0.529)
	TI-E2b	**0.266** (0.045–0.954)
	Hyla **sp. nov.**	
	Y	**0.208** (0.012–1.848)
	JS	**0.273** (0.038–1.581)
	JS1	**0.258** (0.037–1.436)
	JS2	**0.229** (0.012–1.219)
	H. orientalis	**0.992** (0.279–2.677)
	CC	**0.405** (0.106–1.149)
	WT	**0.907** (0.277–2.507)

probabilities, the results were consistent across all five runs in each analysis, resulting in the same inferred haplotypes.

Between the two nuclear genes, rhodopsin showed in its 276 bp long fragment substantially lower variability (18 variable characters, of which 5 parsimony informative; 8/3 in the ingroup) than tyrosinase in its 496 bp long fragment (54 variable characters, of which 38 parsimony informative; 27/17 in the ingroup). However, both genes provided sufficient information for species discrimination, with one exception in tyrosinase (see below). ML analyses under the $TrN+G_{0.010}$ (rhodopsin) and $SYM+G_{0.114}$ (tyrosinase) models yielded the most likely trees with ln*L* = -509.31 and ln*L* = -1139.99, respectively (Fig. 2b,c). The SYM+G (AIC) tyrosinase ML phylogram had exactly the same topology as the K80+G (Kimura, 1980; BIC) ML tree (not shown). The Bayesian consensus tree resulted in polytomy of the ingroup haplotypes in rhodopsin (mean ln*L* = -532.41), while the topology was similar to the ML tree in tyrosinase (mean ln*L* = -1275.80). MP analyses produced 27 and 512 most-parsimonious trees for rhodopsin and tyrosinase, respectively, with a length of 21 and 69 steps (rhodopsin: CI = 0.857, RI = 0.727; tyrosinase: CI = 0.826, RI = 0.898). All phylogenetic methods produced similar tree topologies for tyrosinase, although with low statistical support for individual clades due to low sequences variation. Sequence variation was very low in rhodopsin, rendering this gene unsuitable for a tree inference. Parsimony haplotype networks, however, revealed clear haplotype structure, which was concordant with the topology of the ML trees (Fig. 2b,c).

At rhodopsin, all species, including *Hyla* sp. nov., can be distinguished according to diagnostic haplotypes in the inferred genealogy (Fig. 2b). In *H. orientalis* and *Hyla* sp. nov., one main haplotype and one (*Hyla* sp. nov.) or four (*H. orientalis*), respectively, derived haplotypes, detected only in heterozygous state with the main haplotype, were found. Only in *H. savignyi* two widespread haplotypes were discovered, which were partly geographically concordant with the two major mtDNA lineages. Another haplotype in *H. savignyi* was found only in a heterozygous state with the most common haplotype (R*sav*1). Individuals from Karkom (NE Israel), a putative hybrid population, carried the most common haplotypes of both, *H. savignyi* (n = 1) and *Hyla* sp. nov. (n = 2). One individual was found to be a heterozygote carrying alleles of both species (Table 2).

For tyrosinase, the genealogical pattern is more complex (Fig. 2c). The highest number of haplotypes (k) was found in *H. savignyi* (k = 15), followed by *H. orientalis* (k = 10), and *Hyla* sp. nov. (k = 6). One additional haplotype (T*fel*7), derived from the most common haplotype of *Hyla* sp. nov., was found in the heterozygous state in an individual from the putative hybrid population (Karkom, Israel). This specimen was, at this locus, found to be an interspecific hybrid with *H. savignyi*. Another individuals from the Karkom population carried alleles of both species (*H. savignyi* haplotypes, n = 1, k = 2; *Hyla* sp. nov. haplotypes, n = 2, k = 3), thus, this population was clearly a hybrid population also according to this marker (Table 2). Tyrosinase haplotypes of *H. savignyi* (T*sav* in haplotype names) form a monophyletic clade, which includes a further monophyletic subclade, which is predominantly limited geographically in the eastern part of the species distribution, while more basal haplotypes occur in the western part. The situation in *H. orientalis* and *Hyla* sp. nov. is more diverse. *Hyla* sp. nov. forms a distinct haplotype cluster but it also shares the most common haplotype (T*fel*3) with *H. orientalis* from south-western Turkey. Also, another three haplotypes derived from T*fel*3 were found exclusively in the south-western Turkish population of *H. orientalis*, while the other populations from northern Turkey and the Caucasus-Caspian region formed a monophylum clearly distinct from *Hyla* sp. nov. One individual from south-western Turkey (Marmaris) was found to be a heterozygote carrying haplotypes derived from both groups, the northern Anatolian populations of *H. orientalis* and *Hyla* sp. nov.

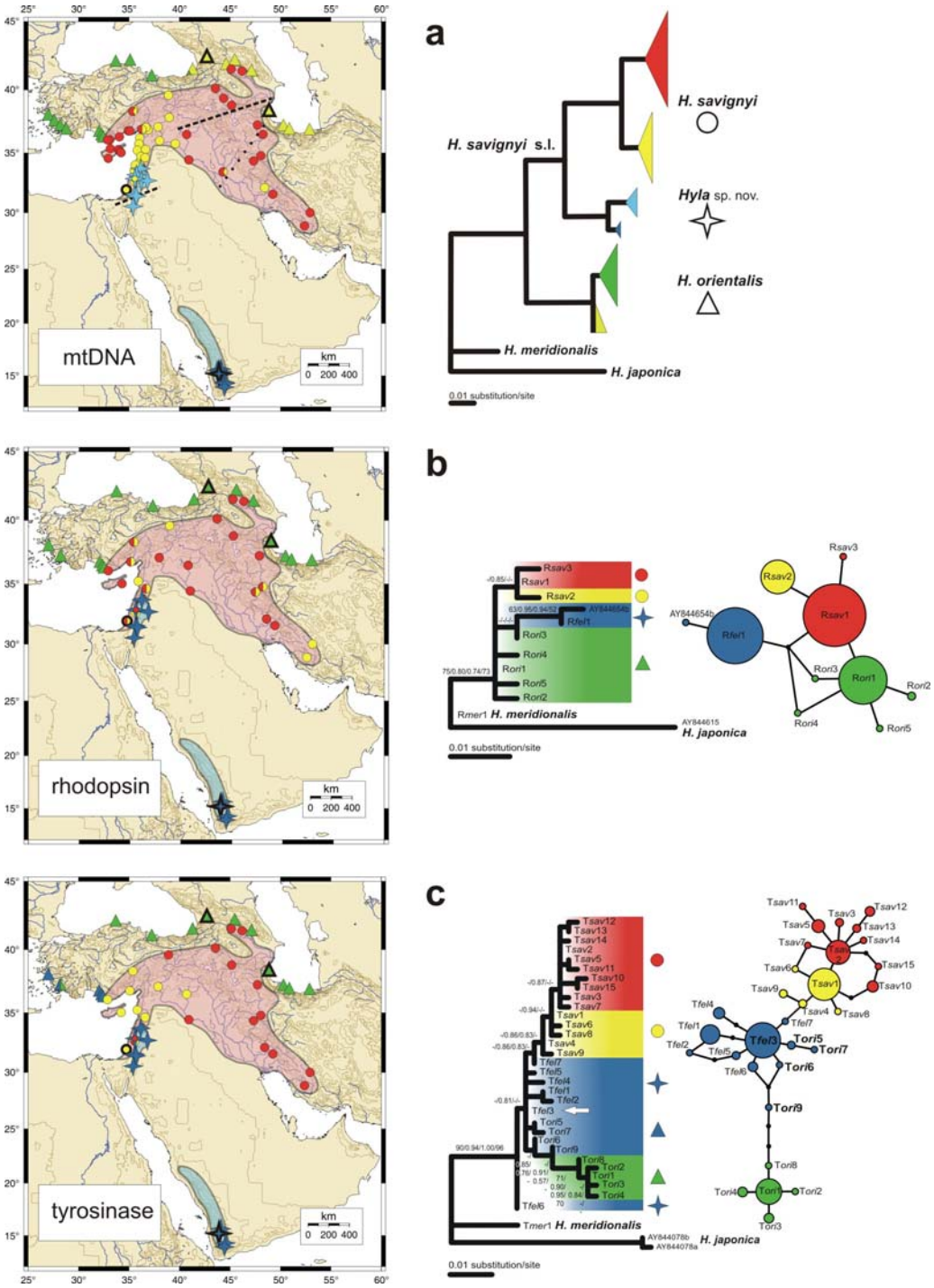

Fig. 2. Comparison of geographic patterns of variation in the mitochondrial (12S and 16S rRNA) and nuclear (rhodopsin, tyrosinase) DNA sequence data of the Middle Eastern tree frogs. (a) Mitochondrial DNA, map and schematic ML tree; dashed lines on the map indicate separation of the E1 (north) and E2 (south) groups of *H. savignyi*, and JS1 (north) and JS2 (south) of *Hyla* sp. nov., respectively, and the dotted line indicates separation of the E2a (west) and E2b (east). (b) Rhodopsin, map, ML tree (nodal support as in Fig. 1, < 0.50 or 50% not shown) and parsimony haplotype network (sizes of circles are proportional to the numbers of specimens). (c) Tyrosinase, map, ML tree (support as above) and parsimony haplotype network; haplotypes in bold belong to the south-western Anatolian population of *H. orientalis*, which are in this locus closer to *Hyla* sp. nov., haplotype T*fel*3 (white arrow) was present in both species. Different symbols correspond to the species: *H. savignyi*, circles; *Hyla* sp. nov., stars; *H. orientalis*, triangles. Colors correspond to different population clusters as indicated in the trees and networks. Bold symbols in the maps indicate type localities (*Hyla* sp. nov. and *H. arborea schelkownikowi*), putative type locality (*H. savignyi*), or a locality close to the type locality (*H. arborea gumilevskii*, Iran). Distributional maps of *H. savignyi* (pink) and *Hyla* sp. nov. (blue) according to the current knowledge are indicated.

3.3. Comparison of mtDNA and nDNA pattern

The three evolutionarily independent markers, mtDNA and two nuclear genes were concordant in that they all defined the same main groups/species (Fig. 2). All species, including the new one, could be distinguished according to unique distinctive haplotypes in all markers. The only exception was haplotype T*fel*3, which was present in two allopatric species, *Hyla* sp. nov. and *H. orientalis*. Populations were markedly geographically structured within the mitochondrial genes. The major mtDNA intraspecific clades were not found to be monophyletic at nuclear markers, although two groups, from which one monophyletic, could be detected in nuclear markers in *H. savignyi*. The geographic pattern of the two intraspecific groups was, however, slightly different among the markers. One of two major mtDNA clades in *Hyla* sp. nov., the Yemeni lineage, could be also distinguished in tyrosinase (T*fel*1 and T*fel*2 haplotypes), while the Levant population (mtDNA JS clade) remained paraphyletic. Interestingly, the Caspian tree frogs were assigned to *H. orientalis* by all markers, and they thus should be considered *H. orientalis*, and not *H. savignyi* as previously thought (Cheatsazan et al., 2005). Also, the population from north-eastern Israel (Karkom) is confirmed to be a hybrid population between northern *H. savignyi* and southern *Hyla* sp. nov. by the comparative analysis of independent markers. A mixture of haplotypes of different species is present at various levels, within the population among individuals, among the markers within an individual, and even within the single marker in an individual (heterozygous states of nDNA) (Table 2).

3.4. Estimation of divergence times

The divergence dates of split events were estimated by relaxed molecular clock approach (Drummond et al., 2006) based on the mitochondrial data set (Table 3). The oldest split within the ingroup, between *H. orientalis* and *H. savignyi-Hyla* sp. nov. occurred probably in the late Miocene, 11.1 Ma, although the range of the 95% highest posterior density (HPD) interval spanned the period from the Early Pliocene through the Miocene, between 4.9 and 23.0 Ma. The divergence between *H. savignyi* and *Hyla* sp. nov. took place also in the late Miocene, 8.4 Ma (HPD between 3.2 and 18.2 Ma). The oldest intraspecific separations in *H. savignyi* and *Hyla* sp. nov. occurred during the Pliocene-Pleistocene boundary 2.8 Ma (HPD 0.02 – 7.5 Ma) and 2.0 Ma (HPD 0.04 – 5.9 Ma), respectively. Model-corrected and uncorrected pairwise genetic distances between the species and major clades based on concatenated mtDNA data set and 12S and 16S separately are given in Table 4.

3.5. Phylogeographic structure and historical demography

3.5.1. Hyla savignyi

H. savignyi forms two distinct clades in the mtDNA markers, Levant (L) and Turkish-Iranian (TI) clades. The TI clade is geographically separated into two subgroups – western (TI-W) and eastern (TI-E). The mismatch distributions (MMD) indicated population growth in both groups, although the *p* value of the L clade was close to 0.05, as tested by the *SSD* and *r* statistics (Table 5). Diagrams of the MMD (Fig. 3a,b) showed a unimodal distribution in the L clade, which suggested a single population under expansion. In contrast, the bi- to slightly tri-modal distribution in the whole TI clade suggested a heterogeneous demographic unit composed of multiple expanding populations. Thus, the MMD was carried out for the TI-W and TI-E separately (Fig. 3c,d) and both were again consistent with the expansion model. Subsequently, the intermatch distribution was estimated between the two subgroups (Fig. 3g). The intermatch provided a pattern concordant with a scenario that the two groups represent different populations (the intermatch peak did not match the mismatch peaks), however, only the TI-W group was unimodal in the MMD, while the TI-E still showed a bimodal distribution. Within the ML phylogram, four haplotypes from Georgia, eastern Turkey and the most north-western part of Iran (hereafter the Transcaucasian lineage, TI-E1) formed a well

Table 4. Genetic distances. ML and uncorrected p-distances based on 12S and 16S rRNA genes, concatenated or separate. In bold on diagonals are within group average genetic distances, below each diagonal are average between groups raw genetic distances, and above each diagonal are net between groups average genetic distances. One outgroup species, *H. meridionalis*, is included for comparison.

Locus **distances**	Species/Group	*H. savignyi*	*H. savignyi* L	*H. savignyi* TI	*Hyla* sp. nov.	*Hyla* sp. nov. Y	*Hyla* sp. nov. JS	*H. orientalis*	*H. orientalis* CC	*H. orientalis* WT	*H. meridionalis*
12+16S rRNA **ML-distances**	*H. savignyi*	**1.5**	-	-	8.3	-	-	12.0	-	-	-
	H. savignyi L	-	**0.3**	2.1	-	-	-	-	-	-	-
	H. savignyi TI	-	2.5	**0.5**	-	-	-	-	-	-	-
	Hyla sp. nov.	9.6	-	-	**1.1**	-	-	10.5	-	-	-
	Hyla sp. nov. Y	-	-	-	-	**0.2**	1.4	-	-	-	-
	Hyla sp. nov. JS	-	-	-	-	1.7	**0.4**	-	-	-	-
	H. orientalis	13.0	-	-	11.4	-	-	**0.6**	-	-	-
	H. orientalis CC	-	-	-	-	-	-	-	**0.3**	0.4	-
	H. orientalis WT	-	-	-	-	-	-	-	0.8	**0.5**	-
	H. meridionalis	18.1	-	-	18.3	-	-	12.9	-	-	**-**
	H. japonica	28.1	-	-	23.5	-	-	24.8	-	-	15.0
12+16S rRNA ***p*-distances**	*H. savignyi*	**1.1**	-	-	3.6	-	-	4.8	-	-	-
	H. savignyi L	-	**0.3**	1.4	-	-	-	-	-	-	-
	H. savignyi TI	-	1.8	**0.5**	-	-	-	-	-	-	-
	Hyla sp. nov.	4.5	-	-	**0.8**	-	-	4.4	-	-	-
	Hyla sp. nov. Y	-	-	-	-	**0.2**	1.0	-	-	-	-
	Hyla sp. nov. JS	-	-	-	-	1.3	**0.4**	-	-	-	-
	H. orientalis	5.7	-	-	5.1	-	-	**0.5**	-	-	-
	H. orientalis CC	-	-	-	-	-	-	-	**0.2**	0.4	-
	H. orientalis WT	-	-	-	-	-	-	-	0.7	**0.4**	-
	H. meridionalis	6.7	-	-	6.4	-	-	5.6	-	-	**-**
	H. japonica	7.6	-	-	7.0	-	-	7.3	-	-	5.8

Locus **distances**	Species/Group	*H. savignyi*	*H. savignyi* L	*H. savignyi* TI	*Hyla* sp. nov.	*Hyla* sp. nov. Y	*Hyla* sp. nov. JS	*H. orientalis*	*H. orientalis* CC	*H. orientalis* WT	*H. meridionalis*
12/16S rRNA ***p*-distances**	*H. savignyi*	**1.0/1.4**	-	-	4.3/3.0	-	-	4.3/5.1	-	-	-
	H. savignyi L	-	**0.5/0.5**	0.7/1.9	-	-	-	-	-	-	-
	H. savignyi TI	-	1.3/2.3	**0.8/0.5**	-	-	-	-	-	-	-
	Hyla sp. nov.	5.2/4.3	-	-	**0.7/1.0**	-	-	4.1/4.6	-	-	-
	Hyla sp. nov. Y	-	-	-	-	**0.3/0.3**	0.6/1.1	-	-	-	-
	Hyla sp. nov. JS	-	-	-	-	0.9/1.5	**0.4/0.5**	-	-	-	-
	H. orientalis	5.2/6.1	-	-	4.8/5.5	-	-	**0.7/0.7**	-	-	-
	H. orientalis CC	-	-	-	-	-	-	-	**0.3/0.4**	0.3/0.4	-
	H. orientalis WT	-	-	-	-	-	-	-	0.8/0.9	**0.7/0.6**	-
	H. meridionalis	6.8/6.7	-	-	6.3/6.5	-	-	5.7/5.6	-	-	**-**
	H. japonica	6.4/8.6	-	-	6.5/7.3	-	-	6.2/8.1	-	-	5.1/6.3

supported monophylum, which showed a unimodal mismatch distribution not different from the expansion model, and the same pattern was shown also by the remaining haplotypes (k = 7) from Iran, Iraq and eastern Syria, haplogroup TI-E2 (Fig. 3e,f). Also the intermatch distribution showed that TI-W, TI-E1 and TI-E2 represent three independently expanding populations (Fig. 3h). However, the TI-E2 group could be geographically further subdivided into two subgroups, the monophyletic TI-E2a from the western part (3 haplotypes from eastern Syria, Iraq and north-western Iran) and the paraphyletic TI-E2b from the east (4 haplotypes from Iran). Within the TI-W population, the isolated population from Cyprus (TI-W-Cy) was of a particular interest. Six haplotypes have been found in Cyprus, of which five formed a monophylum with a haplotype from the Turkish coast (mt*sav*TR10, n = 1). One haplotype (mt*sav*TR1) outside the monophylum was present both on the southern Turkish coast (n = 6) and in Cyprus (n = 1). In general, the mismatch distributions of all particular (sub)populations in all markers (except of the SSD_R of the TI in rhodopsin) indicated population growth according to *SSD* for both expansion models and the raggedness *r* statistics (Table 5). Results of three tests of neutrality significantly rejected the null hypothesis of selective neutrality and constant population size, suggesting possible population growth for the L and TI-W lineage (all tests; mtDNA marker), in the TI-E2 haplogroup and the Cypriote haplotypes (Fu's F_S and R_2 tests), in the TI-E2a (Tajima's *D* and Fu's F_S tests), and in the mitochondrial marker in the whole TI clade (Fu's F_S test only).

The Bayesian skyline plots (BSP) provided a support for demographic growth (started ca. 0.1 – 0.2 Ma) in all main phylogeographic groups (Fig. 4a-c) according to the median values of population size estimates. However they did not give significant results, because the constant population size could not be rejected in all groups due to the wide 95% HPD interval. Times to most recent common ancestor (t_{MRCA}) of particular groups are shown in Table 3.

3.5.2. Hyla sp. nov.

The new species showed two well supported lineages in the mtDNA, the Yemeni (Y) and Jordanian-Syrian (JS) lineage. *SSD* and *r* statistics of the mismatch distribution indicated expansions in both groups (Table 5). However, graphs showed unimodal distribution in the Y lineage only, while the distribution was strongly bimodal in the JS lineage (Fig. 5a,b). On closer inspection, the latter lineage (JS) included two groups – four haplotypes from the northern part of the species range (JS1), and a well supported clade of three haplotypes from the southernmost localities in Jordan (Wadi Fidan, Wadi Mujib; JS2). Separate MMD analyses supported the expansion model for both groups (Fig. 5c,d) and the intermatch distributions (Fig. 5e) had a peak that did not match the MMD peaks, indicating that each groups expanded from a different ancestral population. The intermatch between the Yemeni haplogroup and the two JS haplogroups (Fig. 5e) also demonstrated a clear difference between the Arabian and Levant tree frog populations.

In general, the MMD of all groups/subgroups indicated population growth according to the *SSD* and raggedness *r* tests (except of the result of the *r* test for whole *Hyla* sp. nov. in tyrosinase; Table 5). On the contrary, results of the Tajima's *D*, Fu's F_S, and Ramos-Onsins and Rozas's R_2 tests showed no significant departure from the null hypothesis of selective neutrality and/or constant population size, with the exception of the Fu's F_S test in the mtDNA marker of the JS1 haplogroup.

The BSPs of the main phylogeographic lineages of *Hyla* sp. nov. did not provide significant results as the credible intervals allowed a variety of possible scenarios of demographic histories, both population expansion as well as constant population size or decline. However, the trend of the median values is rather constant or even indicates a declining population size (Fig. 4d,e). Median values of t_{MRCA} of different phylogeographic sets ranged between 0.2 – 0.3 Ma (Table 3).

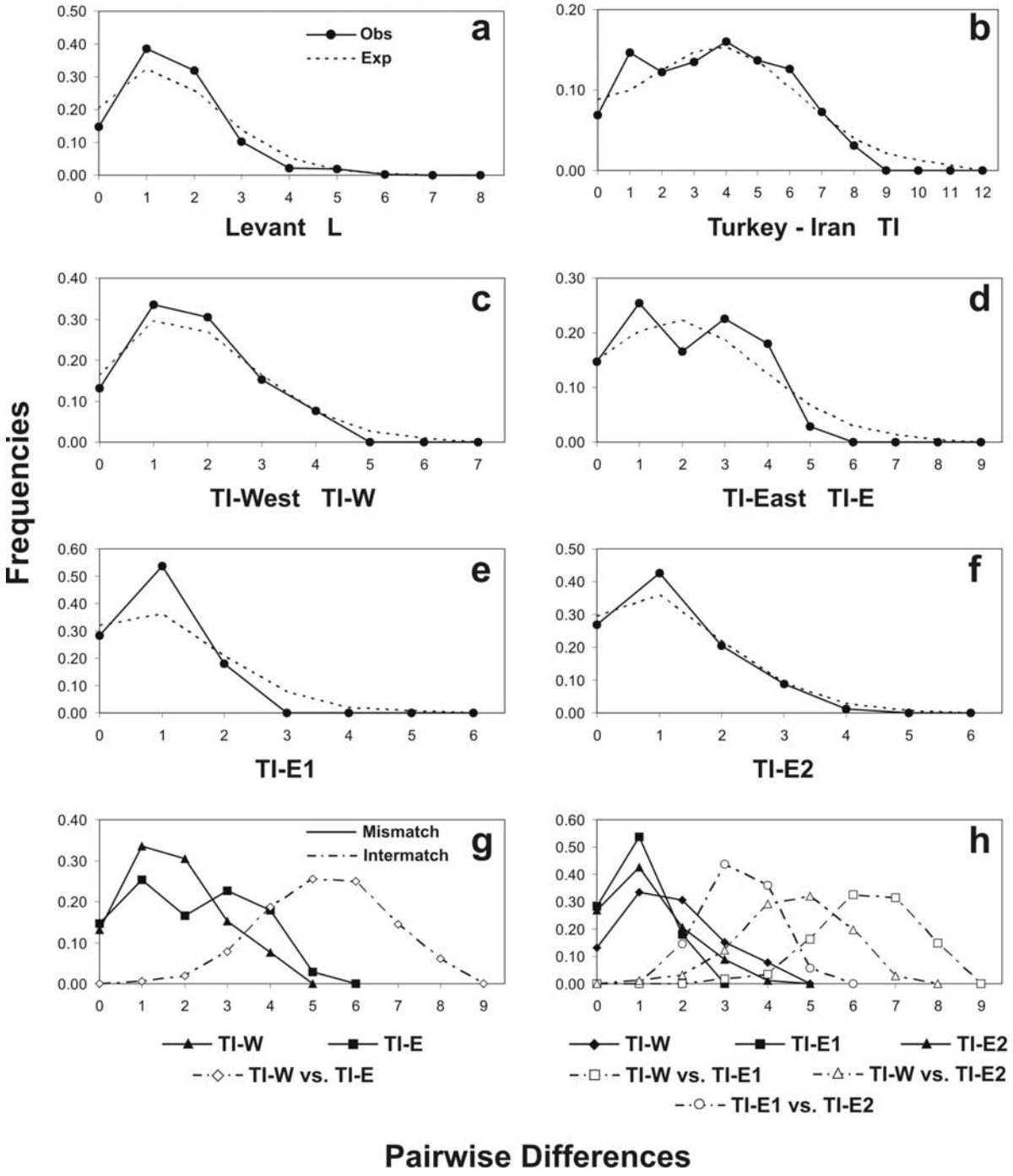

Fig. 3. Mismatch distributions for different population subsets of *H. savignyi* compared to the expected frequencies under the demographic expansion model. Intermatch distributions between different population subsets are also shown (g, h).

3.5.3. Hyla orientalis

According to the ML tree, *H. orientalis* may be subdivided into two haplogroups, the Caucasus-Caspian (CC) and Western Turkish (WT). The CC, however, forms a paraphylum, thus all studied samples of *H. orientalis* could be as well considered a single unit. The *SSD* and *r* tests of the MMD were not in contradiction with the expansion model neither in the whole *H. orientalis* data set nor in the two haplogroups separately. However, the scenario of a single unit is not well supported by the shape of the MMD, which showed a bimodal curve

(Fig. 6a). The two haplogroups examined separately showed a clear unimodal distributions in the mismatches (Fig. 6b,c), both concordant with the expansion model, but the CC population expanded more recently. A differently positioned peak in the intermatch (Fig. 6d) confirmed that at least two expansions from two different ancestral populations occurred within the studied samples of *H. orientalis*.

Partly discordant results were obtained by the neutrality tests, where only Fu's F_S significantly rejected the null hypothesis of selective neutrality and constant population size. Nevertheless, the Fu's F_S tests were significant in all groups and markers (with only exception in tyrosinase in *H. orientalis* as a single unit). The only other significant output was found in the Tajima's *D* test in rhodopsin within all *H. orientalis* samples.

According to the median values of population size estimates from the BSP, a population expansion started app. 0.25 Ma (Fig. 4f). However, they did not show significant results and the constant size model could not be rejected due to the wide 95% HPD interval. Time to most recent common ancestor (t_{MRCA}) of the CC haplogroup is estimated at ca. 0.4 Ma while that of the WT haplogroup or of all samples would be at ca. 0.9 – 1.0 Ma, respectively (Table 3).

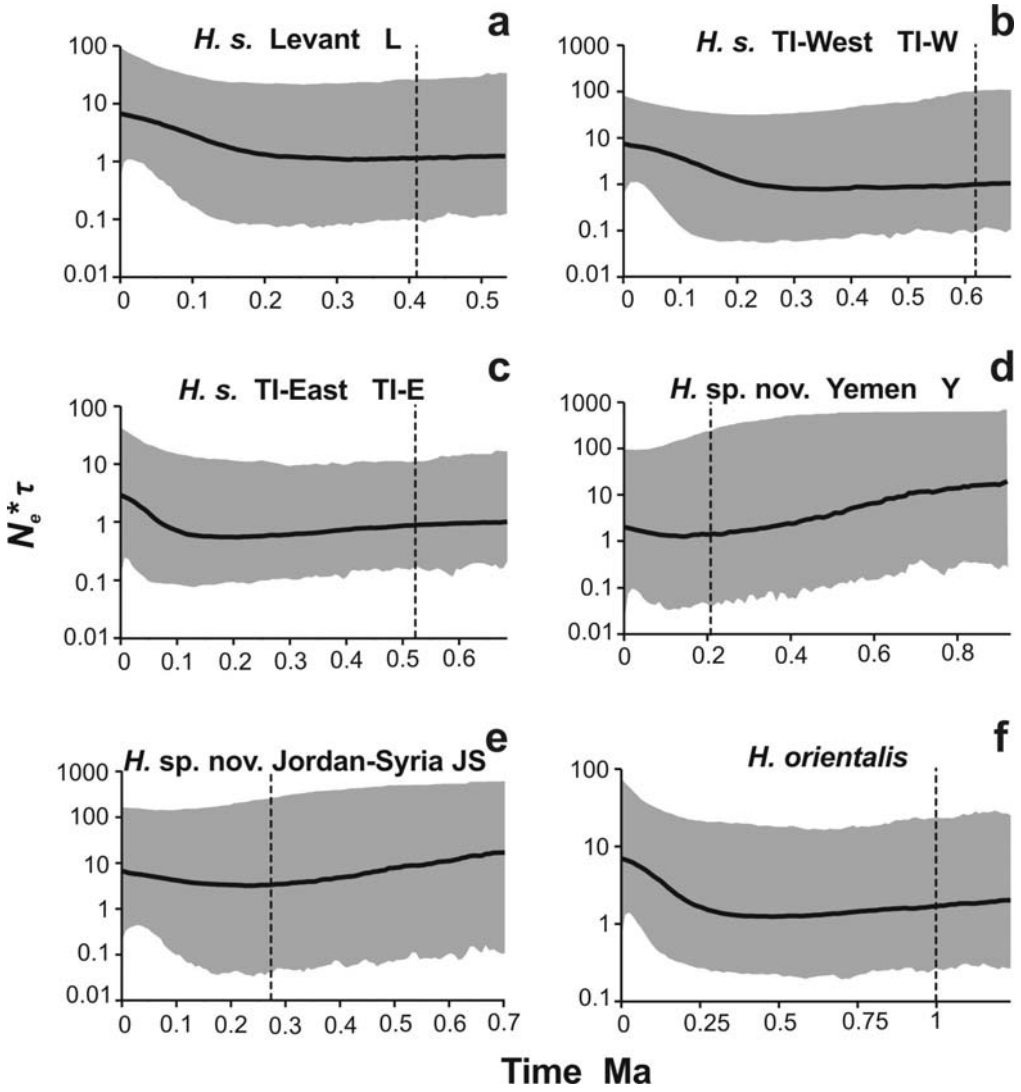

Fig. 4. Demographic history of main groups of *H. savignyi* (a–c), *Hyla* sp. nov. (d, e) and Middle Eastern populations of *H. orientalis* (f) as estimated with Bayesian skyline plots. Thick line shows the median value for the population size (N_e*τ; τ = generation length in units of time) on logarithmic scale and the shaded area represents the 95% highest posterior density.

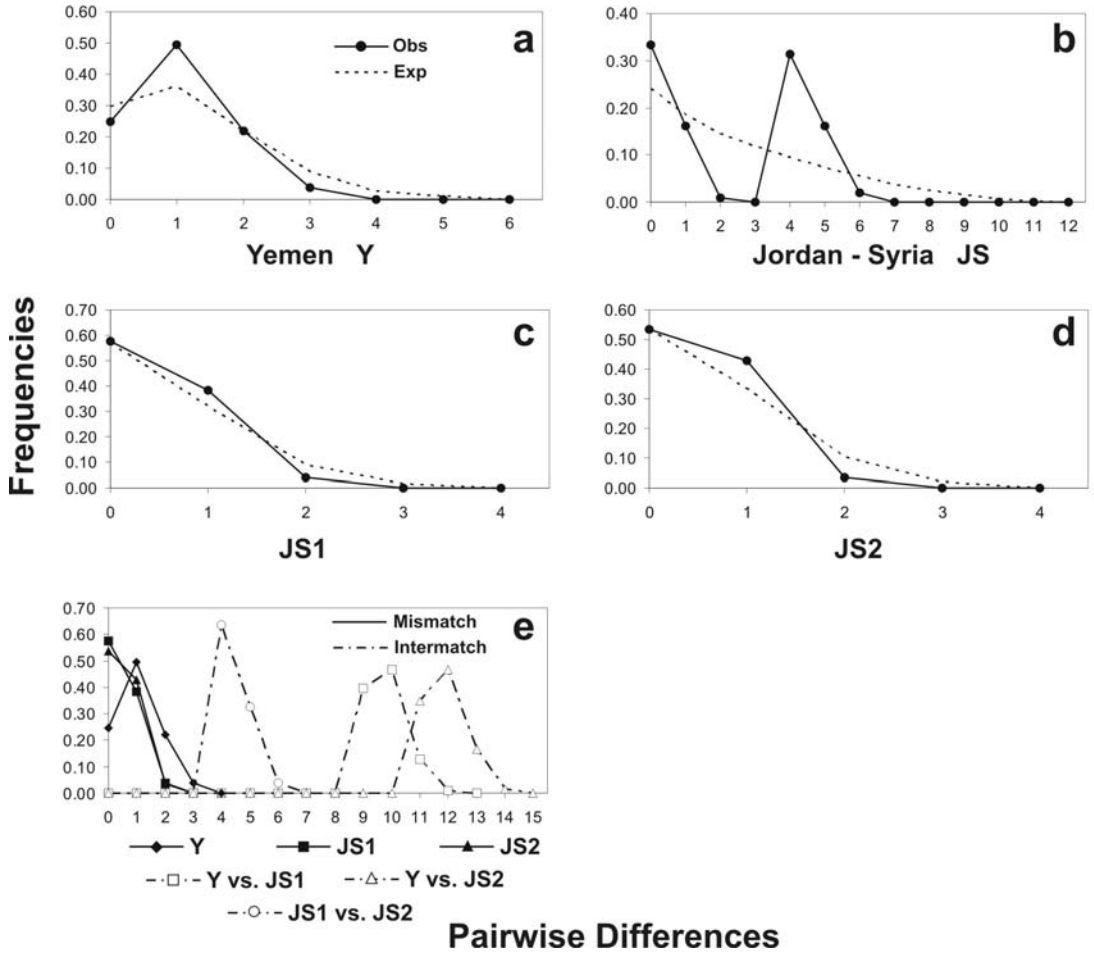

Fig. 5. Mismatch distributions for observed frequencies of different population subsets of *Hyla* sp. nov. compared to the expected frequencies under the demographic expansion model of population size. Intermatch distributions between different population subsets are also provided (e).

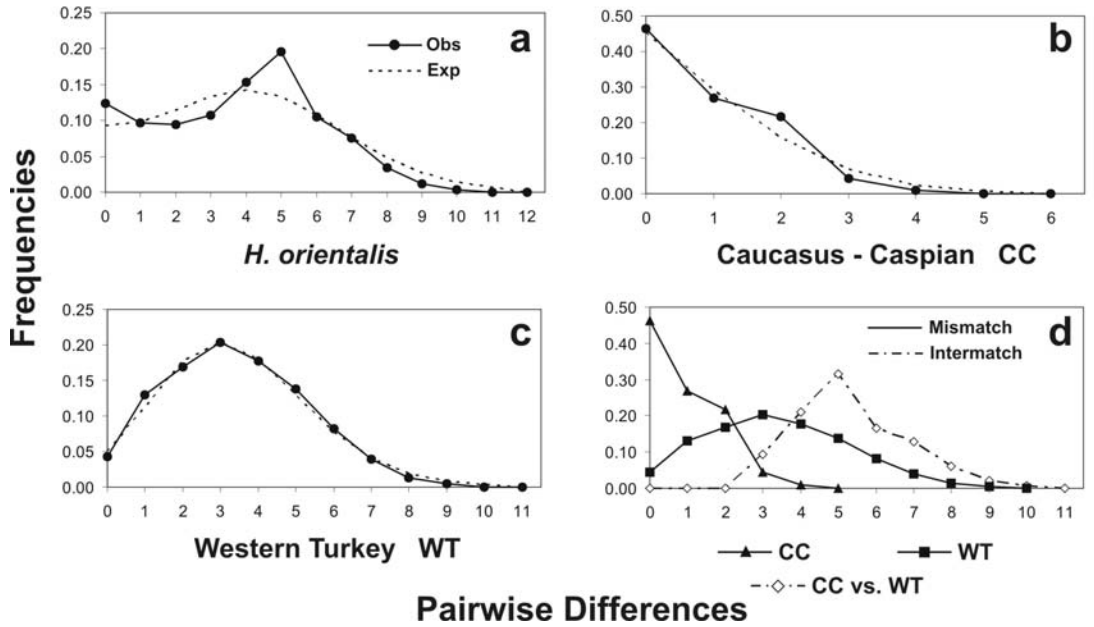

Fig. 6. Mismatch distributions for observed frequencies of *H. orientalis* populations under study (a) and their two main groups (b, c) compared to the expected frequencies under the demographic expansion model of population size. Intermatch distribution between the two groups is also provided (d).

Table 5. Summary of genetic polymorphism and tests of population expansion for different subsets of tree frog populations. Sample size (n), sequence length (L), number of different haplotypes (k), number of polymorphic – segregating – sites (S), nucleotide diversity (π), haplotype diversity (h), Watterson's theta per site (θ_W), Fu's F_S statistics (F_S), Tajima's D statistics (D), Ramos-Onsins and Rozas's R_2 statistics (R_2), SSD statistics for pure demographic (SSD_D) and range expansion model (SSD_R), Harpending's raggedness index statistics (r), and minimum number of recombination events in nuclear loci (R_{min}) are given. The interspecific hybrid population (Karkom, Israel) was not included in evaluation of nuclear markers, as well as the inter-lineage hybrid population of *H. savignyi* s.s. (Ovaçiftliği, Turkey) in evaluation at the population level. Mt = mitochondrial marker (12S/16S rRNA fragments concatenated); Rhod = rhodopsin; Tyr = tyrosinase; SD = standard deviation.

Species	Population	Locus	n	L+ (bp)	k++	S	π ± SD (%)	h ± SD	θ ± SD (%)	F_S	D	R_2	SSD_D	SSD_R	r	R_{min}
H. savignyi		mt	116	893	40	49	1.02±0.02	0.948±0.009	1.03±0.28	-8.856	-0.019	0.0903	-	-	-	
		Rhod	52		3	2	0.18±0.02	0.486±0.044	0.16±0.12	0.399	0.229	0.134	-	-	-	0
		Tyr	52		15	13	0.37±0.04	0.847±0.035	0.58±0.22	-7.786***/x	-1.105	0.067	-	-	-	1
	L	mt	55	895	17	18	0.17±0.02	0.852±0.034	0.44±0.16	-12.195***	-1.892**	0.040**	0.0132	0.0132	0.1157	
		Rhod	16		2	1	0.18±0.03	0.500±0.074	0.11±0.11	1.247	1.309	0.250	0.0219	0.0219	0.2500	0
		Tyr	16		6	5	0.22±0.06	0.675±0.117	0.30±0.17	-2.477*/x	-0.963	0.126	0.0039	0.0039	0.0798	1
	TI	mt	61	894	23	24	0.41±0.02	0.931±0.014	0.57±0.19	-10.170**	-0.919	0.070	0.0034	0.0065	0.0137	
		Rhod	34		3	2	0.14±0.03	0.383±0.086	0.18±0.13	-0.313	-0.377	0.119	0.0087	0.0087*	0.1890	0
		Tyr	34		10	10	0.39±0.04	0.875±0.028	0.49±0.21	-2.950	-0.636	0.093	0.0016	0.0016	0.0487	0
	TI-W	mt	29	896	13	13	0.19±0.03	0.869±0.043	0.37±0.15	-8.260***	-1.609*	0.059**	0.0041	0.0041	0.0777	
	TI-W-Cy	mt	15	896	6	5	0.10±0.02	0.705±0.114	0.17±0.10	-3.235**	-1.451	0.0949**	0.0302	0.0302	0.2171	
	TI-E	mt	32	894	10	10	0.24±0.02	0.853±0.035	0.28±0.12	-2.713	-0.455	0.097	0.0121	0.0137	0.0488	
	TI-E1	mt	13	896	4	3	0.10±0.02	0.718±0.089	0.11±0.07	-0.747	-0.227	0.153	0.0325	0.0325	0.2268	
	TI-E2	mt	19	894	6	5	0.11±0.02	0.702±0.080	0.16±0.09	-2.558*	-1.076	0.096*	0.0056	0.0056	0.0939	
	TI-E2a	mt	11	896	3	2	0.04±0.02	0.345±0.172	0.08±0.06	-1.246**	-1.430*	0.1928	0.0040	0.0040	0.2030	
	TI-E2b	mt	8	894	3	2	0.06±0.03	0.464±0.200	0.09±0.07	-0.999	-1.310	0.2165	0.0136	0.0136	0.1671	
Hyla sp. nov.		mt	36	896	11	19	0.70±0.04	0.848±0.036	0.51±0.19	1.181	1.211	0.1579	-	-	-	
		Rhod	28		2	1	0.03±0.02	0.071±0.065	0.09±0.09	-1.155	-1.151	0.186	-	-	-	0
		Tyr	28		6	6	0.34±0.04	0.714±0.055	0.31±0.15	-0.243	0.240	0.137	-	-	-	2
	Y	mt	15	896	5	4	0.12±0.02	0.752±0.076	0.14±0.08	-1.406	-0.476	0.129	0.0231	0.0231	0.1718	
	JS	mt	21	896	6	8	0.26±0.03	0.667±0.085	0.25±0.12	0.291	0.207	0.133	0.0986	0.0682	0.1955	
	JS1	mt	13	896	3	2	0.03±0.02	0.295±0.156	0.07±0.05	-2.206**	-1.468	0.1804	0.0068	0.0068	0.1583	
	JS2	mt	8	896	3	2	0.06±0.03	0.464±0.200	0.09±0.07	-0.999	-1.310	0.2165	0.0136	0.0136	0.1671	
H. orientalis		mt	44	895	21	25	0.42±0.04	0.876±0.044	0.64±0.22	-9.947**	-1.153	0.069	0.0066	0.0062	0.0162	
		Rhod	32		5	4	0.11±0.04	0.290±0.103	0.36±0.20	-3.873***	-1.740*	0.074	0.0060	0.0013	0.2563	0
		Tyr	32		10	11	0.61±0.07	0.798±0.057	0.55±0.23	-1.254	0.347	0.134	0.0419	0.0396	0.0709	1
	CC	mt	22	896	6	6	0.10±0.03	0.537±0.123	0.18±0.09	-2.552*	-1.469	0.091	0.0050	0.0031	0.0719	
	WT	mt	22	895	15	17	0.38±0.05	0.957±0.026	0.52±0.21	-8.374***	-0.999	0.085	0.0006	0.0009	0.0182	

+ Sequence length (bp); only mtDNA length without gaps is given; full aligned length of mtDNA = 899 bp, Rhod = 276 bp, Tyr = 496 bp

++ haplotypes differing only by deleted site(s) (mtDNA) are not considered here

x not significant if recombinations are considered

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; for Fu's FS P < 0.02 was taken as a threshold at the 5% level

4. Discussion

4.1. Phylogeny and species limits

Our study based on mitochondrial and nuclear DNA combined with a dense sampling covering wide areas of the Middle East and the Eastern Mediterranean substantially extends the knowledge of genetic variation of tree frogs in these regions. Two currently recognized species, *H. savignyi* s.l. and *H. orientalis*, were analyzed and confirmed to represent two reciprocally monophyletic lineages with 5.7 % uncorrected *p*-distances (12S+16S rRNA) separating them in mtDNA. Additionally, both also possess unique haplotypes of nuclear genes (with exception of *H. orientalis* from south-western Turkey, as discussed below). However, one further deep split (4.5 %, northern – southern groups) was confirmed within *H. savignyi* s.l. following our preliminary results (Gvoždík et al., 2007a) and in concordance with results of Stöck et al. (2008). According to the Bayesian coalescent analysis, both splits, that between the ancestors of *H. orientalis* and *H. savignyi* s.l. and that within the latter species, occurred during the late Miocene, 11.1 and 8.4 Ma, respectively. Such deep divergence suggests the existence of two distinct species rather than intraspecific divergence. We found also good agreement between the mitochondrial and nuclear markers, when the putative species carried unique diagnostic nuclear haplotypes. However, the southern group, which we describe in this paper as a new species, possesses the same or similar alleles of tyrosinase gene as a south-western Turkish population of *H. orientalis*. Because there is no direct relationships between the new species and *H. orientalis*, neither genetic nor geographic [mtDNA, rhodopsin (present study); RAG1 (Stöck et al., 2008); closest populations are in southern Syria and south-western Turkey, respectively], this is probably a case of retention of ancestral polymorphism due to incomplete lineage sorting at this gene (Avise and Robinson, 2008; Degnan and Rosenberg, 2009). Reciprocal monophyly in mtDNA and diagnostic exclusivity of nuclear markers, supported additionally by mutual parapatric distributions and by differences in phenotypes (acoustic, and to a lesser degree also morphological features; as described below) justify us to distinguish not two, but three species within the studied material, *H. savignyi*, *H. orientalis* and a new species which until now has been considered conspecific with *H. savignyi*. Further splits in mtDNA phylogeny of *H. savignyi* and of the new species into two distinct subclades in each species (L, TI and Y, JS) were not matched by nuclear loci. Two main alleles of rhodopsin and two haplotype groups of tyrosinase in *H. savignyi* do not closely match geographic distributions of the two main mtDNA haplogroups in this species. These results suggest firstly a recent break of gene flow between currently geographically isolated populations of the new species, and secondly, continued gene flow or incomplete lineage sorting between the main mtDNA haplogroups of *H. savignyi*. Moreover, we found three localities, where specimens from both mtDNA haplogroups were present and one individual of the Levant lineage was present within the distribution range of the Turkish-Iranian lineage (Fig. 2). Neither acoustic data for the two subgroups of *H. savignyi* (cf. Egiasarian and Schneider, 1990; Kaya and Simmons, 1999; Schneider and Nevo, 1972) provided any evidence of their differentiation, nor did a recent study about their morphological characters (Gvoždík et al., 2008). Thus, we suggest the subgroups of *H. savignyi* and *Hyla* sp. nov. be recognized as population units with possible importance for conservation, but without taxonomic status.

4.2. Taxonomic implications

In the following sections we provide a brief discussion of the taxonomic status of the tree frog species occurring in the Middle East and formally describe the new species.

4.2.1. Hyla savignyi

Detailed discussion of the original description, type specimen(s) and type locality was provided by Grach et al. (2007) and Schneider (2009). No type locality was stated in the original description (Audouin, 1827 “1809”) and different type localities have been listed by

various authors during the last century (see Frost, 2009). However, Grach et al. (2007) argued that the type specimen, a female depicted in the Supplement to the original description (and reproduced in Schneider, 2009), was probably collected somewhere in today's western Israel. As we sequenced specimens from western coastal Israel we can conclude that nominal *H. savignyi* belongs to the Levant clade. The new species is not known to occur in western Israel, neither in the Judean Hills (V. Gvoždík et al., new additional unpubl. data), which is the distribution area of genetically distinct *H. heinzsteinitzi* (Stöck et al., 2008), and we therefore describe it as a new species.

4.2.2. Hyla sp. nov. Gvoždík, Kotlík, Moravec

Synonymy. See Supplementary data.

Holotype. NMP6V 72076/1 (Fig. 7a,b), adult male, from 15 km SW of Matnah, 15°12' N, 43°59' E, 2790 m a.s.l., Governorate Sana'a, Yemen, collected by P. Benda and A. Reiter on 1 May 2004, GenBank Acc. Nos. GQ916741, GQ916785, GQ916814, GQ916706 (mt*fel*YE1, R*fel*1, T*fel*1).

Paratypes. Fifteen specimens from Yemen: NMP6V 72076/2, 7–8, 10–11, five subadult males and NMP6V 72076/3–6, 9, five subadult females (Fig. 7c,d), the same locality and collecting data as holotype; ZMH A04131, ZFMK 37039, adult female and male, Sana'a, collected by Rathjes and Wissmann in June 1931, and by Erdelen in 1980, respectively; ZFMK 42847, 42849, adult males, 31 km from Sana'a in direction to Hodeida, collected by Schütte and Fritz on 24 February 1985; ZFMK 32272, adult female, 130 km S of Sana'a, 2300 m a.s.l., collected by Erdelen in August 1980.

Referred material. See Supplementary data (morphologically examined material in the supplementary text and DNA analyzed material in Table S1).

Diagnosis and comparisons. Hyla sp. nov. is a medium sized member of the genus *Hyla* as revealed from general morphology and genetics, distinguished from other species by 1) genetic data, 2) acoustic data – advertisement calls, and 3) morphology.

1) *Hyla* sp. nov. occurs as a distinctive and monophyletic lineage in respect to sister *H. savignyi* (Fig. 1; Gvoždík et al., 2007a) and all Western Palearctic members of *Hyla* on the basis of mtDNA (Gvoždík et al., 2007b). It is distinguished from other Western Palearctic species (data not shown) by three diagnostic nucleotide substitutions in the studied 355 bp fragment of the 12S rRNA gene [position 167, T (thymine) → C (cytosine); position 263, A (adenine) → G (guanine); position 289, G → A (Yemeni lineage)/C (Jordanian-Syrian lineage); GenBank Acc. Nos. GQ916739, GQ916741–GQ916744] and by three unique nucleotide substitutions in the studied 544 bp fragment of the 16S rRNA gene (position 158, G → A; position 202, C/T → A; position 227, A/T → C; GenBank Acc. Nos. GQ916782–GQ916789). Monophyly and uniqueness were further confirmed on the basis of mitochondrial cytochrome oxidase I, III, tRNA Lysine, ATP synthase subunits 6 and 8, and cytochrome *b* genes (Stöck et al., 2008). In addition, nuclear DNA sequence data provide diagnostic haplotypes that clearly delimit *Hyla* sp. nov. from its sister species, *H. savignyi* (Fig. 2, Gvoždík et al., 2007a), and from all Western Palearctic species of tree frogs (Gvoždík et al., 2007b) as has been confirmed for rhodopsin, GenBank Acc. No. GQ916814, and for recombination associated gene 1, RAG1 (Stöck et al., 2008). The sequenced individual of *H. heinzsteinitzi* (from the type locality) possessed mtDNA of *H. japonica*, and thus, might represent an introduced population of this Far Eastern species (Stöck et al., 2008).

2) Acoustic data obtained at two localities in Jordan (Wadi Fidan, Wadi Mujib, 17.5 – 23 °C, n = 12) indicate that *Hyla* sp. nov. resembles *H. savignyi* (Ash Shuna, northern Jordan Valley, Jordan, 17 – 22 °C, n = 5) in a general structure of the advertisement call, although, compared at the same temperature, differs by shorter call segments (pulse group sensu Schneider, 2004) in the sense of number of pulses (mean 15.6, range 13 – 18 vs. 19.7, 19 – 23) as well as duration (mean 105 ms, range 85 – 124 ms vs. 150 ms, 127 – 188 ms) and slightly also by a higher dominant frequency (mean 3.2 kHz, range 2.8 – 3.7 kHz vs. 2.9 kHz,

2.7 – 3.1 kHz). The waveform of the call segment rises gradually (Fig. 8). The here reported advertisement call of Jordanian *H. savignyi* corresponds to the data for this species from different areas (Armenia: Egiasarian and Schneider, 1990; Turkey: Kaya and Simmons, 1999; Schneider, 2001; Israel: Schneider and Nevo, 1972; and V. Gvoždík, unpubl. data from Syria, Iran, Turkey, Cyprus). Advertisement calls of *H. heinzsteinitzi* differ by segments with energy peaks near their temporal beginning (Grach et al., 2007). Neither number of pulses nor dominant frequency were provided by the authors of the original description, however pulses seem to be rather blurred, indistinct, according to the published oscillograms.

3) Morphologically, *Hyla* sp. nov. differs from *H. savignyi* (character states in parentheses) by more truncate snout in lateral view (round in lateral view), snout barely protruding the anterior margin of maxilla in ventral view (markedly protruding the anterior margin of maxilla in ventral view), frequent disruption of dark line separating dorsal and ventral coloration on tibia and tarsus into the irregular marbling (usually straight dark line on tibia and tarsus), whitish outline of dorsal coloration reaching cloacal sheath because of reduced dark supracloacal streak or spot (dark horizontal supracloacal streak or spot separating cloacal sheath from whitish outline of the dorsal coloration usually present), frequent presence of an irregular longitudinal loop-like spot or streak in the groins connected in some cases to the dark lateral stripe (spots in the groins, if present, only rarely of a loop-like shape). The new species differs from *H. heinzsteinitzi* by absence of strong fragmentation of dark lateral stripe (dark lateral stripe highly disrupted into irregular spots; Grach et al., 2007).

Description of holotype and variation. Holotype measurements (mm): SVL 43.5; SUL 41.5; FmL 18.8; TbL 18.7; WL 9.2; T4L 16.2; T1L 4.5; IMTL 1.8; TrL 10.9; HW 14.8; HLt 12.0; ES 5.0; NL 3.3; IND 3.5; EAD 6.8; IOD 3.0; EPD 12.0; ED 4.1; TD 2.7. For detailed description of the holotype, measurements of the type series, morphometric variation of the Jordanian-Syrian lineage and further details see Supplementary data. The morphological variation of *Hyla* sp. nov. from the Arabian Peninsula was described under the name *H. savignyi* in Balletto et al. (1985) and Gvoždík et al. (2008, Supplementary material).

Karyotype. Al-Shehri and Al-Saleh (2005) described the karyotype of *Hyla* sp. nov. from Saudi Arabia and referred to it as *H. savignyi*. Martirosyan and Stepanyan (2007) provided comparison to the karyotype of *H. savignyi* from Armenia and found only slight difference in the karyotype formula, although diploid number of chromosomes, 2n = 24, was the same.

Tadpoles. Properly fixed larval stages are not at our disposal to allow detail morphological description. However, general morphology is similar to other Western Palearctic species of *Hyla* (V. Gvoždík, unpubl. field data).

Distribution, hybridization and ecology. In the Arabian Peninsula, *Hyla* sp. nov. inhabits the regions above 1400 m a.s.l. from about 21°30'N in the south-western Saudi Arabia to about 14°13'N in south-western Yemen (Balletto et al., 1985; Klütsch et al., 2004). In the Levant, *Hyla* sp. nov. seems to be distributed eastward the Dead Sea Rift (Wadi Arabah, Jordan Valley, Huleh Valley, Beqaa Valley), in which a contact and possible hybrid zone with *H. savignyi* is situated (new data from Israel, V. Gvoždík et al., unpubl. data). *Hyla* sp. nov. has been confirmed in the Levant in western Jordan, southern Syria and in extreme north-eastern Israel (Fig. 2). Nevo and Yang (1979) reported genetically distant tree frogs from the Golan Heights, which were apparently *Hyla* sp. nov.. Presence of the new species in south-eastern Lebanon in the Anti-Lebanon Mts. is expected.

The new species is distributed parapatrically to *H. savignyi* and the only sympatric and syntopic locality has been found in the southern Huleh Valley (Karkom, Israel), where hybridization was documented. Individuals with a mixture of alleles of different species among different loci and even within a single locus (Table 2) demonstrate that the hybrids detected were not F1 hybrids, which indicates that the population probably forms a part of

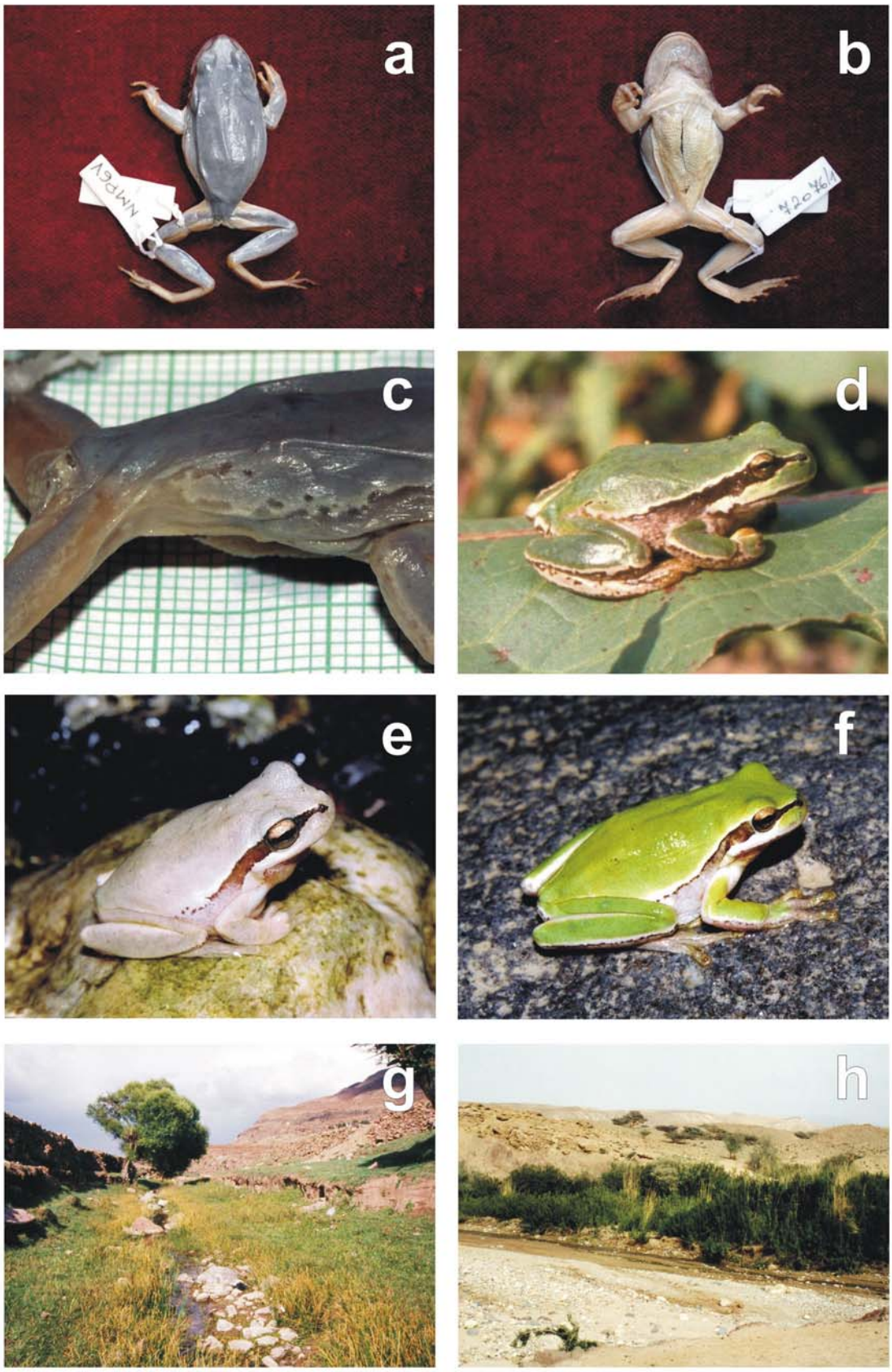

Fig. 7. *Hyla* sp. nov. Holotype, NMP6V 72076/1, adult male, from (a) dorsal and (b) ventral views. (c) Photograph demonstrating inguinal irregular longitudinal loop-like streak on the paratype female, NMP6V 72076/4. (d) Paratype subadult female in life, NMP6V 72076/6. Adult males from the southern Levant in (e) pale beige color phase, Wadi Fidan, Jordan, and (f) bright green color phase, Wadi Mujib, Jordan. (g) Type locality, 15 km SW of Matnah, 15°12' N, 43°59' E, 2790 m a.s.l., Governorate Sana'a, Yemen. (h) One of the southernmost localities in the Levant, isolated spring in the desert, Wadi Fidan, Jordan. Photographs (d) and (g) courtesy of A. Reiter.

a hybrid zone. However, a more detailed study of mutual distributional, evolutionary and ecological relationships is needed.

All type specimens were collected along a small stream springing forth the rocky mountain slope. By day, tree frogs were hidden in low herbaceous vegetation surrounding the stream. The close vicinity of the type locality (Fig. 7g) had a typical rocky character with few bush growths and trees. "*Bufo*" *arabicus* was the only amphibian species observed to occur syntopically with *Hyla* sp. nov. in this place (P. Benda, in verb.). The Jordanian-Syrian populations are usually connected with permanent or temporal wet habitats situated in deeper valleys and indicated typically by presence of the natural growths of oleanders (*Nerium oleander*). Less frequently, the new species was observed also in the surroundings of artificial ponds and tanks in settled desert regions (e.g. Busra as Sham, Syria). Some desert populations seem to be locally isolated and tied to small natural springs (e.g. Wadi Fidan in Wadi Arabah, Jordan).

Threat status. According to the sparse data available we here classify *Hyla* sp. nov. as "Data Deficient" according to the IUCN red list of threatened species criteria. The Jordanian-Syrian population might be threatened due to its limited distribution, especially then its marginal subpopulations located in the spring areas in the desert, which are often over-exploited by local settlers.

Etymology. The specific epithet is derived from the Latin *Felix Arabia* (Fruitful or Happy Arabia) – the expression used by ancient geographers to describe what is now modern-day Yemen.

Proposed English name. Arabian Tree Frog

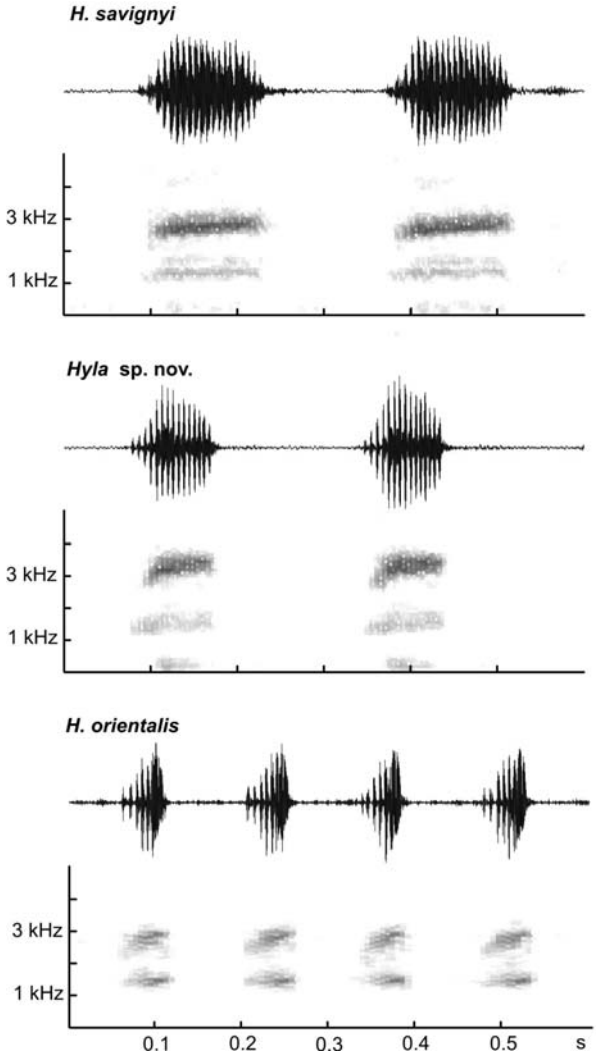

Fig. 8. Advertisement calls of *H. savignyi* (Ash Shuna, Jordan, loc. 20), *Hyla* sp. nov. (Wadi Fidan, Jordan, loc. 55) and *H. orientalis* (Telavi, Georgia, loc. 71) as represented by oscillograms and respective spectrograms of the call segments (pulse groups) at the same time scale (section of 0.6 s) and temperature (20 °C).

4.2.3. Hyla orientalis

Stöck et al. (2008) assigned species rank to the Pontic (and Middle Eastern) populations of *H. arborea* and resurrected the name *H. orientalis* Bedriaga, 1890 for them. We found that all our *H. orientalis* samples form a compact cluster with substantial genetic variation, although without any deep divergences. A sample from the type locality of *H. arborea schelkownikowi* (Kutaisi, Georgia) as well as other samples from the Caucasus area carried alleles closely related to alleles of the Western Anatolian tree frogs, which were by Stöck et al. (2008) shown to be *H. orientalis*. In addition, the specimen from the type locality of *H. arborea schelkownikowi* possessed the same haplotypes as specimens originated from the area close to the type locality of *H. arborea gumilevskii*, previously synonymized with *H. orientalis* (Stöck et al., 2008). Thus, we consider *H. arborea schelkownikowi* Chernov, 1926 as a junior subjective synonym of *H. orientalis*.

4.3. Historical biogeography

The three studied species occur in mutual parapatry. In our phylogeny, *H. orientalis* seems to be the sister species to the clade comprising *H. savignyi* and *Hyla* sp. nov.. However, we did not include other European species (except of *H. meridionalis*), which appear phylogenetically more closely related to *H. orientalis*, such as *H. arborea* (Gvoždík et al., 2007b; Stöck et al., 2008), and therefore we cannot consider *H. orientalis* as a sister taxon to *H. savignyi-Hyla* sp. nov.. Nevertheless, time to their common ancestor dated back to the late Miocene, which may indicate that ancestors of these two lineages could be separated by the Paratethys Sea and today's *H. orientalis* could reach Anatolia and Caucasus from the north, and getting there into secondary contact with *H. savignyi*. This hypothesis needs to be further tested with more extensive sampling including European populations of tree frogs.

The other deep split in our tree, that between *H. savignyi* and *Hyla* sp. nov., dated back also to the late Miocene could be coincident with formation of the Dead Sea Rift (Garfunkel, 1988; Westaway, 2003; Zain Eldeen et al., 2002). *H. savignyi* has been restricted westward the Rift while *Hyla* sp. nov. eastward. Nowadays, they probably meet each other at various places in the Rift, where they can hybridize as was confirmed from the Huleh Valley.

Major intraspecific splits within the two species coincide with the Plio-Pleistocene boundary. The division within *H. savignyi* separated the Levant and the Turkish-Iranian (including Cyprus) lineages, while within *Hyla* sp. nov. the Jordanian-Syrian and the Yemeni lineages. If the split in the latter coincides with the current isolation of the Arabian population, which has started with aridification in the north-western Arabian Peninsula only about 5000 – 6000 years ago (Davies, 2006; Klütsch et al., 2004), or if the split occurred within the southern Arabian Peninsula is yet an unresolved issue. It is necessary to study samples from Saudi Arabia to answer this question. The genetic structuring within the lineages presumably mirrors the Pleistocene climate fluctuations, which were in the Middle East characterized by periods of cold dry (corresponding to the glacial periods in Europe) and warm wet (corresponding to the interglacials) climates (Abed and Yaghan, 2000). During the cold and dry periods tree frogs' populations were restricted into glacial refugia with suitable climate and habitats, such as in deep valleys. The TI lineage of *H. savignyi* is structured into the western subpopulation with a higher genetic variation and several eastern subpopulations, west and east of the Euphrates River, respectively. The Last Glacial was cold and arid in this region (Çiner, 2004; Stevens et al., 2001; Wick et al., 2003; Wright, 2004) and corresponding glacial refugia with suitable conditions were probably located in southern coastal Anatolia, Transcaucasia, Mesopotamia and south-western Persia, as can be judged from the genetic variation. The Cypriote population seems to be a result of recent colonization from southern Anatolia (t_{MRCA} = 0.27 Ma), which originated possibly by accidental transfer by ancient human or by natural overseas dispersal as there was no land bridge between the mainland and Cyprus during the Pleistocene (Böhme and Wiedl, 1994; Marra, 2005). The Jordanian-Syrian lineage is composed of two subgroups that are possibly derived from two distinct refugia

where tree frogs probably survived the cold and dry climatic phase of the last glacial maximum (Abed and Yaghan, 2000; Robinson et al., 2006), one subgroup is found in southern Jordan wadis and another in the Jordanian-Syrian boundary area.

We are not aware of any other vertebrate species with similar phylogeographic pattern to *H. savignyi – Hyla* sp. nov.. Within mice, genus *Mus*, some similarities might be seen in the Yemeni taxon *M.* (*musculus*) *gentilulus*, which forms a distinct clade in respect to all other *Mus* taxa (Prager et al., 1998). It is probably distributed northward as far as the Dead Sea, although the detailed phylogeographic pattern is not known. A similar area for the zone of parapatry within the southern Levant may be observed in two species of bats, the Arabian *Hypsugo ariel* and its Mediterranean vicariant, *H. savii* (Benda and Aulagnier, 2008; Hutson et al., 2008). Some similarities in the phylogeographic pattern of *H. savignyi* might be seen in the pattern of *Mus macedonicus* with a distinct Levant lineage *M. m. spretoides* (Macholán et al., 2007). However, this mouse lineage is restricted only to the Levant and is not spread northward as in the case of the Levant *H. savignyi*. In contrast, the situation of Cypriote tree frogs, which phylogenetically relate to the southern Anatolian populations, is completely different to that of *Mus*. Cypriote mice form a distinct clade, a separate species, *M. cypriacus* (Macholán et al., 2007). Among amphibians similar separate position of Cyprus has also been demonstrated in water frogs, *Pelophylax* sp. (Lymberakis et al., 2007; Plötner et al., 2001; Plötner, 2005), while the phylogeographic position of the green toads, *Pseudepidalea variabilis*, is similar to that of tree frogs (Stöck et al., 2006). Overall phylogeography of these two within the Middle East co-distributed anuran species is different both between each other and in respect to *Hyla* phylogeography. *Pelophylax* cf. *bedriagae* seems to be highly structured with several diverged lineages, while *Pseudepidalea variabilis* occurs in the region as a single evolutionary lineage. *H. savignyi* is intermediate in this respect with its two main lineages in the region.

According to the current fragmented knowledge, *H. orientalis* is a species with Pontic affinity (Gvoždík et al., 2007b; Stöck et al., 2008). It is coming into contact with *H. savignyi* in Anatolia, in the Caucasus, and as we have shown also in Persia. Tree frogs from the southern Caspian coast are clearly assignable according to all molecular markers to *H. orientalis*. This species occurs in Iran in the Caspian lowlands, while *H. savignyi* is distributed southward the Alborz Mts' ridge. If they come into contact somewhere in Iran is presently unknown. The Caspian population of *H. orientalis* is presumably geographically isolated from the Caucasian populations, but genetically the two populations are very close. The easternmost examined Caspian samples possess unique and diverse haplotypes suggesting this region was a possible glacial refugium. The Caucasus-Caspian cluster is mixing with the Western Turkish group in north-eastern Turkey, which is a similar pattern to that of the Caucasian brown frog, *Rana macrocnemis* (Veith et al., 2003). Anatolia formed probably other important glacial refugia for *H. orientalis* as a substantial genetic variation was found there.

Similarities in the mutual distributional pattern of *H. orientalis* vs. *H. savignyi* in Asia Minor and Caucasus can be detected e.g. in tortoises *Testudo graeca ibera* vs. *T. g. terrestris* (Fritz et al., 2007) or bats *Miniopterus s. schreibersii* vs. *M. s. pallidus* (Furman et al., 2009). However, most of phylogeographically studied vertebrates possessed a different pattern, including another amphibious vertebrate, the *Mauremys* terrapin: *M. rivulata* occupies the Mediterranean coastal regions from the Balkans as far as the Levant, while *M. caspica* is distributed inland of Asia Minor and further eastward (Fritz et al., 2008).

See also Supplementary data for several distributional notes.

4.4. Demographic history

4.4.1. Hyla savignyi

According to Fu's F_S, as the most powerful neutrality test for detecting expansions on non-recombining genomic regions (Ramírez-Soriano et al., 2008), the two main lineages (L,

TI) might have undergone expansions, which was found to be in concordance with the reconstruction of population size histories as inferred by the BSPs. Within the Turkish-Iranian lineage, past population expansion was detected in the western group (TI-W), while within the eastern group the neutrality tests suggested population growth only for the TI-E2 subgroup, and, in particular, for the TI-E2a (Mesopotamian) population. Population growth was detected by the neutrality tests also in the Cypriote population. The BSPs suggested similar ages of the beginning of the expansions for the L and TI-W at around 0.2 Ma, while the TI-E, or the Mesopotamian subgroup respectively, started to expand at about 0.1 Ma. The Levant group had probably its refugia in the coastal plain of the eastern Mediterranean, and when started to expand it dispersed along the Euphrates River valley northward, where it probably separated the western and eastern groups of the TI lineage. The TI-W group had its refugia during climatically unfavorable periods of the Pleistocene most likely in the southern coastal Anatolia, from where it colonized Cyprus. The TI-E group expanded mostly in the Mesopotamian plain, where a suitable climate persisted during the Pleistocene (Wright, 2004), while periodically glaciated Iranian Highlands (Stevens et al., 2001) and the Caucasus region (Gobejishvili, 2004) probably did not allow significant expansion of local tree frogs' populations.

4.4.2. Hyla sp. nov.

In contrast to *H. savignyi*, the new species did not show significant signs of a population growth in most of the methods used. The only exception was the population from the northernmost part of the species range, from the Jordanian-Syrian border area (JS1). This population might have undergone slight population expansion in environmentally suitable areas of this eastern Mediterranean region. In contrast, the southern populations have probably remained constant or even declined in size during the late Pleistocene, as suggested by the BSP (Fig. 4), facing the arid and cold climate during the last glacial maximum (Abed and Yaghan, 2000). Time to the most recent common ancestor of haplotypes of the two main lineages (0.21 – 0.27 Ma) is approx. 10 x smaller than that of divergence between them (2.0 Ma), which may suggest that both lineages experienced bottlenecks during the Pleistocene.

4.4.3. Hyla orientalis

H. orientalis populations from the studied area probably underwent population expansion as was shown by significant results of all three different approaches. The growth began approx. 0.25 Ma as evidenced by the BSP, nevertheless, the modes of the mismatch distributions suggested that the Western Turkish group started to grow earlier in size than the Caucasus-Caspian group. It can be inferred from the phylogenetic tree that the WT group could have originated from the Caucasus region and afterwards expanded throughout western Anatolia, where it would have survived the last glacial maximum in several refugia [as demonstrated in, e.g. *Rana macrocnemis* (Veith et al., 2003)], while the CC group might have survived in refugia in Transcaucasia and its expansion therefore probably started later due to harder climate in the Caucasus during the late Pleistocene (Gobejishvili, 2004).

4.4.4. The Middle East

We detected clear signals of population expansions in *H. savignyi* and *H. orientalis*, but no distinct expansion in the most southerly distributed species, *Hyla* sp. nov. could be found. The few studies on demographic histories of vertebrates in the region usually found population growth as the best-fit model, as documented in bats (Furman et al., 2009), insectivores (Dubey et al., 2007a), rodents (Gündüz et al., 2005, 2007; Macholán et al., 2007), or other anurans (Stöck et al., 2006; Veith et al., 2003). Contrary to the former, but consistent to our results of *Hyla* sp. nov., no particularly strong signals of expansion were detected in the eastern (mostly Anatolian) lineage of the shrew *Crocidura leucodon* (Dubey et al., 2007b), and in the ground squirrel *Spermophilus taurensis* (Gündüz et al., 2007). The former is

hypothesized to have subsisted in several refugia from which the subpopulations expanded only at a regional scale, while the second is a restricted taxon from the Taurus Mts., where the species has stayed localized without any strong population expansion. The first case of several geographically distant refugia could be applicable also to our case of *Hyla* sp. nov.. Our study, therefore, adds to a growing body of evidence that refugial and speciation properties of the Middle East may derive from its topographic variety, which allows habitats and lineages to persist by latitudinal shifts and also diverge because of distributional restrictions. Conditions during the glacial periods were colder and drier than at present, extending deserts and steppe while reducing warm wet habitats, hence species associated with different environments would respond differently. Therefore, we suggest that compararative phylogeographic studies of a wide variety of species and fine-scale sampling, as we have described here, in connection with the use of molecular data will be the only way to reconstruct post-glacial colonization in the Middle East in detail.

5. Conclusions

Our study of tree frogs in the Middle East using mitochondrial and nuclear sequence data in combination with a phylogeographic approach has discovered a new species, *Hyla* sp. nov., which is distributed in the Arabian Peninsula and southern Levant, eastward the Dead Sea Rift. This points to a biogeographic connection between south-western Arabia and southern Levant, and highlights the importance of the Dead Sea fault system, which probably played a primary role as a barrier when formed in the late Miocene. Genetic structure of the new species as well as *H. savignyi* consists of two main mitochondrial lineages in each species, which originated presumably during the Plio-Pleistocene boundary. However, persisting gene flow and/or incomplete lineage sorting resulted in discordant intraspecific phylogeographic pattern of the nuclear markers. The Anatolian and Caucasus-Caspian populations of *H. orientalis* demonstrated high genetic variation suggesting these regions were important Pleistocene refugia. However, it will be necessary to study also European populations to infer complete evolutionary history of this species, which will be a subject of a forthcoming study.

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SUPPLEMENTARY DATA to

Phylogeography of the Middle Eastern tree frogs (*Hyla*, Hylidae, Amphibia) as inferred from nuclear and mitochondrial DNA variation, with a description of a new species

by

Václav Gvoždík, Jiří Moravec, Cornelya Klütsch, Petr Kotlík

Fig. S1. Map of localities of *H. savignyi* s.l. (circles) and *H. orientalis* (triangles) in the Middle East with the distribution area of *H. savignyi* s. l. as originally thought when the study started. However, the localities 72–75 harbor *H. orientalis* and not *H. savignyi* as inferred in this study on the genetic basis. Localities 52–66 harbor *Hyla* sp. nov., and the locality 67 is occupied by a hybrid population between *H. savignyi* s.s. and *Hyla* sp. nov. Stars indicate putative type locality of *H. savignyi* and type locality of *H. arborea schelkownikowi*, respectively.

Table S1. Specimens examined, localities (numbers correspond to those in Fig. S1), museum voucher numbers (reference if sample from GenBank), corresponding mitochondrial group, and haplotype names of the mitochondrial marker (12S/16 S rRNA fragments concatenated) and nuclear loci (rhodopsin and tyrosinase; alleles A and B). Holotype of *Hyla* sp. nov. (loc. 63), putative type locality of *H. savignyi* (loc. 19), type locality of *H. arborea schelkownikowi* (loc. 69) and region close to the type locality of *H. arborea gumilevskii* (loc. 72) are in bold. For GenBank accession numbers of the haplotypes and composition of mitochondrial concatenated haplotypes see Table S2.

Table S2. GenBank accession numbers of the haplotypes and composition of mitochondrial composite haplotypes.

Supplementary data to the description of *Hyla* sp. nov. Gvoždík, Kotlík, Moravec (including **Table S3**).

Fig. S1. Map of localities.

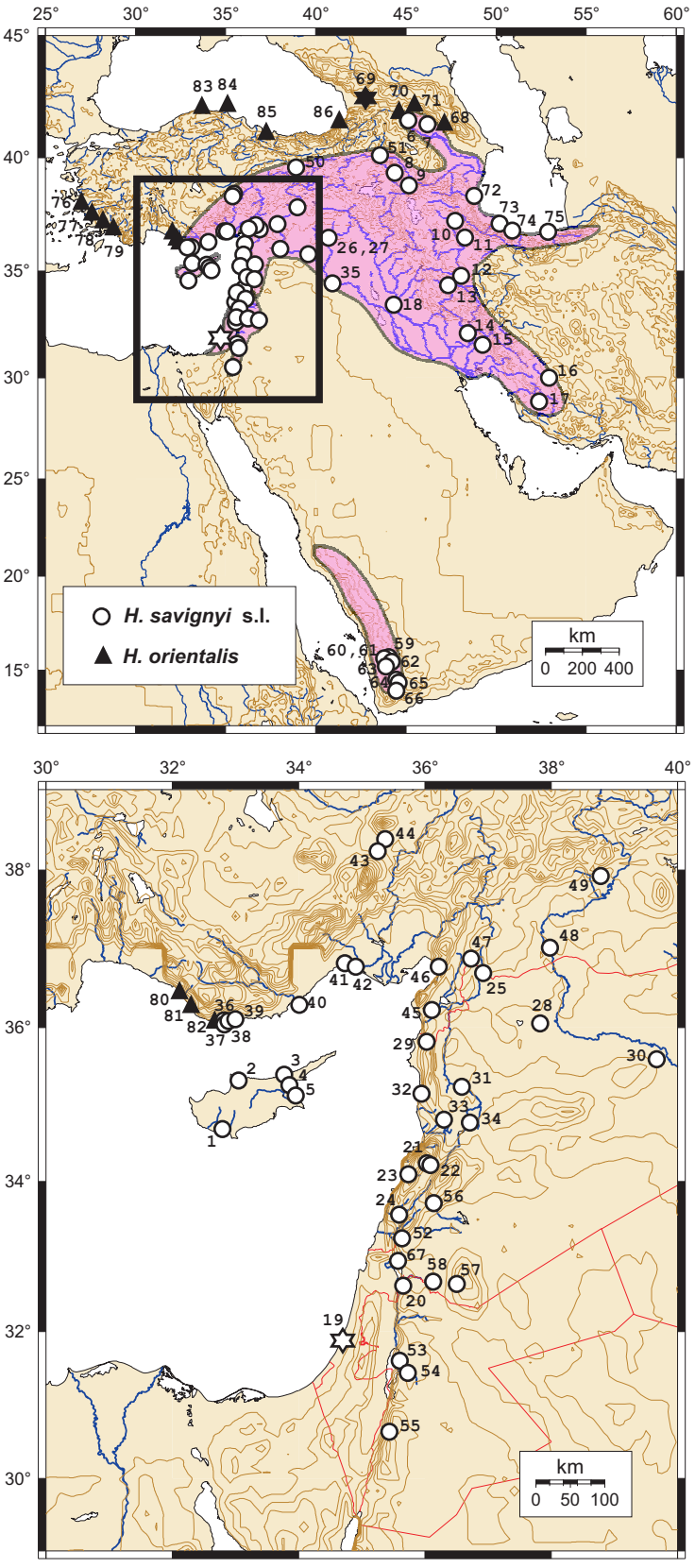

Table S1. Specimens examined.

Hyla	Sample ID	Map	Country	Locality	N	E	Museum/Reference	mt group	mtDNA concatenated	Haplotype Rhodopsin A	Rhodopsin B	Tyrosinase A	Tyrosinase B
savignyi	H262	1	Cyprus	Paramali, 3 km SE	34.70	32.80	NMP6V 72584/1	TI-W	mtsav CY2	-	-	-	-
savignyi	H263	1	Cyprus	Paramali, 3 km SE			NMP6V 72584/3	TI-W	mtsav CY4	-	-	-	-
savignyi	H264	1	Cyprus	Paramali, 3 km SE			NMP6V 72584/4	TI-W	mtsav CY5	-	-	-	-
savignyi	H110	2	Cyprus	Geçitköy (Panagra)			NMP6V 72102/4	TI-W	mtsav TR1	-	-	-	-
savignyi	H108	2	Cyprus	Geçitköy (Panagra)	35.34	33.07	NMP6V 72102/1	TI-W	mtsav CY2	-	-	-	-
savignyi	H109	2	Cyprus	Geçitköy (Panagra)			NMP6V 72102/2	TI-W	mtsav CY3	-	-	-	-
savignyi	H078	3	Cyprus	Yalı (Neraides)	35.39	33.75	NMP6V 71572/1	TI-W	mtsav CY2	-	-	-	-
savignyi	H079	3	Cyprus	Yalı (Neraides)			NMP6V 71572/2	TI-W	mtsav CY2	-	-	-	-
savignyi	H080	3	Cyprus	Yalı (Neraides)			NMP6V 71572/3	TI-W	mtsav CY2	-	-	-	-
savignyi	H076	4	Cyprus	Sınırüstü (Sygkrasi)	35.28	33.85	NMP6V 71571/1	TI-W	mtsav CY2	-	-	-	-
savignyi	H077	4	Cyprus	Sınırüstü (Sygkrasi)			NMP6V 71571/2	TI-W	mtsav CY1	-	-	-	-
savignyi	H062	5	Cyprus	Gazimağusa (Ammochostos)	35.13	33.95	NMP6V 72539/1	TI-W	mtsav CY1	Rsav 1	Rsav 1	Tsav 4	Tsav 4
savignyi	H072	5	Cyprus	Gazimağusa (Ammochostos)			NMP6V 71570/1	TI-W	mtsav CY1	-	-	-	-
savignyi	H063	5	Cyprus	Gazimağusa (Ammochostos)			VG H063	TI-W	mtsav CY2	-	-	-	-
savignyi	H073	5	Cyprus	Gazimağusa (Ammochostos)			NMP6V 71570/2	TI-W	mtsav CY2	-	-	-	-
savignyi	H425	6	Georgia	Rustavi, Mariin Channel	41.49	45.12	-	TI-E1	mtsav IR5	Rsav 1	Rsav 1	Tsav 2	Tsav 14
savignyi	H426	6	Georgia	Rustavi, Mariin Channel			-	TI-E1	mtsav IR5	-	-	-	-
savignyi	H427	6	Georgia	Rustavi, Mariin Channel			-	TI-E1	mtsav IR5	-	-	-	-
savignyi	HC394	6	Georgia	Rustavi, Mariin Channel			ZFMK coll. (CK H394)	TI-E1	mtsav IR5	Rsav 1	Rsav 1	Tsav 2	Tsav 12
savignyi	H402	7	Georgia	Dedop'lis Tskaro	41.43	46.10	-	TI-E1	mtsav GE1	-	-	-	-
savignyi	H404	7	Georgia	Dedop'lis Tskaro			-	TI-E1	mtsav GE1	Rsav 1	Rsav 1	Tsav 2	Tsav 12
savignyi	H504	8	Iran	Bazargan	39.38	44.45	-	TI-E1	mtsav TR13	-	-	-	-
savignyi	H241	9	Iran	Qareh Ziya Eddin, 12 km E	38.89	45.02	-	TI-E1	mtsav IR5	Rsav 1	Rsav 1	Tsav 2	Tsav 12
savignyi	H242	9	Iran	Qareh Ziya Eddin, 12 km E			-	TI-E1	mtsav IR5	-	-	-	-
savignyi	H243	9	Iran	Qareh Ziya Eddin, 12 km E			-	TI-E1	mtsav IR6	-	-	-	-
savignyi	H246	10	Iran	Mianeh, 10 km W	37.41	47.72	-	TI-E2a	mtsav SY5	Rsav 1	Rsav 1	Tsav 13	Tsav 13
savignyi	H247	10	Iran	Mianeh, 10 km W			NMP6V 72668/2	TI-E2a	mtsav SY5	-	-	-	-
savignyi	H248	10	Iran	Mianeh, 10 km W			NMP6V 72668/1	TI-E2a	mtsav IR7	-	-	-	-
savignyi	H391	11	Iran	Sa'idabad, 7km E	36.38	48.18	NMP6V 72830	TI-E2b	mtsav IR2	-	-	-	-
savignyi	H180	12	Iran	Alanje	34.75	47.97	CUP\AMPH\IRA\304	TI-E2b	mtsav IR2	Rsav 1	Rsav 2	Tsav 5	Tsav 11
savignyi	H179	13	Iran	Bisotun	34.40	47.43	CUP\AMPH\IRA\306	TI-E2b	mtsav IR2	Rsav 1	Rsav 2	Tsav 5	Tsav 5
savignyi	H182	14	Iran	Choqa Zanbil	32.02	48.53	CUP\AMPH\IRA\337	L	mtsav SY4	Rsav 1	Rsav 1	Tsav 2	Tsav 15[a]
savignyi	H181	15	Iran	Simili	31.70	49.40	CUP\AMPH\IRA\314	TI-E2b	mtsav IR2	Rsav 1	Rsav 3	Tsav 10	Tsav 10
savignyi	H183	15	Iran	Simili			CUP\AMPH\IRA\315	TI-E2b	mtsav IR4	-	-	-	-
savignyi	H066a	16	Iran	Sivand	30.06	53.03	VG H066	TI-E2b	mtsav IR1	Rsav 2	Rsav 2	Tsav 5	Tsav 5
savignyi	H067	16	Iran	Sivand			VG H067	TI-E2b	mtsav IR2	-	-	-	-
savignyi	H177	17	Iran	Firuz Abad	28.93	52.53	CUP\AMPH\IRA\411	TI-E2b	mtsav IR3	Rsav 2	Rsav 2	Tsav 10	Tsav 10
savignyi	HC094	18	Iraq	Bagdad	33.50	44.33	ZFMK coll. (CK H094)	TI-E2a	mtsav SY5	-	-	-	-
savignyi	HC095	18	Iraq	Bagdad			ZFMK coll. (CK H095)	TI-E2a	mtsav IQ1	-	-	-	-
savignyi	HC096	18	Iraq	Bagdad			ZFMK coll. (CK H096)	L	mtsav TR14	-	-	-	-
savignyi	H341	**19**	**Israel**	**Rishon LeZiyon, 5km SW**	31.93	34.71	-	L	mtsav JO2	-	-	-	-
savignyi	H342b	**19**	**Israel**	**Rishon LeZiyon, 5km SW**			-	L	mtsav JO2	Rsav 1	Rsav 2	Tsav 1	Tsav 1
savignyi	H344	**19**	**Israel**	**Rishon LeZiyon, 5km SW**			-	L	mtsav LB1	-	-	-	-
savignyi	H345	**19**	**Israel**	**Rishon LeZiyon, 5km SW**			-	L	mtsav JO2	-	-	-	-
savignyi	H090	20	Jordan	Ash Shuna	32.61	35.61	VG H090	L	mtsav JO1	Rsav 2	Rsav 2	Tsav 6	Tsav 7
savignyi	H091	20	Jordan	Ash Shuna			NMP6V 72087/1	L	mtsav JO2	Rsav 2	Rsav 2	Tsav 1	Tsav 1
savignyi	H092	20	Jordan	Ash Shuna			NMP6V 72087/3	L	mtsav JO3	-	-	-	-
savignyi	H093	20	Jordan	Ash Shuna			NMP6V 72087/2	L	mtsav LB1	-	-	-	-
savignyi	H360	21	Lebanon	Kadisha Valley, S slope	34.24	36.00	NMP6V 74015/1	L	mtsav LB2	-	-	-	-
savignyi	H361	21	Lebanon	Kadisha Valley, S slope			NMP6V 74015/2	L	mtsav LB2	-	-	-	-
savignyi	H037	22	Lebanon	Bsharri	34.22	36.05	NMP6V 70517	L	mtsav LB1	-	-	-	-
savignyi	H465	22	Lebanon	Bsharri			-	L	mtsav LB3	-	-	-	-
savignyi	H466	23	Lebanon	Adonis, Nahr Ibrahim	34.08	35.73	NMP6V 74018	L	mtsav LB2	-	-	-	-
savignyi	H464	24	Lebanon	Jezzine, Pont Al Khalass	33.55	35.60	NMP6V 74016	L	mtsav LB1	-	-	-	-
savignyi	H499	25	Syria	Cyrrhus, Sabon River	36.74	36.95	-	L	mtsav SY7	-	-	-	-
savignyi	H102	26	Syria	Al Hasakah 1	36.48	40.75	NMP6V 72100	TI-E2a	mtsav SY5	Rsav 1	Rsav 1	Tsav 1	Tsav 1
savignyi	H103	27	Syria	Al Hasakah 2	36.48	40.75	NMP6V 72101/1	TI-E2a	mtsav SY5	-	-	-	-
savignyi	H104	27	Syria	Al Hasakah 2			NMP6V 72101/3	TI-E2a	mtsav SY5	-	-	-	-
savignyi	H193	28	Syria	Mishrifah	36.10	37.85	CUP\AMPH\SUR\008	L	mtsav SY4	-	-	-	-
savignyi	H194	28	Syria	Mishrifah			CUP\AMPH\SUR\009	L	mtsav SY4	-	-	-	-
savignyi	H018	29	Syria	Rabi'ah	35.82	36.03	NMP6V 70713/3	L	mtsav SY1	-	-	-	-
savignyi	H186	29	Syria	Rabi'ah			CUP\AMPH\SUR\050	L	mtsav SY1	-	-	-	-
savignyi	H192	30	Syria	Halabiyah	35.65	39.67	CUP\AMPH\SUR\020	L	mtsav SY4	-	-	-	-
savignyi	H187	31	Syria	Shayzar	35.27	36.57	CUP\AMPH\SUR\048	L	mtsav LB1	-	-	-	-
savignyi	H188	31	Syria	Shayzar			CUP\AMPH\SUR\047	L	mtsav LB1	-	-	-	-
savignyi	H189	31	Syria	Shayzar			CUP\AMPH\SUR\046	L	mtsav SY1	-	-	-	-
savignyi	H096	32	Syria	Al 'Adimah, Baniyas	35.15	35.92	NMP6V 72094/5	L	mtsav SY3	Rsav 2	Rsav 2	Tsav 1	Tsav 1
savignyi	H097	32	Syria	Al 'Adimah, Baniyas			NMP6V 72094/1	L	mtsav SY4	-	-	-	-
savignyi	H098	32	Syria	Al 'Adimah, Baniyas			NMP6V 72094/4	L	mtsav LB1	-	-	-	-
savignyi	H094	33	Syria	Qal'at al Hisn	34.77	36.32	-	L	mtsav SY2	-	-	-	-
savignyi	H095	33	Syria	Qal'at al Hisn			NMP6V 72093	L	mtsav LB1	-	-	-	-
savignyi	H190	34	Syria	Homs	34.73	36.72	CUP\AMPH\SUR\040	L	mtsav SY6	Rsav 1	Rsav 2	Tsav 1	Tsav 1
savignyi	H191	34	Syria	Homs			CUP\AMPH\SUR\037	L	mtsav LB1	-	-	-	-
savignyi	H099	35	Syria	Abu Kamal	34.45	40.92	NMP6V 72095/3	TI-E2a	mtsav SY5	Rsav 1	Rsav 1	Tsav 2	Tsav 3
savignyi	H100	35	Syria	Abu Kamal			NMP6V 72095/4	TI-E2a	mtsav SY5	-	-	-	-
savignyi	H101	35	Syria	Abu Kamal			NMP6V 72095/2	TI-E2a	mtsav SY5	-	-	-	-
savignyi	H005	36	Turkey	Anamur, 5 km NW	36.10	32.79	NMP6V 71574/4	TI-W	mtsav TR1	Rsav 1	Rsav 1	Tsav 1	Tsav 1
savignyi	H055	37	Turkey	Anamurium	36.03	32.81	NMP6V 72537/1	TI-W	mtsav TR2	Rsav 1	Rsav 1	Tsav 1	Tsav 1
savignyi	H056	37	Turkey	Anamurium			NMP6V 72537/2	TI-W	mtsav TR1	-	-	-	-
savignyi	H057a	37	Turkey	Anamurium			NMP6V 72537/3	TI-W	mtsav TR3	-	-	-	-
savignyi	H058	37	Turkey	Anamurium			-	TI-W	mtsav TR1	-	-	-	-
savignyi	H068	38	Turkey	Anamur	36.06	32.84	NMP6V 71573/1	TI-W	mtsav TR4	-	-	-	-
savignyi	H061	39	Turkey	Bozyazi	36.10	32.97	NMP6V 72538	TI-W	mtsav TR1	-	-	-	-
savignyi	H069	40	Turkey	Kurtuluş, Silifke	36.33	34.00	NMP6V 71575/1	TI-W	mtsav TR1	-	-	-	-
savignyi	H070	41	Turkey	Kazanli, 10 km E of Mersin	36.83	34.75	NMP6V 71576/1	TI-W	mtsav TR1	-	-	-	-
savignyi	H071	41	Turkey	Kazanli, 10 km E of Mersin			NMP6V 71576/2	TI-W	mtsav TR5	-	-	-	-
savignyi	H119	42	Turkey	Kulak, Tarsus	36.80	34.90	-	TI-W	mtsav TR10	Rsav 1	Rsav 2	Tsav 1	Tsav 1
savignyi	H111	43	Turkey	Ovaçiftliği	38.24	35.20	NMP6V 72107/2	TI-W	mtsav TR7	Rsav 1	Rsav 2	Tsav 9	Tsav 9
savignyi	H112	43	Turkey	Ovaçiftliği			NMP6V 72107/5	L	mtsav LB1	-	-	-	-
savignyi	H113	43	Turkey	Ovaçiftliği			NMP6V 72107/4	TI-W	mtsav TR7	-	-	-	-
savignyi	H114	44	Turkey	Soysallı	38.38	35.38	NMP6V 72108/1	L	mtsav LB1	-	-	-	-
savignyi	H115	44	Turkey	Soysallı			NMP6V 72108/3	L	mtsav LB1	-	-	-	-
savignyi	H116	44	Turkey	Soysallı			NMP6V 72108/2	L	mtsav LB1	-	-	-	-
savignyi	H503	45	Turkey	Antakya	36.22	36.12	-	L	mtsav TR14	-	-	-	-
savignyi	H117	46	Turkey	Payas	36.75	36.20	-	TI-W	mtsav TR8	-	-	-	-
savignyi	H118	46	Turkey	Payas			VG H118	L	mtsav TR9	-	-	-	-
savignyi	H467	47	Turkey	Tahtaköprü Dam	36.87	36.70	-	L	mtsav SY4	-	-	-	-
savignyi	H468	47	Turkey	Tahtaköprü Dam			-	L	mtsav SY4	-	-	-	-
savignyi	H469	47	Turkey	Tahtaköprü Dam			-	L	mtsav SY4	-	-	-	-
savignyi	H105	48	Turkey	Birecik	37.03	37.99	NMP6V 72105/1	L	mtsav LB1	-	-	-	-
savignyi	H106	48	Turkey	Birecik			NMP6V 72105/2	L	mtsav LB1	Rsav 1	Rsav 1	Tsav 1	Tsav 8
savignyi	H107	48	Turkey	Birecik			NMP6V 72105/3	L	mtsav TR6	-	-	-	-
savignyi	H120	49	Turkey	Karadut, Nemrut Dağı	37.93	38.78	-	L	mtsav LB1	-	-	-	-
savignyi	H121	49	Turkey	Karadut, Nemrut Dağı			-	L	mtsav TR11	-	-	-	-
savignyi	H122	49	Turkey	Karadut, Nemrut Dağı			-	L	mtsav SY4	-	-	-	-
savignyi	H231	50	Turkey	Kemah	39.60	39.04	-	L	mtsav TR12	Rsav 2	Rsav 2	Tsav 2	Tsav 2
savignyi	H232	50	Turkey	Kemah			-	L	mtsav TR12	-	-	-	-
savignyi	H233	50	Turkey	Kemah			-	L	mtsav TR12	-	-	-	-
savignyi	H236	51	Turkey	Cincevat River, Tuzluca	40.05	43.66	-	TI-E1	mtsav TR13	-	-	-	-
savignyi	H237	51	Turkey	Cincevat River, Tuzluca			-	TI-E1	mtsav TR13	Rsav 1	Rsav 1	Tsav 2	Tsav 2

savignyi	H238	51	Turkey	Cincevat River, Tuzluca			-	TI-E1	mts*av* TR13	-	-	-	-
sp. nov.	HD10A	52	Israel	Tel Dan	33.25	35.63	-	JS1	mt*fel* SY1	-	-	-	-
sp. nov.	HD10B	52	Israel	Tel Dan			-	JS1	mt*fel* SY1	-	-	-	-
sp. nov.	HD10C	52	Israel	Tel Dan			-	JS1	mt*fel* SY1	-	-	-	-
sp. nov.	H505	53	Jordan	Zara, Dead Sea	31.58	35.56	-	JS1	mt*fel* JO4	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 6
sp. nov.	H506	53	Jordan	Zara, Dead Sea			-	JS1	mt*fel* SY1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 3
sp. nov.	H086	54	Jordan	Wadi Mujib	31.44	35.70	NMP6V 72085/3	JS2	mt*fel* JO1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 3
sp. nov.	H087	54	Jordan	Wadi Mujib			NMP6V 72085/1	JS2	mt*fel* JO1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 3
sp. nov.	H088	54	Jordan	Wadi Mujib			NMP6V 72085/4	JS2	mt*fel* JO3	-	-	-	-
sp. nov.	H089	54	Jordan	Wadi Mujib			NMP6V 72085/2	JS2	mt*fel* JO1	-	-	-	-
sp. nov.	H082	55	Jordan	Wadi Fidan	30.65	35.40	NMP6V 72082/2	JS2	mt*fel* JO1	R*fel* 1	R*fel* 1	T*fel* 4	T*fel* 4
sp. nov.	H083	55	Jordan	Wadi Fidan			NMP6V 72082/3	JS2	mt*fel* JO2	R*fel* 1	R*fel* 1	T*fel* 4	T*fel* 4
sp. nov.	H084	55	Jordan	Wadi Fidan			NMP6V 72082/4	JS2	mt*fel* JO1	-	-	-	-
sp. nov.	H085	55	Jordan	Wadi Fidan			NMP6V 72082/1	JS2	mt*fel* JO1	-	-	-	-
sp. nov.	H195	56	Syria	Bludan	33.73	36.13	CUP\AMPH\SUR\030	JS1	mt*fel* SY1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 5
sp. nov.	H196	56	Syria	Bludan			CUP\AMPH\SUR\031	JS1	mt*fel* SY1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 3
sp. nov.	HC009	57	Syria	Jabal Druz	32.66	36.73	ZFMK coll. (CK H009)	JS1	mt*fel* SY1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 3
sp. nov.	HC010	57	Syria	Jabal Druz			ZFMK coll. (CK H010)	JS1	mt*fel* SY1	-	-	-	-
sp. nov.		58	Syria	Amman-Damascus road, 10 km N of the border	32.65	36.12	KU207344; Moriarty Lemmon et al. (2007, Suppl.)	JS1	EF566954	-	-	-	-
sp. nov.		58	Syria	Amman-Damascus road, 10 km N of the border			KU207346; Smith et al. (2005)	JS1	DQ055843	-	-	-	-
sp. nov.	HC162	59	Yemen	Amran	15.72	43.97	ZFMK coll. (CK H162)	Y	mt*fel* YE1	R*fel* 1	R*fel* 1	T*fel* 1	T*fel* 2
sp. nov.	HC002	60	Yemen	Habiba	15.55	43.87	ZFMK coll. (CK H002)	Y	mt*fel* YE1	-	-	-	-
sp. nov.	HC120	61	Yemen	Wadi Annaim, West Kawkaban	15.50	43.88	ZFMK coll. (CK H120)	Y	mt*fel* YE4	-	-	-	-
sp. nov.	HC144	61	Yemen	Wadi Annaim, West Kawkaban			ZFMK coll. (CK H144)	Y	mt*fel* YE5	-	-	-	-
sp. nov.	HC019	62	Yemen	Sana'a	15.30	44.20	ZFMK coll. (CK H019)	Y	mt*fel* YE3	-	-	-	-
sp. nov.	**H167**	**63**	**Yemen**	**Matnah, 15 km SW**	**15.20**	**43.98**	**NMP6V 72076/1**	Y	**mt*fel* YE1**	**R*fel* 1**	**R*fel* 1**	**T*fel* 1**	**T*fel* 1**
sp. nov.	H168	63	Yemen	Matnah, 15 km SW			NMP6V 72076/3	Y	mt*fel* YE2	R*fel* 1	R*fel* 1	T*fel* 1	T*fel* 1
sp. nov.	H169	63	Yemen	Matnah, 15 km SW			NMP6V 72076/2	Y	mt*fel* YE2	-	-	-	-
sp. nov.	H170	63	Yemen	Matnah, 15 km SW			NMP6V 72076/4	Y	mt*fel* YE1	-	-	-	-
sp. nov.	H171	63	Yemen	Matnah, 15 km SW			NMP6V 72076/5	Y	mt*fel* YE1	-	-	-	-
sp. nov.	HC044	64	Yemen	Dhamar	14.55	44.35	ZFMK coll. (CK H044)	Y	mt*fel* YE3	-	-	-	-
sp. nov.	HC013	65	Yemen	Yarim, 2813 m	14.45	44.40	ZFMK coll. (CK H013)	Y	mt*fel* YE3	-	-	-	-
sp. nov.		65	Yemen	Yarim, 2813 m			ZFMK coll. (CK H074); Faivovich et al. (2005)	Y	AY843665 = mt*fel* YE3	AY844654a = R*fel* 1	AY844654b	AY844107 = T*fel* 1	AY844107 = T*fel* 1
sp. nov.	HC199	66	Yemen	Mawah, S of Yarim	14.22	44.40	ZFMK coll. (CK H199)	Y	mt*fel* YE3	R*fel* 1	R*fel* 1	T*fel* 1	T*fel* 1
sp. nov.	HC205	66	Yemen	Mawah, S of Yarim			ZFMK coll. (CK H205)	Y	mt*fel* YE3	-	-	-	-
hybrid *sav-fel*	H301	67	Israel	Karkom	32.91	35.60	-	L	mts*av* JO2	-	-	-	-
hybrid *sav-fel*	H303	67	Israel	Karkom			-	L	mts*av* JO2	-	-	-	-
hybrid *sav-fel*	H305	67	Israel	Karkom			-	L	mts*av* LB1	-	-	-	-
hybrid *sav-fel*	H306	67	Israel	Karkom			-	L	mts*av* JO2	-	-	-	-
hybrid *sav-fel*	H299	67	Israel	Karkom			-	L	mts*av* IL1	R*fel* 1	R*fel* 1	T*sav* 2	T*sav* 3
hybrid *sav-fel*	H300	67	Israel	Karkom			-	JS1	mt*fel* SY1	R*fel* 1	R*sav* 1	T*fel* 6	T*fel* 6
hybrid *sav-fel*	H302	67	Israel	Karkom			-	JS1	mt*fel* SY1	R*fel* 1	R*fel* 1	T*fel* 3	T*fel* 5
hybrid *sav-fel*	H304	67	Israel	Karkom			-	JS1	mt*fel* IL1	R*sav* 1	R*sav* 1	T*sav* 2	T*fel* 7
orientalis	H392	68	Azerbaijan	Kiş, Şəki	41.26	47.19	NMP6V 73709/1	CC	mt*ori* AZ1	R*ori* 1	R*ori* 1	T*ori* 1	T*ori* 3
orientalis	H393	68	Azerbaijan	Kiş, Şəki			NMP6V 73709/2	CC	mt*ori* AZ1	-	-	-	-
orientalis	H394	68	Azerbaijan	Kiş, Şəki			NMP6V 73709/3	CC	mt*ori* TR8	-	-	-	-
orientalis	H420	**69**	**Georgia**	**Kutaisi**	42.26	42.72	-	CC	mt*ori* TR8	R*ori* 1	R*ori* 1	T*ori* 1	T*ori* 4
orientalis	H421	70	Georgia	Tsodoreti Lake, 10 km W of Tbilisi	41.76	44.65	-	CC	mt*ori* TR8	-	-	-	-
orientalis	H422	70	Georgia	Tsodoreti Lake, 10 km W of Tbilisi			-	CC	mt*ori* TR8	-	-	-	-
orientalis	H423	70	Georgia	Tsodoreti Lake, 10 km W of Tbilisi			-	CC	mt*ori* GE1	-	-	-	-
orientalis	H407	71	Georgia	Telavi	41.92	45.49	NMP6V 73713/2	CC	mt*ori* TR8	R*ori* 1	R*ori* 1	T*ori* 3	T*ori* 3
orientalis	H408	71	Georgia	Telavi			NMP6V 73713/1	CC	mt*ori* TR8	-	-	-	-
orientalis	H409	71	Georgia	Telavi			NMP6V 73713/3	CC	mt*ori* TR8	-	-	-	-
orientalis	H257	**72**	**Iran**	**Motalla Sara-ye Lemir**	38.20	48.87	NMP6V 72677/2	CC	mt*ori* TR8	R*ori* 1	R*ori* 1	T*ori* 1	T*ori* 1
orientalis	H258	**72**	**Iran**	**Motalla Sara-ye Lemir**			NMP6V 72677/3	CC	mt*ori* TR8	-	-	-	-
orientalis	H259	**72**	**Iran**	**Motalla Sara-ye Lemir**			NMP6V 72677/1	CC	mt*ori* TR8	-	-	-	-
orientalis	H390	73	Iran	Chayjan, Gilan	37.01	50.50	NMP6V 72842	CC	mt*ori* TR8	R*ori* 1	R*ori* 3	T*ori* 1	T*ori* 1
orientalis	H251	74	Iran	Tonekabon	36.81	50.88	NMP6V 72676/4	CC	mt*ori* IR1	R*ori* 1	R*ori* 2	T*ori* 1	T*ori* 1
orientalis	H252	74	Iran	Tonekabon			VG H252	CC	mt*ori* IR2	-	-	-	-
orientalis	H253	74	Iran	Tonekabon			-	CC	mt*ori* IR3	-	-	-	-
orientalis	H362a	75	Iran	Babol Sar	36.68	52.65	-	CC	mt*ori* IR2	R*ori* 1	R*ori* 2	T*ori* 1	T*ori* 2
orientalis	H160a	76	Turkey	Selçuk - Efes	37.95	27.37	NMP6V 72534/1	WT	mt*ori* TR1	R*ori* 1	R*ori* 1	T*fel* 3	T*ori* 5
orientalis	H204	76	Turkey	Selçuk - Efes			NMP6V 72534/2	WT	mt*ori* TR1	R*ori* 1	R*ori* 1	T*ori* 5	T*ori* 7
orientalis	H205	76	Turkey	Selçuk - Efes			NMP6V 72534/3	WT	mt*ori* TR1	-	-	-	-
orientalis	H461	77	Turkey	Bafa Gölü	37.47	27.50	-	WT	mt*ori* TR14	-	-	-	-
orientalis	HD14A	78	Turkey	Marmaris	36.87	28.27	-	WT	mt*ori* TR16	R*ori* 1	R*ori* 5	T*ori* 8	T*ori* 9
orientalis	HD14B	78	Turkey	Marmaris			-	WT	mt*ori* TR14	-	-	-	-
orientalis	HD14C	78	Turkey	Marmaris			-	WT	mt*ori* TR17	-	-	-	-
orientalis	H388	79	Turkey	Dalaman	36.77	28.80	NMP6V 70833	WT	mt*ori* TR9	-	-	-	-
orientalis	H161a	80	Turkey	Syedra, 15 km E of Alanya	36.48	32.12	NMP6V 72535/1	WT	mt*ori* TR2	R*ori* 1	R*ori* 1	T*ori* 6	T*fel* 3
orientalis	H162	80	Turkey	Syedra, 15 km E of Alanya			-	WT	mt*ori* TR3	-	-	-	-
orientalis	H163	80	Turkey	Syedra, 15 km E of Alanya			-	WT	mt*ori* TR2	-	-	-	-
orientalis	H164a	81	Turkey	Gazipaşa	36.27	32.32	NMP6V 72536/1	WT	mt*ori* TR4	R*ori* 1	R*ori* 1	T*fel* 3	T*fel* 3
orientalis	H165	81	Turkey	Gazipaşa			NMP6V 72536/2	WT	mt*ori* TR5	-	-	-	-
orientalis	H166a	82	Turkey	Melleç	36.07	32.70	VG H166	WT	mt*ori* TR6	R*ori* 1	R*ori* 1	T*fel* 3	T*fel* 3
orientalis	H446	83	Turkey	İnebolu	41.97	33.76	NMP6V 73700/2	WT	mt*ori* TR14	R*ori* 1	R*ori* 1	T*ori* 1	T*ori* 1
orientalis	H447	83	Turkey	İnebolu			NMP6V 73700/1	WT	mt*ori* TR15	-	-	-	-
orientalis	H448	83	Turkey	İnebolu			-	WT	mt*ori* TR15	-	-	-	-
orientalis	H429	84	Turkey	Sinop	42.03	35.16	NMP6V 73698/2	WT	mt*ori* TR11	-	-	-	-
orientalis	H430	84	Turkey	Sinop			NMP6V 73698/1	WT	mt*ori* TR12	-	-	-	-
orientalis	H431	84	Turkey	Sinop			NMP6V 73698/3	WT	mt*ori* TR13	-	-	-	-
orientalis	H428	85	Turkey	Kumru, 5 km NE	40.87	37.26	NMP6V 73697	WT	mt*ori* TR10	R*ori* 1	R*ori* 1	T*ori* 4	T*ori* 4
orientalis	H216	86	Turkey	Hopa	41.41	41.44	-	WT	mt*ori* TR7	R*ori* 1	R*ori* 4	T*ori* 1	T*ori* 1
orientalis	H217	86	Turkey	Hopa			NMP6V 72688/1	CC	mt*ori* TR8	-	-	-	-
orientalis	H218	86	Turkey	Hopa			NMP6V 72688/2	CC	mt*ori* TR8	-	-	-	-
orientalis	H219	86	Turkey	Hopa			NMP6V 72688/3	CC	mt*ori* TR8	-	-	-	-
orientalis	H220	86	Turkey	Hopa			-	CC	mt*ori* TR8	-	-	-	-
meridionalis	HE101A	-	Spain	Puerto de la Cruz, Tenerife	28.38	-16.55	-		mt*mer* ES1	R*mer* 1	R*mer* 1	T*mer* 1	T*mer* 1
japonica		-	Japan	Yasufutuichi-cho, Aito, Hiroshima			LSUMZ H-230; Faivovich et al. (2005)		AY843633	AY844615		AY844078	

[a] gametic haplotypes inferred with mean probability 0.66 in one from two heterozygous sites
CK, C. Klütsch et al. collection
VG, V. Gvoždík collection

Table S2. GenBank accession numbers.

mtDNA haplotype	GenBank (composite 12S/16S rRNA)	Rhodopsin haplotype	GenBank	Tyrosinase haplotype	GenBank
mt*sav* CY1	GQ916723 / GQ916758	R*sav* 1	GQ916811	T*sav* 1	GQ916691
mt*sav* CY2	GQ916723 / GQ916759	R*sav* 2	GQ916812	T*sav* 2	GQ916692
mt*sav* CY3	GQ916730 / GQ916759	R*sav* 3	GQ916813	T*sav* 3	GQ916693
mt*sav* CY4	GQ916736 / GQ916759	R*fel* 1	GQ916814	T*sav* 4	GQ916694
mt*sav* CY5	GQ916723 / GQ916777	R*ori* 1	GQ916815	T*sav* 5	GQ916695
mt*sav* GE1	GQ916735 / GQ916778	R*ori* 2	GQ916816	T*sav* 6	GQ916696
mt*sav* IL1	GQ916724 / GQ916790	R*ori* 3	GQ916817	T*sav* 7	GQ916697
mt*sav* IQ1	GQ916727 / GQ916781	R*ori* 4	GQ916818	T*sav* 8	GQ916698
mt*sav* IR1	GQ916726 / GQ916754	R*ori* 5	GQ916819	T*sav* 9	GQ916699
mt*sav* IR2	GQ916727 / GQ916754	R*mer* 1	GQ916820	T*sav* 10	GQ916700
mt*sav* IR3	GQ916733 / GQ916754			T*sav* 11	GQ916701
mt*sav* IR4	GQ916734 / GQ916754			T*sav* 12	GQ916702
mt*sav* IR5	GQ916735 / GQ916774			T*sav* 13	GQ916703
mt*sav* IR6	GQ916735 / GQ916775			T*sav* 14	GQ916704
mt*sav* IR7	GQ916727 / GQ916776			T*sav* 15	GQ916705
mt*sav* JO1	GQ916724 / GQ916761			T*fel* 1	GQ916706
mt*sav* JO2	GQ916724 / GQ916762			T*fel* 2	GQ916707
mt*sav* JO3	GQ916740 / GQ916763			T*fel* 3	GQ916708
mt*sav* LB1	GQ916724 / GQ916756			T*fel* 4	GQ916709
mt*sav* LB2	GQ916737 / GQ916756			T*fel* 5	GQ916710
mt*sav* LB3	GQ916738 / GQ916756			T*fel* 6	GQ916711
mt*sav* SY1	GQ916724 / GQ916755			T*fel* 7	GQ916712
mt*sav* SY2	GQ916724 / GQ916764			T*ori* 1	GQ916713
mt*sav* SY3	GQ916724 / GQ916765			T*ori* 2	GQ916714
mt*sav* SY4	GQ916724 / GQ916766			T*ori* 3	GQ916715
mt*sav* SY5	GQ916727 / GQ916767			T*ori* 4	GQ916716
mt*sav* SY6	GQ916724 / GQ916771			T*ori* 5	GQ916717
mt*sav* SY7	GQ916724 / GQ916779			T*ori* 6	GQ916718
mt*sav* TR1	GQ916723 / GQ916754			T*ori* 7	GQ916719
mt*sav* TR10	GQ916732 / GQ916759			T*ori* 8	GQ916720
mt*sav* TR11	GQ916724 / GQ916770			T*ori* 9	GQ916721
mt*sav* TR12	GQ916724 / GQ916772			T*mer* 1	GQ916722
mt*sav* TR13	GQ916735 / GQ916773				
mt*sav* TR14	GQ916724 / GQ916780				
mt*sav* TR2	GQ916725 / GQ916754				
mt*sav* TR3	GQ916723 / GQ916757				
mt*sav* TR4	GQ916723 / GQ916760				
mt*sav* TR5	GQ916728 / GQ916754				
mt*sav* TR6	GQ916729 / GQ916766				
mt*sav* TR7	GQ916731 / GQ916768				
mt*sav* TR8	GQ916731 / GQ916754				
mt*sav* TR9	GQ916724 / GQ916769				
mt*fel* IL1	GQ916743 / GQ916791				
mt*fel* JO1	GQ916739 / GQ916782				
mt*fel* JO2	GQ916739 / GQ916783				
mt*fel* JO3	GQ916739 / GQ916784				
mt*fel* JO4	GQ916744 / GQ916787				
mt*fel* SY1	GQ916743 / GQ916787				
mt*fel* YE1	GQ916741 / GQ916785				
mt*fel* YE2	GQ916742 / GQ916786				
mt*fel* YE3	GQ916742 / GQ916785				
mt*fel* YE4	GQ916741 / GQ916788				
mt*fel* YE5	GQ916741 / GQ916789				
mt*ori* AZ1	GQ916749 / GQ916803				
mt*ori* GE1	GQ916751 / GQ916798				
mt*ori* IR1	GQ916749 / GQ916799				
mt*ori* IR2	GQ916749 / GQ916800				
mt*ori* IR3	GQ916749 / GQ916801				
mt*ori* TR1	GQ916745 / GQ916792				
mt*ori* TR10	GQ916746 / GQ916804				
mt*ori* TR11	GQ916746 / GQ916805				
mt*ori* TR12	GQ916746 / GQ916806				
mt*ori* TR13	GQ916746 / GQ916807				
mt*ori* TR14	GQ916746 / GQ916792				
mt*ori* TR15	GQ916746 / GQ916808				
mt*ori* TR16	GQ916752 / GQ916809				
mt*ori* TR17	GQ916750 / GQ916809				
mt*ori* TR2	GQ916746 / GQ916793				
mt*ori* TR3	GQ916746 / GQ916794				
mt*ori* TR4	GQ916746 / GQ916795				
mt*ori* TR5	GQ916747 / GQ916793				
mt*ori* TR6	GQ916748 / GQ916796				
mt*ori* TR7	GQ916746 / GQ916797				
mt*ori* TR8	GQ916749 / GQ916798				
mt*ori* TR9	GQ916750 / GQ916802				
mt*mer* ES1	GQ916753 / GQ916810				

Supplementary data to the description of *Hyla* sp. nov. Gvoždík, Kotlík, Moravec

Synonymy

Hyla arborea var. *meridionalis* Boettger, 1874 (partim): Boettger (1880: p. 212)

Hyla arborea var. *savignyi* Audouin, 1827 ["1809"] (partim): Boulenger (1882: p. 380)

Hyla arborea (Linnaeus, 1758) (partim): Tristram (1885: p. 160)

Hyla arborea savignyi Audouin, 1827 ["1809"] (partim): Nieden (1924: p. 199); Nevo and Yang (1979: p. 47)

Hyla arborea savignyi Audouin, 1827 ["1809"]: Parker (1938: p. 492)

Hyla savignyi Audouin, 1827 ["1809"] (partim): Riehl et al. (1995: p. 247); Disi et al. (2001: p. 97); Gvoždík et al. (2008: p. 541)

Hyla savignyi Audouin, 1827 ["1809"]: Balletto et al. (1985: p. 363); Klütsch et al. (2004: p. 47); Al-Shehri and Al-Saleh (2005: p. 768); Faivovich et al. (2005: p. 177); Smith et al. (2005: p. 2441); Moriarty Lemmon et al. (2007: Suppl. p. 2)

Hyla sp.: Gvoždík et al. (2007a: p. 33); Stöck et al. (2008: p. 1023)

Note: For a discussion about the date of description of *Hyla savignyi* Audouin, 1827 ["1809"] see Grach et al. (2007).

Referred material – morphologically examined, DNA not analyzed

Yemen: ZMH A04130, ZFMK 37040, Sana'a, collected by Rathjes and Wissmann in June 1931 and by Erdelen in 1980, respectively; ZFMK 42848, 42850–52 (42850–51 subadults), 31 km from Sana'a in direction to Hodeida, 24.II.1985, leg. Schütte and Fritz; ZFMK 43108, 3 km W of Shibam, 22.III.1985, leg. Schütte and Fritz; Saudi Arabia: CAS 139732-33, Wadi Amaq, 2100 m a.s.l., 23.VIII.1974, leg. Gasperetti; CAS 136516-27, Barahara, 2000 m a.s.l., 8.,11.VIII.1973, leg. Gasperetti; CAS 145320-22, Wadi Mahra, 19°38' N, 41°54' E, 2000 m a.s.l., 21.IV.1977, leg. Gasperetti; CAS 145328, 145347, Wadi Mahra, 19°38' N, 42°38' E, 2000 m a.s.l., 29.VI.1977, 15.IX.1977, leg. Gasperetti; MHNG 1213.5-11 (all subadults), Taif, 20.VIII.1971, leg. Müller; Jordan: NMP6V 71088/1–7 (/5–7 subadults), Al Ayna, 18.VI.2000, leg. D. Modrý; NMP6V 71089/1–3 (all subadults), Wadi Fidan, 18.VI.2000, leg. D. Modrý; Syria: NMP6V 34820–21, Busra ash Sham, 30.V.1994, leg. J. Moravec, and 25.IV.1994, leg. D. Modrý, respectively; NMP6V 71366/1–3, Sia, Suweida Dam, 24.V.2001, leg. P. Benda; ZFMK 21006, 25 km N of Dar'a, 25.III.1977, leg. Kinzelbach; ZFMK 42818, 10 km N of Syria-Jordan border, 31.III.1985, leg. Schütte and Fritz; ZFMK 60854, 12 km E of Suweida, Jabal Druz; Israel: NMP6V 70803/1–5, Huleh Valley, collected by H. Steinitz in April 1940;

Golan Heights: NMW 26791:5, Golan Heights, 29.I.1980, leg. Kollenberger (only body length and coloration were examined).

Description of holotype

Adult male, snout-vent length 43.5 mm (Fig. 7a,b). Head narrower than body, slightly shorter than wide; snout rounded in dorsal view, truncate in lateral view; distance from nostril to eye shorter than diameter of eye; canthus rostralis barely distinct, rounded in cross-section; loreal region posterior to nostril slightly concave; internarial area slightly depressed; nostrils slightly protuberant, directed anterolaterally; interorbital area flat, interorbital distance 78.9% of the eyelid width; eye large, strongly protuberant; tympanic membrane round, about two thirds of eye length, separated from eye by ca. 44% of its diameter; tympanic annulus indistinct; supratympanic fold conspicuous, continuing above insertion of arm. Arm slender, relative length of fingers 1<2<4<3; fingers bearing small, oval discs, that of third finger about half of tympanum diameter; subarticular tubercles prominent, round, single; supernumerary tubercles present; palmar tubercle large, elongate; prepollical tubercle large, elliptical; prepollex enlarged; webbing between fingers rudimental. Legs moderately long, slender; heels slightly overlapping when limbs flexed perpendicular to the axis of body; distinct fold along the inner edge of tarsus; toes moderately long, bearing oval discs slightly smaller than those of fingers; relative length of toes 1<2<5<3<4; outer metatarsal tubercle distinct, small, round; inner metatarsal tubercle large, ovoid, protuberant; toes half webbed; webbing formula of toes I2—2^{+}II2^{-}—3III2—3IV3—2V (sensu Myers and Duellman, 1982). Skin on head, dorsum, flanks and dorsal surfaces of limbs smooth; skin on venter and lower surfaces of thighs coarsely granular; skin on throat slightly granular. Cloacal opening directed posteriorly at upper level of thighs; cloacal opening covered by short simple cloacal sheath; rounded white tubercles around vent. Tongue ovoid, posterior part and posterior two thirds of lateral margins not attached to floor of mouth; vomerine odontophores prominent, separated medially, between choanae, bearing 4 and 7 (left/right) vomerine teeth; choanae oval; vocal slits long, extending from anterior third of lateral base of tongue to angle of jaws; vocal sac subgular.

Measurements as follow (in mm): SVL 43.5; SUL 41.5; FmL 18.8; TbL 18.7; WL 9.2; T4L 16.2; T1L 4.5; IMTL 1.8; TrL 10.9; HW 14.8; HLt 12.0; ES 5.0; NL 3.3; IND 3.5; EAD 6.8; IOD 3.0; EPD 12.0; ED 4.1; TD 2.7.

Coloration: In alcohol, head, dorsum and dorsal surfaces of limbs bluish grey. A darker stripe edged dorsally and ventrally by a narrow whitish line runs from nostril to axilla and continues to groin as a wider lateral stripe edged by whitish line dorsally and being “washed

out" ventrally. Dorsal margin of the lateral stripe forms a low indistinct inguinal loop on the left side and becomes fragmented on the right side. Supracloacal area light with a minute dark spot on the left side. Dorsal coloration of forearm, femur, tibia and tarsus edged by a narrow whitish line and indistinct dark pigmentation. Throat whitish with densely scattered melanophores. Belly, ventral surfaces of legs and hidden surfaces of thighs yellowish white.

General morphology and variation

Hyla sp. nov. is characterized by the following combination of morphological characters: (1) medium size, SVL 34.9–44.4 mm in males, 38.0–54.1 mm in females; (2) snout rounded in dorsal view, truncate to slightly rounded in lateral view; (3) canthus rostralis barely distinct, rounded in cross-section; loreal region posterior to nostril slightly concave; (4) tympanum round, about 48–74% of eye diameter, tympanic annulus indistinct; supratympanic fold distinct; (5) vomerine odontophores prominent, separated medially, between oval choanae; (6) skin on dorsal surfaces smooth; (7) distinct fold along the inner edge of tarsus; (8) basal webbing on hand, toes about half webbed; (9) fingers and toes bearing small round discs, diameter of disc on third finger about half to two thirds of the size of tympanum; (10) in alcohol, dorsum bluish grey, ventral surfaces yellowish white; (11) in alcohol, a dark stripe edged dorsally and ventrally by a narrow whitish line runs from nostril to axilla, a lateral dark stripe edged by whitish line dorsally and "washed out" ventrally continues to the groin; dorsal margin of the lateral dark stripe becomes irregular or fragmented into several small spots in the inguinal area, in some individuals the larger longitudinal spots are situated above the dorsal margin of the stripe and form a narrow low inguinal loop; (12) in alcohol, dark supracloacal streak or spot, and dark line separating dorsal and ventral coloration of tibia and tarsus reduced.

In general, the subadult paratypes closely resemble the holotype (measurements of the paratypes and morphometric variation of the referred Jordanian-Syrian lineage's adult specimens is given in Table S3; for further data to the population from the Arabian Peninsula see Balletto et al. (1985) and Gvoždík et al. (2008)). The most considerable variation is evident in the shape of the dark lateral stripe, which is usually fragmented into several small irregular spots in the inguinal area. In some paratypes the larger longitudinal spots are in contact with the dorsal margin of the stripe and form a narrow low inguinal loop on one (NMP6V 72076/2–7) or both sides of the body (NMP6V 72076/8–9). In remaining specimens the spots are clearly isolated from the stripe (NMP6V 72076/10) or indistinct (NMP6V 72076/11). Some variation can be detected in size and shape of palmar tubercle, which is smaller and round in all paratypes except of the male NMP6V 72076/2. In comparison with

the type series, the referred Jordanian, Syrian and Israeli specimens of *Hyla* sp. nov. resemble more closely *H. savignyi* in less truncate snout in lateral view, having snout more protruding the anterior margin of maxilla in ventral view, less fragmented lateral stripe, lower frequency of small longitudinal loop-like spots in inguinal area, and sharper delimitation of dorsal and ventral coloration by light and dark lines. It can suggest a phenotypic variation influenced by different environmental conditions as was documented in the case of the Mediterranean and Caucasian populations of *H. savignyi* and *H. orientalis* (referred to as *H. arborea*) (Gvoždík et al., 2008).

In life, dorsal coloration of *Hyla* sp. nov. varies from pale beige, through bright yellowish green (most common) to dark green (Fig. 7d-f). Dark color-changeable spots and/or pigmented dots may be present on dorsum. Ventral surfaces vary from cream white to yellowish white and hidden parts of thighs from yellow to orange yellow.

Distributional notes

In Anatolia, it was shown that *H. orientalis*/*H. savignyi* boundary occurs in the southern coast just a few kilometers west of Anamur (Kaya, 2001; Schneider, 2001, 2009). Although, boundary in the inland is not clear, e.g. the Sultansazlığı marshes have been cited by several authors (Kaya, 2001; Schneider, 2000; Schneider and Grosse, 2009) as a locality of *H. arborea* (*H. orientalis* respectively) based on M. Kasparek's record. However, identification was probably erroneous as morphology of the Kasparek's voucher specimen, ZDEU 182/1982, corresponds to *H. savignyi*, and we identified only *H. savignyi* in the Sultansazlığı region (our localities 43, 44) based on both genetics and bioacoustics (data not shown).

Similarly, Stöck et al. (2008) apparently erroneously quoted his locality No. 41 (Yeniköy, Mersin, Turkey) as a locality of *H. orientalis*. We found only *H. savignyi* within our relatively dense sampling of this area.

The isolated population of tree frogs from the Golestan Province, Iran, which was reported as *H. savignyi* (Cheatsazan et al., 2005; Kami, 2005), is probably *H. orientalis* according to its geographic position and our genetic identification of *H. orientalis* within the geographically closest population from the southern Caspian lowland. However, further genetic or acoustic confirmation is needed.

Museum abbreviations

CAS, California Academy of Sciences, San Francisco, CA, USA; CUP, Charles University Prague, Czech Republic; MHNG, Muséum d'Histoire Naturelle Genève, Switzerland; NMP6V, National Museum Prague, Czech Republic; NMW, Naturhistorisches Museum Wien, Austria; ZDEU, Zoology Department, Ege University, Izmir-Bornova, Turkey; ZFMK, Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; ZMH, Zoologisches Institut und Zoologisches Museum der Universität Hamburg, Germany.

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Table S3. *Hyla* sp. nov. – Measurements of holotype (NMP6V 72076/1) and paratypes (Yemen), and morphometric variation (mean ± SD, range) of referred material from the Jordanian-Syrian lineage (adults only). For further data of the population from Arabian Peninsula see Gvoždík et al. (2008, Supplementary material). Measurements (in mm) were taken as in Gvoždík et al. (2008), abbreviations as follow: SVL, snout–vent length; SUL, snout–urostyle length; FmL, femur length; TbL, tibia length; WL, webbing length; T4L, fourth toe length; T1L, first toe length; IMTL, inner metatarsal tubercle length; TrL, tarsus length; HW, head width; HLt, head length (including tympanum); ES, eye-snout distance; NL, nostril-upper lip distance; IND, internarial distance; EAD, distance between the anterior corners of eyes; IOD, interorbital distance; EPD, distance between the posterior corners of eyes; ED, eye diameter; TD, horizontal tympanum diameter; M = male, F = female, ad = adult, sad = subadult, asterisk * denotes bilateral characters measured on both sides and averaged.

	Sex	SVL	SUL	FmL*	TbL*	WL*	T4L*	T1L*	IMTL*	TrL*	HW	HLt	ES	NL	IND	EAD	IOD	EPD	ED	TD
Type series - Yemen																				
NMP6V 72076/1	M ad	43.5	41.5	18.8	18.7	9.2	16.2	4.5	1.8	10.9	14.8	12.0	5.0	3.3	3.5	6.8	3.0	12.0	4.1	2.7
ZFMK 37039	M ad	38.3	36.5	16.7	16.1	8.9	14.7	4.0	2.0	9.0	13.2	10.2	3.9	3.2	3.3	6.3	3.5	11.5	4.1	2.4
ZFMK 42847	M ad	38.8	37.4	17.2	17.3	8.7	15.0	3.9	1.7	9.9	13.1	11.2	4.6	3.3	3.3	6.6	4.0	11.6	4.4	2.6
ZFMK 42849	M ad	35.2	34.6	15.8	16.0	8.1	13.8	3.8	1.7	9.0	12.4	10.4	4.5	3.0	3.0	6.0	3.5	10.5	3.8	2.1
NMP6V 72076/2	M sad	35.0	33.6	15.7	15.7	8.3	13.8	3.6	1.7	9.3	13.1	10.1	4.6	3.0	3.2	6.6	2.8	10.4	3.5	2.1
NMP6V 72076/7	M sad	32.3	31.0	14.5	14.7	6.8	-	3.4	1.7	7.9	11.6	9.4	4.4	2.7	2.7	5.8	2.3	9.3	3.3	2.2
NMP6V 72076/8	M sad	29.6	28.1	12.9	13.0	6.5	11.0	3.3	1.3	7.5	10.7	9.0	4.1	2.5	2.5	5.5	2.6	9.0	3.0	1.9
NMP6V 72076/10	M sad	28.6	27.0	12.6	12.8	6.6	10.9	2.9	1.4	7.3	10.4	8.8	4.0	2.5	2.4	5.3	2.7	8.6	3.0	2.0
NMP6V 72076/11	M sad	29.4	28.0	12.4	12.4	6.3	10.9	3.1	1.4	7.0	10.2	8.7	3.9	2.5	2.5	5.2	2.6	8.9	3.0	1.8
ZFMK 32272	F ad	42.8	40.6	18.6	18.6	9.5	17.8	4.8	2.1	10.3	15.0	12.5	4.1	3.5	3.6	7.5	3.7	13.0	4.9	2.6
ZMH A 04131	F ad	40.9	38.9	18.1	18.2	8.9	15.4	4.5	1.9	10.3	14.7	11.1	5.0	3.4	3.3	7.4	3.9	11.6	4.0	2.9
NMP6V 72076/3	F sad	32.6	31.1	14.9	15.1	7.9	13.8	3.8	2.1	8.4	11.6	9.8	4.8	2.9	2.7	6.2	2.8	9.7	3.2	2.3
NMP6V 72076/4	F sad	36.5	35.1	16.8	16.9	8.7	14.8	4.2	1.6	9.4	12.4	10.5	4.8	3.1	2.8	6.8	3.0	10.9	3.8	2.5
NMP6V 72076/5	F sad	32.2	31.1	14.8	15.3	7.5	13.0	3.6	1.3	8.3	11.1	9.3	3.8	2.7	2.7	6.0	2.7	9.2	3.6	2.1
NMP6V 72076/6	F sad	31.3	30.4	14.1	14.4	7.6	13.3	4.0	1.5	8.7	10.6	9.9	4.6	2.8	2.6	5.9	2.9	9.3	3.1	2.3
NMP6V 72076/9	F sad	29.6	28.2	13.0	13.5	6.5	10.9	3.1	1.3	7.6	11.2	8.5	4.0	2.5	2.3	5.5	2.5	9.3	3.1	2.2
Jordan-Syria	Males (n = 13)	38.5±3.4	37.1±3.3	18.2±1.6	18.8±1.7	9.3±1.0	15.9±1.5	4.4±0.4	1.7±0.1	10.4±1.0	12.5±1.2	10.3±0.9	4.4±0.5	2.9±0.2	2.9±0.3	6.9±0.5	3.4±0.5	10.9±0.8	3.8±0.2	2.3±0.2
		34.9-44.4	33.0-42.8	16.3-20.9	16.9-22.1	8.1-11.2	14.1-18.7	3.9-5.3	1.5-2.0	9.2-12.2	10.8-14.4	9.2-12.0	4.0-5.4	2.7-3.2	2.6-3.4	6.2-7.8	2.8-4.3	10.0-12.4	3.4-4.2	2.0-2.7
	Females (n = 4)	44.3±1.9	42.2±1.5	21.1±0.6	21.8±1.1	10.6±0.7	18.3±1.0	5.0±0.4	1.9±0.1	11.7±0.5	13.9±0.6	11.5±0.4	4.8±0.2	3.3±0.2	3.3±0.1	7.6±0.4	3.6±0.2	11.9±0.5	4.1±0.3	2.5±0.3
		41.9-46.1	40.5-43.7	20.3-21.6	20.7-23.2	9.8-11.4	17.4-19.2	4.6-5.4	1.8-2.1	11.2-12.3	13.2-14.5	11.1-12.0	4.5-5.0	3.1-3.4	3.1-3.4	7.1-8.0	3.4-3.8	11.6-12.6	3.7-4.5	2.2-2.8

Kapitola V.

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Osteocephalus castaneicola, San Antonio de Filadelfia, Bolívie (Bolivia)
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A new species of *Osteocephalus* (Anura: Hylidae) from Amazonian Bolivia: first evidence of tree frog breeding in fruit capsules of the Brazil nut tree

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Abstract

A new species of *Osteocephalus* is described from lowland Amazonia of the Departamento Pando, northern Bolivia. The new species is most similar to *Osteocephalus planiceps* but differs by its smaller size (SVL 47.8–51.3 mm in males, 47.7–63.3 mm in females), absence of vocal slits, lack of sexual dimorphism in dorsal tubercles, single distal subarticular tubercle on the fourth finger, absence of dark spots on flanks, and by bicoloured iris with fine dark reticulate to radiate lines. The new species inhabits terra firme rainforest, breeds in water-filled fruit capsules of the Brazil nut tree and has oophagous tadpoles. Estimations of phylogenetic relationships within *Osteocephalus* based on mitochondrial DNA sequences show that the new species is closely related to *O. planiceps* and *O. deridens*.

Key words: Amphibia, Anura, Bolivia, Hylidae, Molecular Phylogeny, New Species, Oophagy, *Osteocephalus castaneicola*

Introduction

Hylid frogs of the genus *Osteocephalus* represent typical anuran forms adapted to arboreal mode of life in rainforests of South America. They are excellent climbers and many of them evolved different reproductive adaptations to decrease competition and predator pressure. In this respect, the most specialized species call from or breed in bromeliads or other phytotelmata and provide biparental care to oophagous tadpoles (Jungfer & Schiesari 1995, Jungfer & Weygoldt 1999, Jungfer *et al.* 2000, Jungfer & Lehr 2001, Jungfer & Hödl 2002). Currently, the genus *Osteocephalus* comprises 20 recognized species distributed in the Amazon basin, Guianas and upper drainages of Río Magdalena and Río Orinoco in Colombia and Venezuela (Frost 2009). Nevertheless, *Osteocephalus* alpha taxonomy is far from stable. Existence of several unnamed species is mentioned by Jungfer & Hödl (2002).

Currently, four species of *Osteocephalus* are known to be present in Bolivia: *O. buckleyi* Goin, *O. pearsoni* Gaige, *O. taurinus* Steindachner and an undescribed *Osteocephalus* sp. (A) (sensu Jungfer & Lehr 2001). The latter one was originally associated with the name *O. leprieurii* (Duméril & Bibron) and its formal description remains under process of publication since long ago (see De la Riva *et al.* 2000, Jungfer & Lehr 2001, Jungfer & Hödl 2002). Apart from this, recent field research in the Departamento Pando (the northernmost region of Bolivia, situated in the south-western Amazonian basin within the zone of tall

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evergreen lowland rainforest) revealed that at least two other taxa of *Osteocephalus* occur in Bolivian Amazonia. One, *Osteocephalus* sp. (B), is morphologically similar to *O. leprieurii* (see Moravec & Aparicio 2004). The second taxon, *Osteocephalus* sp. (C) represents a morphologically well differentiated, still unnamed species, which reproduces in abandoned water-filled fruit capsules of the Brazil nut tree. This contribution is aimed at the description of the latter.

Materials and methods

Collected specimens (for exact localities see type specimens, Appendix, and Fig. 4) were fixed and stored in 70 % ethanol. Measurements are given in millimetres (mm) and were taken to the nearest 0.1 mm using a dissecting microscope and electronic digital calipers. Notes on colour in life were taken from field notes and colour photographs. Webbing formulae follow the standards of Myers & Duellman (1982), whereas all other terminology is that of Duellman (1970). Measurement abbreviations used throughout the text are: EN, eye to nostril distance; ED, horizontal eye diameter; ELW, upper eyelid width; FL, foot length as the distance from the heel to the tip of the fourth toe; HL, head length as the straight line distance from the posterior edge of the jaw articulation to the tip of the snout; HW, greatest head width; IOD, interorbital distance; SVL, snout-vent length; TD, horizontal tympanum diameter; and TL, tibia length. Specimens morphologically examined are listed in the Appendix. Institutional acronyms used are those listed in Leviton *et al.* (1985) with the following additions and corrections: CBF, Colección Boliviana de Fauna, La Paz; NMP6V and NMP6d, National Museum Prague.

For purpose of genetic analyses, tissue samples from 13 specimens of five *Osteocephalus* species, including *O.* sp. (B) and *O.* sp. (C), were taken from preserved voucher specimens (Table 1). We targeted a 1943 bp fragment of mitochondrial DNA (mtDNA) comprising partial 12S rRNA (12S), complete transfer RNA-Valin (tRNA-Val) and partial 16S rRNA (16S) genes. Our own DNA sequences were compared to and evaluated together with sequences of comparable mtDNA fragments of nine *Osteocephalus* species (14 individuals) obtained from GenBank. Additional sequences from five individuals of four species of genera *Tepuihyla*, *Itapotihyla*, *Osteopilus* and *Acris* were also taken from GenkBank and used as outgroups (for their outgroup position see Faivovich *et al.* 2005, Wiens *et al.* 2006, Moen & Wiens 2009). For overview of all samples, their coverage, and GenBank accession numbers see Table 1.

Total genomic DNA was extracted from tissue samples using a commercial kit following the manufacturer's protocol. The whole portion of the targeted mtDNA was amplified using primers 12Sa [5'-CTGGGATTAGATACCCCACTA-3'; adapted from Kocher *et al.* (1989)] and 16SH1 [5'-CCGGTCTGAACTCAGATCACGT-3'; Palumbi *et al.* (1991)]. However, we were able to obtain only shorter separate fragments of the 12S (352 bp) and 16S (549 bp) genes in five samples due to low quality of their DNA using two pairs of primers: 12Sa / 12Sbs [12Sbs: 5'-TGAGGAGGGTGACGGGCGGT-3', adapted from Kocher *et al.* (1989)] and 16SL1 / 16SH1 [16SL1: 5'-CGCCTGTTTAACAAAAACAT-3', adapted from Palumbi *et al.* (1991)]. Amplification of all fragments involved an initial cycle of denaturation at 94 °C for 15 min, and 35 subsequent cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Sequencing was carried out using the 12Sa and 16SH1 primers and newly designed internal primers 16SLin (5'-AGTACCGYAAGGGAAAG-3') and 16SinH (5'-TCTTCTTGTTACTAGTT-3') by Macrogen Inc. (Seoul, Korea, http://www.macrogen.com). The sequences obtained have been deposited in GenBank (FJ965291–FJ965308).

Alignment was made by ClustalW (Thompson *et al.* 1994) as implemented in BioEdit 7.0 (Hall 1999) and checked by eye. The best-fit model of sequence evolution was selected using jModelTest 0.1.1 (Posada 2008) using maximum likelihood optimized trees calculated by the implemented PhyML algorithm (Guindon & Gascuel 2003). Both, the Akaike information criterion (AIC; Akaike, 1974) and the Bayesian information criterion (BIC; Schwarz 1978) selected the same best-fit model: transitional model 2 with gamma rate variation among sites (TIM2+G; Posada 2003). Phylogenetic trees were built using maximum likelihood

TABLE 1. *Osteocephalus* and outgroup species included in the molecular phylogenetic analyses.

Species	Code	Country	Locality	Museum Number	GenBank Accession Number			Note/Reference
					12S rRNA	tRNA-Val	16S rRNA	
O. alboguttatus		Ecuador	Sucumbios	KU 143119	DQ380347	-	-	Wiens *et al.* 2006
O. buckleyi		Ecuador	Napo: Jatun Sacha, 420 m	LAC 2216	DQ380378	-	EU034082	Wiens *et al.* 2006; Moen & Wiens 2009
O. cabrerai		Brazil	Acre, 5 km N Porto Walter, inland from Rio Jurua	JPC 13178; LSUMZ H-13720	AY843705	AY843705	AY843705	Faivovich *et al.* 2005
O. deridens	der1	Peru	Loreto: 40 km SW of Iquitos	NMP6V 71262/2	FJ965304	-	FJ965291	this study
O. leprieurii		French Guiana	Creek of Margot		-	-	EF376066	Salducci *et al.* 2005
O. "leprieurii"		Venezuela	Amazonas: Neblina Base Camp on Río Mawarinuma (= Río Baria)	AMNH-A 1312546	AY549361	AY549361	AY549361	Faivovich *et al.* 2004
O. mutabor		Peru	Loreto: 1.5 km N Teniente López, elev. range 310–340 m	KU 221930	DQ380379	-	-	Wiens *et al.* 2006
O. "oophagus"		French Guiana	Kaw Road, 04°42' N / 52°18' W	MNHN 2001.0828	AY843708	AY843708	AY843708	Faivovich *et al.* 2005
O. "oophagus"		French Guiana	Mountain of Kaw		-	-	AF467267	Salducci *et al.* 2002
O. planiceps	pla1	Peru	Loreto: Puerto Almendras	NMP6V 71174/1	FJ965305	-	FJ965292	this study
O. planiceps	pla2	Peru	Loreto: Anguilla	NMP6V 71264/1	FJ965306	-	FJ965293	this study
O. planiceps	pla3	Peru	Loreto: Anguilla	NMP6V 71264/2	FJ965307	-	FJ965294	this study
O. planiceps		Peru	Loreto: San Jacinto: 175 m	KU 221933	DQ380380	-	-	Wiens *et al.* 2006
O. taurinus	tau1	Bolivia	Pando: Santa Crucito	CBF collections	FJ965296	FJ965296	FJ965296	this study
O. taurinus		French Guiana	Saül		-	-	EF376067	Salducci *et al.* 2005
O. taurinus		Peru	Loreto: Teniente López, 310 m	KU 221941	AY819380	-	AY819512	Wiens *et al.* 2005
O. taurinus		Peru	Madre de Dios: Cusco Amazónico	KU 205406; WED 55452	AY326041	AY326041	AY326041	Darst & Cannatella 2004

continued next page.

TABLE 1. (continued)

Species	Code	Country	Locality	Museum Number	GenBank Accession Number			Note/Reference
O. taurinus		Venezuela	Amazonas, Neblina Base Camp on Río Mawarinuma (= Río Baria), 140 m	AMNH-A 131245	-	-	'AY843709' **	Faivovich *et al.* 2005
O. verruciger		Ecuador	Napo: Río Azuela, 9.5 km W Reventador, 1630 m	KU 217751	DQ380381	-	-	Wiens *et al.* 2006
O. sp. (B)	spB1	Bolivia	Pando: Palmira	NMP6d 41/2009	FJ965297	FJ965297	FJ965297	this study
O. sp. (B)	spB2	Bolivia	Pando: Canadá	NMP6V 73105	FJ965298	FJ965298	FJ965298	this study
O. sp. (B)	spB3	Bolivia	Pando: Nacebe	NMP6V 72173/1	FJ965299	FJ965299	FJ965299	this study
O. sp. (B)	spB4	Bolivia	Pando: Nacebe	NMP6V 72173/3	FJ965308	-	FJ965295	this study
O. sp. (C) = *castaneicola*	spC1	Bolivia	Pando: San Antonio de Filadelfia	CBF 6051	FJ965300	FJ965300	FJ965300	**holotype**; this study
O. sp. (C) = *castaneicola*	spC2	Bolivia	Pando: San Antonio de Filadelfia	NMP6V 73810/3	FJ965301	FJ965301	FJ965301	this study
O. sp. (C) = *castaneicola*	spC3*	Bolivia	Pando: San Antonio de Filadelfia	NMP6d 28/2009	FJ965302	FJ965302	FJ965302	this study
O. sp. (C) = *castaneicola*	spC4	Bolivia	Pando: San Antonio del Matti	NMP6V 73820	FJ965303	FJ965303	FJ965303	this study
Tepuihyla edelcae		Venezuela	Estado Bolívar, Auyantepui (2015 m)	MNHNP 1998–311	AY843770	AY843770	AY843770	Faivovich *et al.* 2005
Osteopilus septentrionalis		Cuba	Guantánamo, Guantánamo bay	USNM 317830	AY843712	AY843712	AY843712	Faivovich *et al.* 2005
Itapotihyla langsdorffii		Argentina	Misiones, General Belgrano, 10 Km N Bernardo de Irigoyen, Salto Andresito	MACN 38643	AY843706	AY843706	AY843706	Faivovich *et al.* 2005
Itapotihyla langsdorffii		Brazil	São Paulo: Estaçao Ecologica de Jureia, N of Jureia	USNM 303287	AY819379	AY819379	AY819511	Wiens *et al.* 2005
Acris crepitans		USA	Alabama, De Kalb Co., Powerline access Rd., 1/10 mi W of Lookout Mt. Boys Camp Rd.	LSUMZ H-2164	AY843559	AY843559	AY843559	Faivovich *et al.* 2005

* tadpole from a water-filled fruit capsule of the Brazil nut tree

** AY843709 sequence is a chimera; the 12S rRNA part corresponds fully to AY843707 (*O. "leprieurii"*; Faivovich *et al.* 2005), which is, moreover, the same sequence of the same individual as AY549361 (Faivovich *et al.* 2004); we used only the 16S rRNA part

method (ML) by PhyML 3.0 (Guindon & Gascuel 2003), and for comparison by RAxML 7.0 (Stamatakis 2006), and using Bayesian analysis (BA) by MrBayes 3.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Parameters were set in PhyML as follows: base frequencies A = 0.35, C = 0.21, G = 0.18, T = 0.26, substitution rate matrix AC = AT = 5.31, AG = 10.77, CG = GT = 1.00, CT = 37.85 and gamma shape rate variation among sites α = 0.151. BioNJ tree was used as an initial tree, the best of the nearest neighbour interchange (NNI), and the 'new' subtree pruning and regrafting algorithm (SPR; Hordijk & Gascuel 2005) of branch swapping was used as a tree topology search, with options to optimize the topology and branch lengths. Bootstrap values were computed based on 1000 resampled data sets (Felsenstein 1985). ML using RAxML was computed with the general time-reversible model with rate heterogeneity (GTR+G; Tavaré 1986), with parameters estimated during the run. The same was done in the case of BA. The BA was performed with two runs and four chains for each run for 6 x 10^6 generations, and sampling every 100_{th} tree. The first 300 trees (burn-in value) were discarded, as log-likelihood scores of sampled trees plotted against the generation time showed that stationarity was fully achieved after the first 20,000 generations. A majority rule consensus tree was then produced from the remaining trees after discarding the burn-in trees, and the posterior probabilities (BPP) calculated as the frequency of samples recovering any particular clade (Huelsenbeck & Ronquist 2001). The BA was run four more times with random starting trees and the results were compared to check for local optima. Genetic uncorrected *p*-distances were calculated in PAUP* (Swofford 2003).

Results

Both ML analyses, PhyML (Guindon & Gascuel 2003) and RAxML (Stamatakis 2006), resulted in most likely trees with the same topology with log likelihoods (ln*L*) = -7486.82 and -7489.98, respectively. Although, the bootstrap support of the relationships among main clades and most of species was very low. All independent BA runs resulted in essentially identical topologies and likelihood estimates. The majority rule consensus Bayesian tree (mean ln*L* = -7534.54; Fig. 1) had the same topology as both ML trees, when branches of the ML trees with the bootstrap support below 50% were collapsed. The estimation of phylogenetic relationships within the genus *Osteocephalus* shows that four main phylogenetic lineages can be distinguished within the studied species: (1) individual lineage formed by *O. alboguttatus* (Boulenger); (2) lineage comprising *O.* "*oophagus*" Jungfer & Schiesari from French Guiana and *O. taurinus* (support 1.00/94 = BPP/ML bootstrap), however, the latter is further structured forming a sublineage from Peru and Bolivia, while next samples from French Guiana and Venezuela form a polytomy within the whole clade (low support of their relationships); (3) clade of not very high statistical support (0.81/68) comprising three well supported sublineages: (i) *O. mutabor* Jungfer & Hödl, (ii) *O. buckleyi*, *O. cabrerai* (Cochran & Goin), *O. verruciger* Werner and another sample of *O.* "*oophagus*" from French Guiana showing remarkably low mutual genetic differentiation (support 1.00/90), and (iii) Bolivian population of *Osteocephalus* sp. (B) clustering close to *O. leprieurii* from French Guiana (support 0.95/91); (4) clade (support 1.00/98) consisting of four sublineages represented by (i) *O. deridens* Jungfer, Ron, Seipp & Almendáriz, (ii) *O.* "*leprieurii*" from Venezuela, (iii) *O. planiceps* Cope, and (iv) the unnamed Bolivian *Osteocephalus* sp. (C).

The representatives of the two known populations of *Osteocephalus* sp. (C) form a well supported (1.00/92) separate lineage within the fourth clade. *O. planiceps* seems to be the closest relative of *Osteocephalus* sp. (C), with 3.0 % of mean uncorrected *p*-distances in 16S rRNA (Table 2), which is concordant with suggested interspecific level in this molecular marker in frogs (Fouquet *et al.* 2007, Vieites *et al.* 2009). This fact also corresponds to the unique morphology and life history of this species and justifies us to describe it as a new species herein.

TABLE 2. Uncorrected *p*-distances in percentage among *Osteocephalus* species and outgroup genera included in the phylogenetic analysis. Below diagonal are genetic distances based on the 352bp 12S rRNA fragment, above diagonal are distances based on the 380 bp 16S rRNA fragment, and on diagonal within species mean uncorrected *p*-distances, if applicable.

	12S/16S rRNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	*O. alboguttatus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*O. buckleyi*	5.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	*O. cabrerai*	4.8	1.4	-	8.2	5.6	6.8	-	5.5	0.8	6.1	-	5.5	5.3	-	5.5	5.8	-	5.7	6.9	11.6	10.0	11.6	-	13.4
4	*O. deridens*	6.8	8.0	8.0	-	8.2	4.5	-	8.4	8.5	3.4	-	8.4	8.7	-	8.4	9.2	-	8.6	5.3	12.4	9.7	13.4	-	15.0
5	*O. leprieurii* F. Guiana	-	-	-	-	-	7.7	-	6.1	5.3	5.8	-	5.3	5.3	-	5.3	5.8	-	1.2	6.8	11.6	10.8	11.6	-	14.5
6	*O. "leprieurii"* Venezuela	5.4	3.7	4.5	5.7	-	-	-	7.9	6.6	2.4	-	7.9	7.6	-	7.9	8.7	-	7.8	4.4	10.5	9.5	12.1	-	14.7
7	*O. mutabor*	4.3	2.8	2.6	8.0	-	4.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	*O. "oophagus"* AY843708	5.1	4.5	4.8	8.2	-	4.8	4.0	-	5.3	7.1	-	2.1	1.6	-	2.1	2.6	-	6.7	7.6	10.8	10.0	11.8	-	14.7
9	*O. "oophagus"* AF467267	-	-	-	-	-	-	-	-	-	5.8	-	5.3	4.7	-	5.3	5.6	-	5.1	6.6	10.8	9.5	10.6	-	12.9
10	*O. planiceps* pla1-pla3	4.8	3.7	4.0	5.4	-	1.7	4.3	4.3	-	**0.0/0.0**	-	6.8	6.9	-	6.8	7.6	-	6.4	3.0	10.3	8.7	11.6	-	14.5
11	*O. planiceps* DQ380380	4.5	3.4	3.7	5.4	-	2.0	4.0	4.0	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	*O. taurinus* tau1	4.5	4.0	4.3	7.4	-	4.3	3.4	0.9	-	3.7	3.4	-	1.6	-	0.0	2.1	-	5.9	7.4	11.1	9.5	10.0	-	14.5
13	*O. taurinus* F. Guiana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6	1.1	-	5.7	7.4	10.8	9.2	10.8	-	14.2
14	*O. taurinus* Peru AY819380	4.5	4.5	4.8	7.4	-	4.8	4.0	1.4	-	4.3	4.0	0.6	-	-	-	-	-	-	-	-	-	-	-	-
15	*O. taurinus* Peru AY326041	4.3	4.3	4.5	7.1	-	4.5	3.7	1.1	-	4.0	3.7	0.3	-	0.3	-	2.1	-	5.9	7.4	11.1	9.5	10.0	-	14.5
16	*O. taurinus* Venezuela	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.4	8.2	11.8	9.7	11.3	-	14.7
17	*O. verruciger*	5.4	0.9	0.6	8.2	-	4.0	3.1	4.8	-	4.0	3.7	4.3	-	4.8	4.5	-	-	-	-	-	-	-	-	-
18	*O.* sp. (B) spB1-spB4	5.5	3.1	3.9	8.6	-	4.9	3.1	4.6	-	4.3	4.0	4.0	-	4.6	4.3	-	3.9	**0.4/0.5**	7.4	12.0	10.7	11.7	-	14.6
19	*O.* sp. (C) = *castaneicola* spC1-spC4	4.5	3.3	4.2	5.3	-	1.9	3.9	3.9	-	1.4	1.1	3.3	-	3.9	3.6	-	3.6	4.0	**0.2/0.6**	10.4	8.6	11.5	-	14.6
20	*Tepuihyla edelcae*	6.3	5.1	5.4	8.5	-	5.7	5.4	5.1	-	5.4	5.1	4.3	-	4.8	4.5	-	5.4	6.0	4.8	-	9.2	11.6	-	13.2
21	*Osteopilus septentrionalis*	4.8	4.8	4.5	8.0	-	5.7	4.8	4.3	-	5.1	4.8	3.7	-	4.3	4.0	-	5.1	5.5	4.8	5.1	-	11.1	-	12.4
22	*Itapotihyla langsdorffii* Argentina	8.0	6.0	6.0	10.2	-	8.0	6.8	6.8	-	7.4	7.1	6.5	-	7.1	6.8	-	6.3	6.6	7.0	5.7	6.0	-	-	12.6
23	*Itapotihyla langsdorffii* Brazil	8.0	6.0	6.0	10.0	-	7.7	6.8	6.8	-	7.1	6.8	6.5	-	7.1	6.8	-	6.3	6.6	6.8	5.7	6.0	0.6	-	-
24	*Acris crepitans*	9.1	8.8	8.8	11.6	-	9.1	9.7	9.9	-	8.8	8.8	9.4	-	9.9	9.7	-	9.1	9.7	9.0	9.1	8.2	10.2	9.9	-

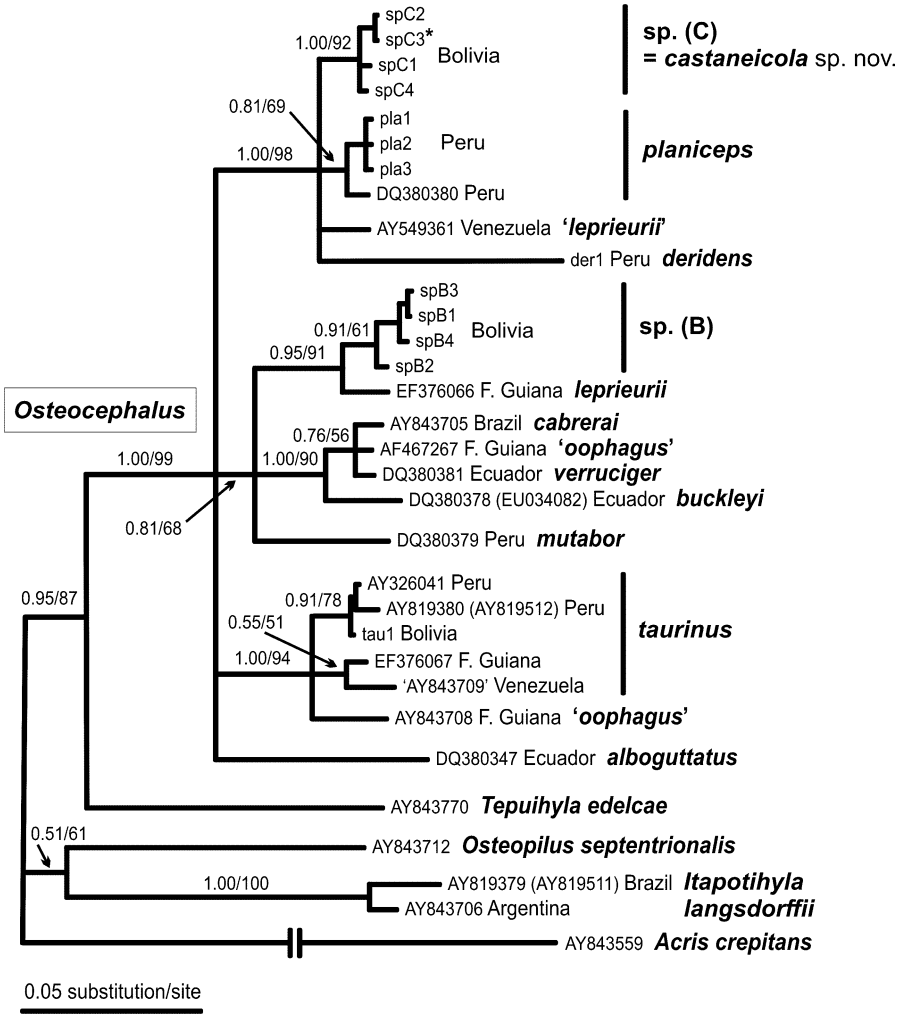

FIGURE 1. The majority rule consensus tree of the Bayesian phylogeny of the frog genus *Osteocephalus*, using mitochondrial 12S rRNA – tRNA-Val – 16S rRNA data. Nodal support, Bayesian posterior probabilities (BPP) and maximum likelihood (PhyML) bootstrap (1000 pseudoreplicates), are indicated. Collapsed branches were supported < 50 % bootstrap and < 0.50 BPP. The asterisk denotes the tadpole sample from a water-filled fruit capsule of the Brazil nut tree. *Osteocephalus* "*oophagus*" no. AF467267 may represent *O. cabrerai* according to Fouquet *et al.* (2007, Supporting Information).

***Osteocephalus castaneicola* sp. n.**
Figs. 2(A–E), 3(A–B)

Holotype. CBF 6051, adult male from the vicinity of the settlement of San Antonio de Filadelfia, 11°18' S, 67°23' W, ca. 200 m a.s.l., Provincia Manuripi, Departamento Pando, Bolivia, collected on 22 November 2007 by J. Moravec, M. Guerrero-Reinhard and G. Calderón.

Paratopotypes. NMP6V 73810/1–3, two adult males and an adult female, same locality and collecting data as holotype; CBF 6052, adult female, same locality and collecting data as holotype;

Paratypes. CBF 6053–6054, adult male and adult female from San Antonio del Matti, 11°30'S, 68°53'W, ca. 270 m a.s.l., Provincia Manuripi, Departamento Pando, Bolivia, collected on 27 November 2007 by J. Moravec, M. Guerrero-Reinhard and G. Calderón; NMP6V 73820, adult female, same locality and collecting data as CBF 6053–6054.

Diagnosis. A medium-sized species of *Osteocephalus* as revealed from mtDNA analyses, which can be distinguished by the following combination of characters: (1) medium size, SVL 47.8–51.3 mm in males,

47.7–63.3 mm in females; (2) snout rounded in dorsal view, rounded and slightly inclined posteroventrally in lateral view; (3) canthus rostralis distinct, angular, distinctly curved medially; loreal region concave; (4) low frontoparietal ridges well-marked in large individuals; (5) tympanum large, round to oval, about 62.5–76.5% of eye diameter, tympanic annulus distinct; supratympanic fold markedly developed; (6) vocal slits absent, vocal sac indistinct; (7) vomerine odontophores large, prominent, angular, narrowly separated or in contact medially, between oblique choanae, bearing 6–14 vomerine teeth each; (8) skin on dorsal surfaces with numerous minute tubercles; (9) low tarsal and ulnar tubercles present, slightly larger than dorsal tubercles; (10) axillary membrane absent; (11) basal webbing on hand [webbing formula II (2^{-}–2^{+})—(3^{-}–3^{+}) III (3^{-}–3)—($2^{2/3}$–3^{-}) IV]; toes about three fourths webbed [webbing formula I (1–$1^{1/4}$)—($1^{2/3}$–2^{-}) II (1–1^{+})—(2^{-}–2) III (1–1^{+})—($1^{2/3}$–2) IV ($1^{2/3}$–2^{-})—(1^{-}–1) V]; (12) single round distal subarticular tubercle under the fourth finger; (13) dark keratinous excrescences restricted to prepollex; (14) in life, dorsum tan, pale brown to purple brown, with scarce narrow irregular dark brown markings; a narrow pale supralabial line expanding in a subocular spot; flanks pale, without markings; hidden surfaces of thighs light brown; throat and belly creamy white; a narrow dark line along the mandible; ventral surfaces of thighs fleshy pink; iris bicoloured with a dark horizontal stripe, golden above, bronze below, both parts with fine dark reticulate to radiate lines; tibiae green or white; (15) in life, newly metamorphosed juveniles light brown dorsally, with a dark interorbital spot, bright orange iris, and creamy white upper arms, knees and heels.

Comparisons. Morphologically, *O. castaneicola* can be distinguished from all other Amazonian species of *Osteocephalus* by absence of vocal slits and by the following combinations of characters: from *O. alboguttatus* by more extensive webbing and by colouration (*O. alboguttatus*: toes two thirds webbed, light brown dorsum with small blackish dots, flanks and upper surface of thighs with small round white spots, beneath whitish with dark reticulation) (Boulenger 1882, Duellman 1978); from *O. buckleyi* by absence of large tarsal tubercles, absence of patagium and by eye colouration (*O. buckleyi*: large tubercles along the tarsus, well developed patagium, light iris without conspicuous dark pattern) (Boulenger 1882, Cochran & Goin 1970; examined specimens listed in the Appendix); from *O. cabrerai* by absence of large dorsal, ulnar and tarsal tubercles, absence of patagium and by colouration (*O. cabrerai*: large wart-like tubercles on head and dorsum, large tubercles along the ulna and tarsus, small patagium, irregularly mottled dorsal pattern, light iris with very fine vermiculation) (Cochran & Goin 1970; examined specimens listed in the Appendix); from *O. carri* (Cochran & Goin) by colouration (*O. carri*: dense large irregular dark spots on the dorsum, black spots on flanks, fuscous throat and chest) (Cochran & Goin 1970); from *O. deridens* by larger size and by colouration (*O. deridens*: SVL up to 34.9 mm in males and 50.6 mm in females, dorsum light or dark tan with or without irregular darker or lighter markings, golden yellow iris with a dark horizontal stripe and regular dark radiation (Jungfer *et al.* 2000; examined specimens listed in the Appendix); from *O. elkejungingerae* (Henle) by skin texture and by colouration (*O. elkejungingerae*: conspicuous tubercles with keratinized tips in breeding males, dorsum with broad light dorsolateral stripes in juvenile and subadult specimens (Henle *et al.* 1983; Jungfer *et al.* 2000; examined specimens listed in the Appendix); from *O. fuscifacies* by larger size and by colouration (*O. fuscifacies*: SVL up to 45.6 mm in males and 53.2 in females, dorsum light or dark tan with or without irregular darker or lighter markings, light subocular spot absent, venter dark with creamy white granules or creamy white, golden iris with a dark horizontal stripe and regular dark radiation (Jungfer *et al.* 2000; examined specimens listed in the Appendix); from *O. heyeri* Lynch by larger size and by colouration (*O. heyeri*: SVL up to 36.1 mm in males and 47.7 mm in females, dorsum brown with darker markings and pale spots, flanks with pale spots, hidden surfaces of limbs dark brown with pale spots, iris dark) (Lynch 2002); from *O. leoninae* Jungfer & Lehr by larger size and by colouration (*O. leoninae*: SVL up to 42.0 mm in males and 53.2 mm in females, upper part of iris yellow without dark markings, unpigmented nuptial pads, bold dorsal pattern) (Jungfer & Lehr 2001, Chávez *et al.* 2008); from *O. leprieurii* by nuptial excrescences restricted to prepollex, skin texture and by colouration (*O. leprieurii*: prepollical and subdigital nuptial excrescences, numerous conspicuous tubercles with keratinized tips in breeding males, golden iris with dark vermiculation, white supralabial stripe in juveniles) (Jungfer & Hödl 2002); from *O. mutabor* by skin texture

and by colouration (*O. mutabor*: numerous conspicuous tubercles with keratinized tips in breeding males, bold dark transverse markings, golden yellow iris with dark vermiculation, white dorsolateral stripes in juveniles) (Jungfer & Hödl 2002; examined specimens listed in the Appendix); from *O. oophagus* by head shape and by colouration (*O. oophagus*: truncate snout in dorsal view, white mottling or reticulation on posterior half of the flanks, golden iris with regular black radiation, orange spots on elbow, knee and heel in juveniles) (Jungfer & Schiesari 1995; examined specimens listed in the Appendix); from *O. pearsoni* by skin texture and by colouration (*O. pearsoni*: small nonspinous tubercles in males, black reticulation on the venter, dark iris) (Trueb & Duellman 1971, Jungfer & Schiesari 1995, Jungfer & Lehr 2001); from *O. planiceps* by smaller size, skin texture, keratinous excrescences restricted on prepollex and by colouration (*O. planiceps*: SVL up to 65.9 mm in males and 88.2 mm in females, numerous conspicuous tubercles with keratinized tips in breeding males, keratinous excrescences extending laterally to disc of thumb, dark spots on flanks, iris with regular black radiation) (Cope 1874, Duellman & Mendelson 1995, Jungfer & Lehr 2001, examined specimens listed in the Appendix); from *O. subtilis* Martins & Cardoso by larger size and by colouration (*O. subtilis*: SVL up to 38.8 mm in males, dark iris) (Martins & Cardoso 1987); from *O. taurinus* by smaller size, less webbing on the hands and by colouration (*O. taurinus*: SVL up to 81.0 mm in males and 94.1 in females, fingers one-half webbed, dark spots on flanks, small brown flecks on the throat, chest and sides of the belly, greenish gold iris with regular black radiation) (Duellman 2005; examined specimens listed in the Aappendix); from *O. verruciger* by skin texture and by colouration (*O. verruciger*: numerous conspicuous tubercles with keratinized tips in breeding males, uniform reddish brown iris) (Trueb & Duellman 1971, Jungfer *et al.* 2000, Jungfer & Hödl 2002); from *O. yasuni* by skin texture and by colouration (*O. yasuni*: numerous conspicuous tubercles with keratinized tips in breeding males, yellow venter in adults, iris with irregular dark reticulation, intense yellow-orange venter and webbing in juveniles) (Ron & Pramuk 1999, Jungfer *et al.* 2000, Jungfer & Hödl 2002, Cisneros-Heredia 2007).

There are seven available names in the synonymy of four *Osteocephalus* species: *Hyla festae* Perraca, 1904 (type locality: Ecuador: "Valle de Santiago" (= lower Río Zamora) Province of Morona-Santiago) in the synonymy of *O. buckleyi*; *Hyla leprieurii britti* Melin, 1941 (type locality: Brazil: "Río Uaupés (north of the Río Japú", Amazonas) and *Osteocephalus ayarzaguenai* Gorzula & Señaris, 1997 (type locality: Venezuela: "Campamento Airo, Valle del Río Karuay", Estado Bolívar) in the synonymy of *O. leprieurii*; *Osteocephalus flavolineatus* Steindachner, 1862 (type locality: Brazil: "Cocuy" (= Cucuí), Amazonas) and *Hyla depressa* Andersson, 1945 (type locality: Ecuador: "Río Pastaza, Watershed") in the synonymy of *O. taurinus*; and *Hyla riopastazae* Andersson, 1945 (type locality: Ecuador: "Baños, Río Pastaza, Provincia Tungurahua") and *Hyla orcesi* Funkhouser, 1956 (type locality: Ecuador: "[Río] Pacayacu, a stream that flows into the Cotapino, drainage of the Suno, Río Napo region") in the synonymy of *O. verruciger.* The new species differs from all of them by the following combination of characters: from *Hyla festae* by smaller size and by colouration (female holotype of *H. festae*: SVL 75.0 mm, large median longitudinal dark brown blotch on the dorsum, dark brown spots on flanks, throat and belly) (Trueb & Duellman 1971); from *Hyla leprieurii britti* by nuptial excrescences restricted to prepollex and by skin texture (male holotype of *H. l. britti*: prepollical and subdigital nuptial excrescences and tuberculate dorsum) (Trueb & Duellman 1971, Jungfer & Hödl 2002), from *Osteocephalus ayarzaguenai* by colouration (*O. ayarzaguenai*: golden iris with dark vermiculation) (Jungfer & Hödl 2002; examined specimen listed in the Appendix); from *Osteocephalus flavolineatus* by smaller size and colouration (female holotype of *O. flavolineatus*: SVL 81.8 mm, light middorsal stripe, spots on the flanks) (Cochran & Goin 1970, Trueb & Duellman 1971); from *Hyla depressa* by smaller size, skin texture, and by colouration (male holotype of *H. depressa*: SVL 68.9 mm, tuberculate dorsum, light middorsal stripe) (Cochran & Goin 1970, Trueb & Duellman 1971); from *Hyla riopastazae* by colouration (*H. riopastazae*: brown spots and mottling on throat, chest and belly) (Trueb & Duellman 1971); and from *Hyla orcesi* by skin texture and by colouration (*H. orcesi*: tuberculate dorsum, ventral surfaces dirty brown) (Cochran & Goin 1970, Trueb & Duellman 1971).

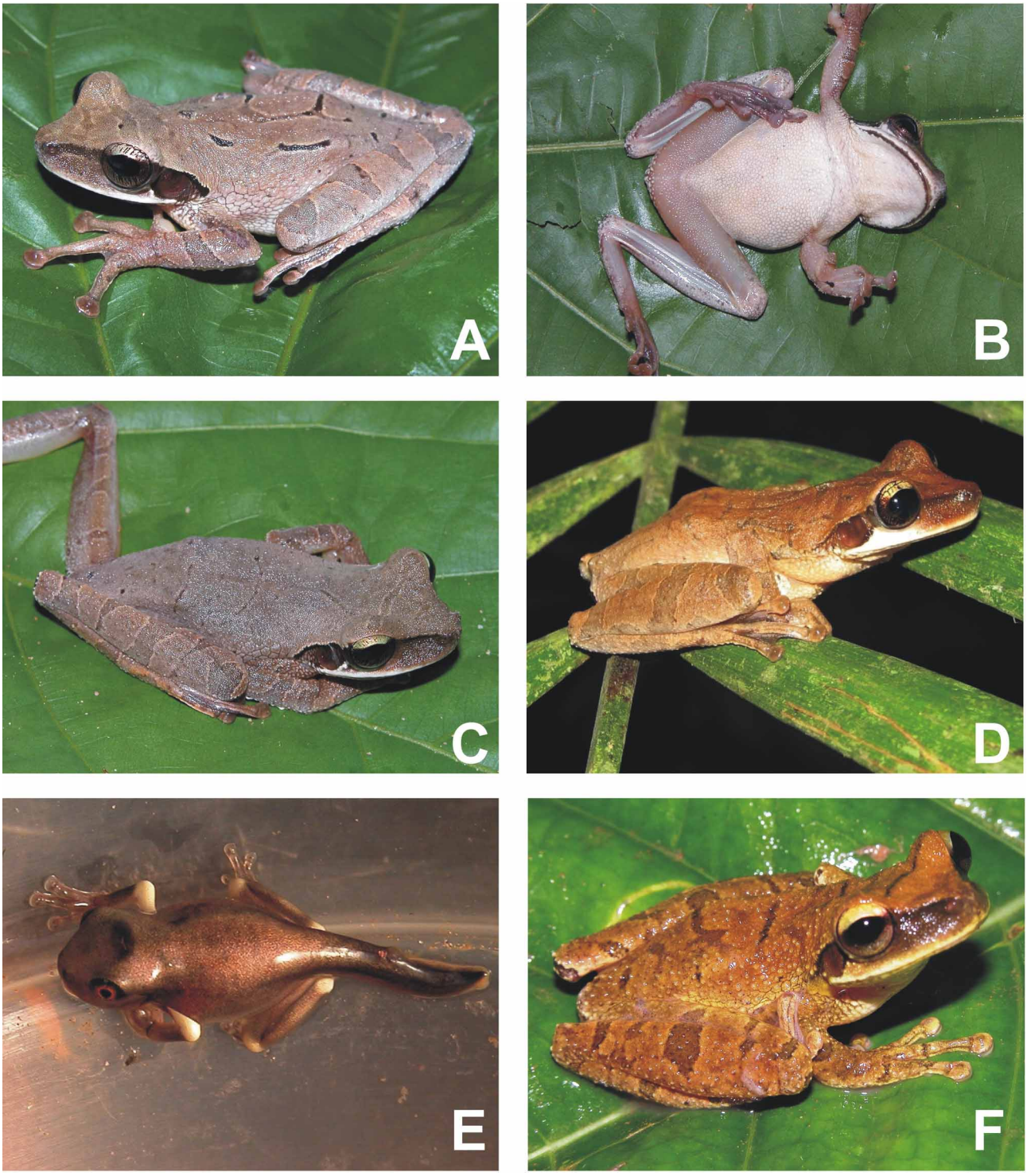

FIGURE 2. Holotype of *Osteocephalus castaneicola* **sp. n.** (CBF 6051) in life, (A) dorsal, and (B) ventral views. (C) Adult female paratype of *Osteocephalus castaneicola* **sp. n.** (CBF 6052) in life. (D) Night colouration of adult male paratype of *Osteocephalus castaneicola* **sp. n.** (NMP6V 73810/2) under natural conditions. (E) Newly metamorphosed juvenile of *Osteocephalus castaneicola* **sp. n.** (F) Adult male of *Osteocephalus* sp**.** (B) (NMP6V 73105) from Canadá (Bolivia, Pando) in life.

Description of the holotype. Adult male 51.3 mm SVL. Head narrower than body, slightly longer than wide; snout rounded in dorsal view, moderately protruding in lateral view; distance from nostril to eye shorter than diameter of eye; canthus rostralis distinct, angular, curved medially; loreal region concave; internarial

area slightly depressed; nostrils moderately protuberant, directed laterally; interorbital area flat, IOD 112.2% of ELW; lateral margins of the frontoparietals barely visible through skin; eye large, strongly protuberant, its diameter about five times depth of lip below eye; tympanic membrane clearly evident, large, slightly wider than high, about two third of eye length, separated from eye by ca. 50% of its diameter; tympanic annulus distinct; supratympanic fold conspicuous, covering upper edge of tympanum, continuing above insertion of arm. Arm slender, axillary membrane absent; small low tubercles scattered along ventrolateral edge of forearm; relative length of fingers I<II<IV<III; fingers bearing large, oval discs, that of third finger about half of tympanum diameter; subarticular tubercles prominent, round, single; supernumerary tubercles present; palmar tubercle large, flat, disunited distally; prepollical tubercle large, flat, elliptical; prepollex enlarged; large dark keratinous nuptial excrescences covering inner surface of prepollex up to subarticular tubercle of thumb (Fig. 3); webbing rudimentary between fingers I and II; webbing formula of fingers II2^{-}—3^{-}III3^{-}—3^{-}IV. Legs moderately long, slender; heels overlapping when limbs flexed perpendicular to the axis of body; small raised tubercles on the outer edge of tibiotarsal articulation; small low tubercles scattered along the ventrolateral edge of foot; toes moderately long, bearing oval discs slightly smaller than those of fingers; relative length of toes I<II<V<III<IV; outer metatarsal tubercle distinct, small, round; inner metatarsal tubercle large, ovoid; subarticular tubercles single, round, protuberant; supernumerary tubercles present; toes three fourths webbed; webbing formula of toes I1^{+}—2^{-}II1—2^{-}III1—2^{-}IV2^{-}—1^{-}V. Skin on dorsum, head, and dorsal surfaces of limbs smooth, with numerous minute tubercles; skin on flanks shagreen; skin on venter coarsely granular; skin on throat slightly granular; proximal two thirds of lower surfaces of thighs slightly granular. Cloacal opening directed posteriorly at upper level of thighs; short simple cloacal sheath covering cloacal opening; rounded tubercles around vent and on posterior surface of proximal third of thigh. Tongue ovoid, widely attached to floor of mouth; vomerine odontophores angular, separated medially, between choanae, bearing 8 and 9 (left/right) vomerine teeth; choanae rhomboidal, oblique; vocal slits absent; vocal sac indistinct.

Measurements of the holotype: SVL 51.3; HL 17.7; HW 16.6; EN 5.3; ED 6.1; TD 4.0; ELW 4.9; IOD 5.4; TL 27.3; FL 33.4.

In alcohol, head and dorsum tan with several narrow irregular darker tan to dark brown markings (including an indistinct interorbital stripe) narrowly outlined by pale brown line; dorsal surfaces of limbs tan with darker tan crossbars outlined by a pale brown line. A narrow pale supralabial line expanding in a subocular spot; a dark canthal stripe extending from nostril to the anterior margin of eye; a broad dark brown postocular stripe extending from posterior margin of eye across the tympanum to insertion of arm. Flanks pale with several inconspicuous small darker markings; a dark supracloacal spot; hidden surfaces of thighs tan. Throat and belly creamy white; a narrow dark line along the lower jaw; ventral surfaces of thighs yellowish white; plantar surfaces pale brown. Tibiae green.

In life, dorsal and lateral colouration differed only slightly from the preserved specimen in having a slight purple-red tint by day. Ventral surfaces of forearms and thighs fleshy pink; tibiae green. Iris bicoloured with dark brown horizontal stripe, golden above, bronze below, both parts with fine dark reticulate to radiate lines (Fig. 2A).

Variation. Variation of measurements of the type series is given in Table 3. *Osteocephalus castaneicola* exhibits sexual dimorphism in body size, but sexual dimorphism of dorsal skin texture is absent. Both breeding males and females bear similar minute flat to round tubercles on dorsal surfaces of head, body and limbs. The most conspicuous dorsal tubercles are present in female paratopotype CBF 6052 (Fig. 2C), having SVL 47.7 mm and containing numerous small immature eggs. The new species shows considerable variation in number of vomerine teeth (6–14 on each odontophore). Vomerine odontophores are separated in holotype, paratopotype NMP6V 72810/1 and paratypes CBF 6054 and NMP6V 73820, but in contact in the remaining types. Some variation seems to be evident in distinctiveness of lateral margins of the frontoparietals. They are not visible through skin in smaller individuals (SVL up to 47 mm; paratopotype CBF 6052 and paratype CBF 6053) and best pronounced in largest individuals (SVL above 59 mm; female paratopotype NMP6V 73810/3

and female paratypes CBF 6054 and NMP6V 73820). Some differences can be found in shape of distal subarticular tubercle of the fourth finger. It is single in holotype and four other type specimens, but it shows a slight tendency to bifidity in the paratopotype NMP6V 73810/3 and paratypes CBF 6053 and NMP6V 73820. The finger and toe webbing formulae vary as follows: II (2^{-}–2^{+})—(3^{-}–3^{+}) III (3^{-}–3)—($2^{2/3}$–3^{-}) IV and I (1–$1^{1/4}$)—($1^{2/3}$–2^{-}) II (1–1^{+})—(2^{-}–2) III (1–1^{+})—($1^{2/3}$–2) IV ($1^{2/3}$–2^{-})—(1^{-}–1) V.

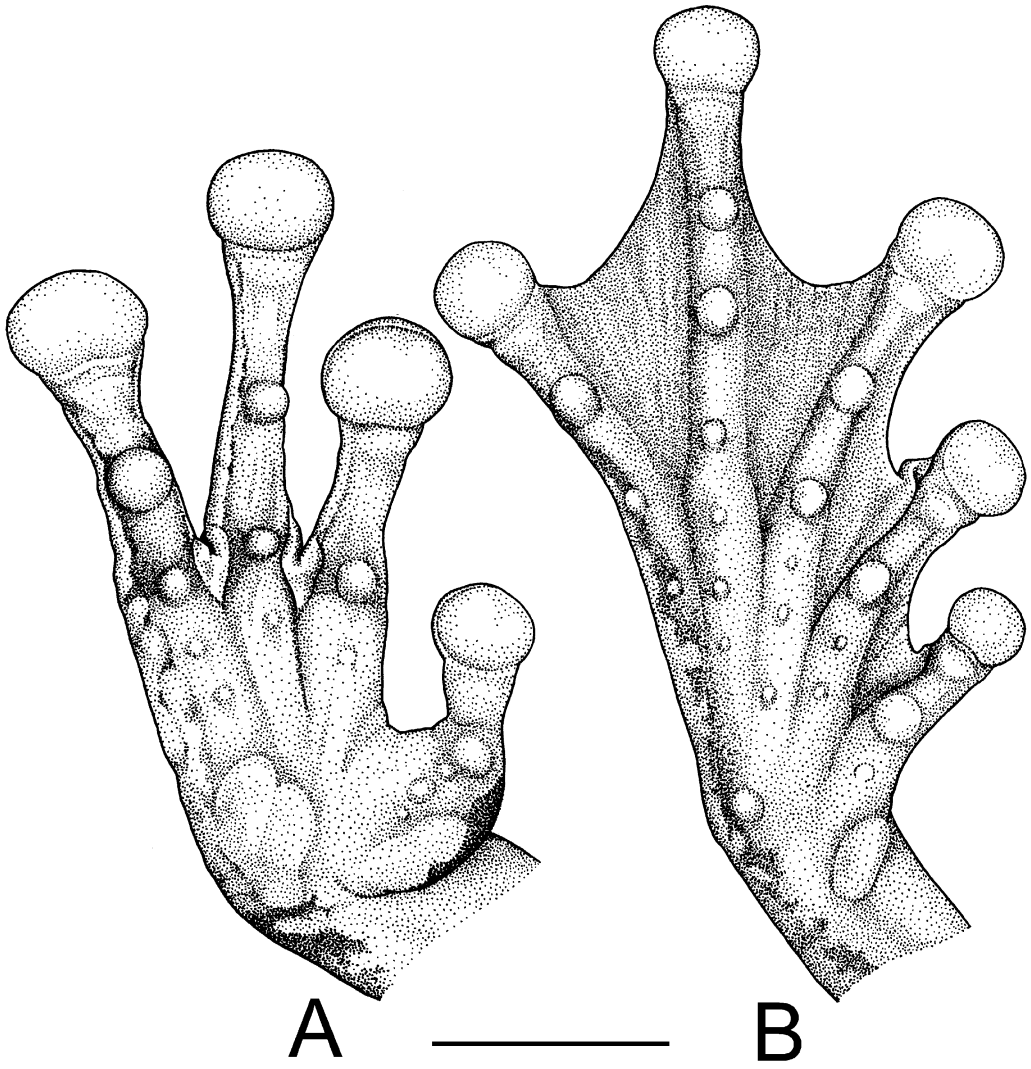

FIGURE 3. (A) Palmar, and (B) plantar views of right hand and foot of the holotype of *Osteocephalus castaneicola* **sp. n.** (CBF 6051). Scale bar equals 5 mm.

General dorsal colouration in alcohol varies from light tan to dark tan with purple-red tint or to reddish-brown. Dorsal pattern varies mostly regarding distinctness and shape of the irregular darker markings. A more or less distinct interorbital streak narrower than the diameter of the eye is present in all individuals. Dorsal markings are fused in a large, irregular, indistinct dorsal spot in the male paratype CBF 6053, whereas dorsal pattern of paratopotypes CBF 6052, NMP6V 73810/1, 73810/3 and paratype 73820 is almost missing. Ventral colouration in alcohol varies from cream white to yellowish-white. A fine dark brown mottling is present on the throat and pectoral area of the female paratype NMP6V 73820. Colour of tibiae seems to vary independently of age or size of individual specimens. The bones are green in the holotype and paratopotypes NMP6V 73810/1–3 (SVL 48.4–59.1 mm) and white in paratopotype CBF 6052 and paratypes CBF 6053, 6054 and NMP6V 73820 (SVL 47.7–63.3 mm).

In life, dorsal colouration varies from tan to brown. A slight purple-red tint observed in most specimens by day turns into ochre by night (Fig. 2D). Newly metamorphosed juveniles are light brown dorsally with a dark interorbital spot, bright orange iris, and creamy white upper arms, knees and heels (Fig. 2E).

TABLE 3. Variation of measurements (in mm) of the type series of *Osteocephalus castaneicola* **sp. n.**). See text for abbreviation.

Measurement	Males (N=4) Mean ± SD; Range	Females (N=4) Mean ± SD; Range
SVL	49.3 ± 1.56; 47.8–51.3	57.6 ± 6.83; 47.7–63.3
HL	16.9 ± 0.87; 15.7–17.7	19.4 ± 2.18; 16.6–21.9
HW	16.2 ± 0.67; 15.4–16.9	18.4 ± 1.92; 15.6–20.0
EN	5.0 ± 0.36; 4.5–5.3	6.1 ± 0.87; 4.9–7.0
ED	5.9 ± 0.29; 5.1–6.1	6.1 ± 0.71; 5.1–6.8
TD	3.7 ± 0.22; 3.5–4.0	4.3 ± 0.70; 3.5–5.2
ELW	4.8 ± 0.08; 4.7–4.9	5.5 ± 0.79; 4.6–6.4
IOD	5.0 ± 0.30; 4.7–5.4	5.8 ± 0.83; 4.8–6.6
TL	26.5 ± 0.70; 25.7–27.3	31.9 ± 3.28; 27.2–32.9
FL	32.5 ± 1.35; 30.5–33.4	38.5 ± 4.46; 31.9–41.3

Distribution, ecology and threat status. The known localities of *Osteocephalus castaneicola* lie in western and central part of the Departamento Pando, northern Bolivia (Fig. 4). This area is located in the south-western Amazon basin within the zone of tall evergreen lowland rainforest. *O. castaneicola* was encountered in more or less undisturbed terra firme forest with frequent occurrence of large climax forest trees [e.g. *Bertholletia excelsa* Humb. & Bonpl., *Ceiba pentandra* (L.) Gaertn., *Cedrela odorata* L., *Ficus* sp.]. The forest was characterised by relatively well defined tree strata and a dense canopy at ca. 25–35 m above the ground. The understory was dominated by various tree seedlings, young trees, herbaceous lianas, palms and ferns. The forest floor was covered by leaf litter with scattered large fruit capsules of the Brazil nut tree (*Bertholletia excelsa*) and other species of Lecythiadaceae. All observed individuals of *O. castaneicola* were sitting on vegetation in ca. 0.5–2 m height. No calling males were located. Other hylid species found in sympatry with *O. castaneicola* included *Hypsiboas lanciformis* Cope, *H. punctatus* (Schneider), *Phyllomedusa camba* De la Riva, *P. tomopterna* (Cope), *P. vaillantii* Boulenger, *Trachycephalus coriaceus* (Peters), and *T. resinifictrix* (Goeldi). *O. castaneicola* is apparently known (as *O.* sp.) to occur also in the Region Madre de Dios (exact localities not provided) in adjacent southern Peru (von May *et al.* 2007).

Life history of *O. castaneicola* is closely associated with fruit capsules of the Brazil nut tree, which are opened by agoutis (*Dasyprocta* sp.) or indigenous Brazil nut collectors and abandoned on the forest floor. At both known localities of *O. castaneicola* some of water-filled capsules contained tadpole assemblages numbering up to tens of individuals. Rarely the same tadpoles were found also in water-filled palm bracts lying on the ground. In some cases the assemblages consisted of larvae of markedly different sizes and different stage of development. The largest tadpoles reached a total length of 33–35 mm. Occasionally, white ingested eggs were visible through the transparent venter of the larger larvae. The tadpoles were raised until metamorphosis (Fig. 2E) and their determination was verified by genetic comparison with the adult specimens (Fig. 1).

According to the sparse data available we here classify *O. castaneicola* as “Data Deficient” according to the IUCN red list criteria. In Peru, the species occurs within protected areas (von May *et al.* 2007).

Etymology. The specific name is a compound from the Latin *castanea* (Horse Chestnut, *Aesculus*) from which the Spanish *castaña* (vernacular name of the Brazil nut tree) was derived and the Latin *colō* (to inhabit). The name is used as a noun in apposition and refers to the life history of the new species.

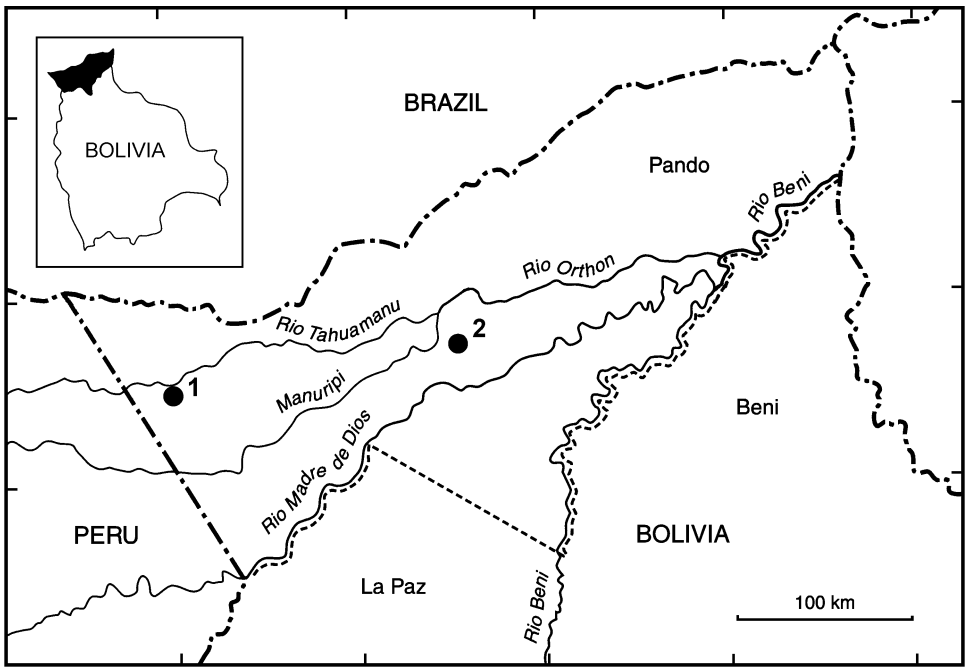

FIGURE 4. Schematic map of northern Bolivia showing the known distribution of *Osteocephalus castaneicola* **sp. n**. in Bolivia: (1) San Antonio del Matti, (2) San Antonio de Filadelfia (type locality).

Discussion

The obtained phylogeny demonstrates existence of several distinct lineages within the genus *Osteocephalus*. Nevertheless, the fact that representatives of two nominal species (*O. leprieurii* and *O. oophagus*) cluster independently within two different clades indicates that more complete sampling and accurate determination of analysed taxa are necessary to obtain a more exact picture of phylogenetic relationships within the given genus. Some of the GenBank sequences represent obviously misidentified species or even a chimerical sequence (see Table 1). Also the high genetic similarity of the GenBank sequences of *O. cabrerai*, *O. verruciger* and *O.* "*oophagus*" argue more likely for erroneous original determination of the sequenced animals than for close or even nearly identical phylogenetic positions of these morphologically well differentiated species. However, despite of the rather preliminary character of the obtained phylogeny of the genus, it is evident that *O. castaneicola* forms a separate monophylum with *O. deridens*, *O. planiceps* and the GenBank sample from Venezuela determined as *O. leprieurii* (sensu Faivovich *et al.* 2004, 2005) (Fig. 1). Both *O. castaneicola* and *O. deridens* lay eggs in phytotelmata and take care of their oophagous tadpoles. Similarly, *O. planiceps* breeds in various phytotelmata (pers. obs. KHJ). In contrary, *O. leprieurii* forms explosive breeding congregations around temporal free waters (Jungfer & Hödl 2002). Considering these huge differences in reproductive mode, the position of Venezuelan "*leprieurii*" in this clade seems to be questionable. In the past, the name *O. leprieurii* has been widely used for many forms of *Osteocephalus* and it is likely that the Venezuelan sample represents a misidentified taxon (this sample was used later also by Wiens *et al.* 2005, 2006, Fouquet *et al.* 2007 and Moen & Wiens 2009). This possibility is supported by the fact that the GenBank sample of *O. leprieurii* from French Guiana (EF376066; Salducci *et al.* 2005), where the type locality of this species (Cayenne) is located, is embedded in a different clade together with morphologically similar *O. mutabor*, *Osteocephalus* sp. (B) and other species. A similarly doubtful situation can be found in the case of two GenBank samples of *O.* "*oophagus*" from French Guiana (Salducci *et al.* 2002, Faivovich *et al.* 2005; the latter sample was used also by Wiens *et al.* 2006, and Moen & Wiens 2009), which cluster separately in two different clades. However, the GenBank sample AF467267 was labelled without further

explanation as *O. cabrerai* in Fouquet *et al.* (2007), contrary to the original (Salducci *et al.* 2002, 2005) and GenBank data. The sample really clusters with *O. cabrerai* (AY843705; Faivovich *et al.* 2005), and thus, may represent rather *O. cabrerai* than *O. oophagus*. Anyway, both clades containing *O.* "*oophagus*" comprise species, which breed in free water bodies and do not take care of their tadpoles, although *O. oophagus* is known by reproducing in phytotelmata and feeding its tadpoles with fertilized eggs (Jungfer & Weygoldt 1999). Considering egg deposition in free water plesiomorphic (Duellman & Trueb 1986), we can suppose the tendency to utilize phytotelmata as breeding place to be a derived state. Therefore, one may expect that *Osteocephalus* species with this derived reproductive mode could cluster together. However, this pattern is not supported by current data considering any of the ambiguous positions of *O.* "*oophagus*" in our tree, suggesting that this reproductive strategy may have evolved more than once within *Osteocephalus*. Nevertheless, specific determination of the "*oophagus*" samples should be verified to have a clearer picture on the life history evolutionary scenario in this genus.

Breeding in phytotelmata is one of many possible ways to avoid competition and predator pressure in the tropics (e.g. Duellman 1978, Krügel & Richter 1995). In this respect, fruit capsules of the Brazil nut tree may offer an excellent shelter. In addition, water trapped in the capsules dries up much more slowly than free water in small puddles appearing on the forest floor after heavy rains. Therefore, frogs specialized to breeding in abandoned Brazil nut tree capsules may profit both from the protection and from relatively stable water conditions provided by these unusual phytotelmata. Although *O. castaneicola* represents the first evidence of a hylid frog breeding in fruit capsules of the Brazil nut tree, at least three other frog species are known to use the same breeding place: two dendrobatids, *Adelphobates castaneoticus* (Caldwell & Myers) and *A. quinquevittatus* (Steindachner), and one bufonid, *Rhinella castaneotica* (Caldwell) (Caldwell 1993, Lötters *et al.* 2007). Complex life history responses to predation are described in two of these species by Caldwell (1993). There are no similar available data in the case of *Osteocephalus castaneicola*.

Absence of vocal slits and absence of an obvious vocal sac is the most characteristic morphological feature of the males of *O. castaneicola*. Jungfer & Hödl (2002) suppose that the relatively small subgular vocal sac of bromeliad-breeding species of *Osteocephalus* might have evolved from ancestral (for the genus) lateral or both lateral and subgular vocal sac as an adaptation towards limited space in a narrow leaf axil. Therefore, an interesting question is, if disappearance of vocal slits as well as a distinct vocal sac can be seen as an advanced adaptation for breeding in limited space inside the Brazil nut tree fruit capsule. Observation of sporadic breeding also in fallen water-filled palm bracts indicates some degree of plasticity in the breeding strategy of the new species. It appears that at least in the case of *Rhinella castaneotica* the fruit capsules are not obligatory for the breeding. This species has subgular vocal sac and can reproduce also in small water-filled holes in the soil (see Köhler & Lötters 1999). Therefore, more detailed research should be further done to understand the unusual life history of *O. castaneicola.*

As mentioned in the introduction, the taxonomic status of the Bolivian populations referred to as *Osteocephalus* sp. (A) (Jungfer & Lehr 2001) remains to be solved. It was reported as *O. leprieurii* or *O.* cf. *leprieurii* from the Departamento Santa Cruz by De la Riva *et al.* (2000). According to the scarce information available, this taxon resembles *O. leprieurii*, but differs from it by its bicoloured iris with dark reticulation, yellow venter and absence of sexual size dimorphism (Jungfer & Hödl 2002). On the other hand, Jungfer & Lehr (2001) mentioned that the bicoloured iris of this species lacks reticulation and also the specimen from Los Fierros figured by De la Riva *et al.* (2000, p. 107) lacks an obvious reticulation in the iris. A formal description of this taxon should be in press since 2000 (see De la Riva *et al.* 2000, Jungfer & Lehr 2001, Jungfer & Hödl 2002). Unfortunately, no tissue samples of *Osteocephalus* sp. (A) were at disposal for our molecular comparison with populations of similar *Osteocephalus* sp. (B) from Pando (Fig. 2F). The latter form shows relatively high variation in iris colouration (bicoloured to uniform with dark horizontal stripe and dark vermiculation). It has creamy to yellowish white venter and it is slightly dimorphic in sexual size (Moravec & Aparicio 2004). Morphologically, *Osteocephalus* sp. (B) cannot be differentiated clearly from *O. leprieurii*, redescribed by Jungfer & Hödl (2002). It appears to be conspecific with the Brazilian population discovered recently ca. 600 km east of the Bolivian border in municipality of Aripuanã (10°09' S, 59°28' W;

state of Mato Grosso) and determined as *O. leprieurii* (Santana *et al.* 2008). According to our molecular results, *Osteocephalus* sp. (B) forms a discrete unit with the sample of *O. leprieurii* from French Guiana. Nevertheless, it is difficult to judge if it is really conspecific with *leprieurii* or not (obtained divergence 1.3–1.8 % uncorrected *p*-distances in 16S rRNA; 1.2 % in the among-all species comparable fragment, Table 2). Therefore, a more thorough study focused on phylogeography and bioacoustics of *O. leprieurii* including populations of *Osteocephalus* sp. (A) and *Osteocephalus* sp. (B) is necessary to solve the systematic status and mutual position of these forms.

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Appendix. Additional specimens examined

Osteocephalus "*ayarzaguenai*": VENEZUELA: Amazonas: Marakapiwei, MBUCV 6632.
Osteocephalus buckleyi: BOLIVIA: Pando: Santa Crucito, NMP6V 73945; Pando: CBF 1262, 2150–51.
Osteocephalus cabrerai: PERU: Loreto: 21 km W of Iquitos, NMP6V 71144/1–2.
Osteocephalus castaneicola sp. n.: BOLIVIA: Pando: San Antonio de Filadelfia, NMP6d 28/2009.
Osteocephalus deridens: PERU: Loreto: Anguilla, NMP6V 71263; 35 km SW of Iquitos, NMP6V 71262/1–5.
Osteocephalus elkejungingerae: PERU: Huánuco/Ucayali: El Boquerón del Padre Abad, ZFMK 33352 (holotype), 36319 (paratype).
Osteocephalus fuscifacies: ECUADOR: Napo: Aliñahuí (5 km W of Ahuano), ZFMK 68660 (paratype).
Osteocephalus mutabor: ECUADOR: Napo: Río Chaloyacu on Carretera Narupa – Coca, ZFMK 66237 (paratype).
Osteocephalus oophagus: BRAZIL: Amazonas: Reserva Forestal Adolfo Ducke, ZFMK 57137–38 (paratypes).
Osteocephalus planiceps: PERU: Loreto: Anguilla, NMP6V, 71264/1–2; 21 km W of Iquitos, NMP6V 71204/1–2; Puerto Almendras, NMP6V 71174/1–5.
Osteocephalus taurinus: BOLIVIA: Pando, Nacebe, NMP6V 72172/1–2; Pando: CBF 1281, 1300–02, 2147–48, 43333; PERU: Loreto: Puerto Almendras, NMP6V 71184.
Osteocephalus sp. (B): BOLIVIA: Pando: Canadá, NMP6V 73105; Nacebe, CBF 5589–93, NMP6V 72173/1–4; Palmira, NMP6d 41/2009.

Shrnutí výsledků a závěr

Z chronologického hlediska výzkum sestával nejprve z morfologických studií, paralelně a průběžně doplňovaných o bioakustické přístupy (většina dat však zůstala nepublikovaných[1]) a vyústil v detailní molekulárně-fylogeografickou studii rosniček (*Hyla*) Blízkého východu, včetně popisu nového druhu z Jemenu až jižní Levanty a v systematickou studii popisující nový druh amazonské rosničky rodu *Osteocephalus*, včetně formulované hypotézy o fylogenetických vztazích uvnitř rodu.

Morfologický fenotypový přístup se ukázal u studovaných zástupců rodu *Hyla* jako nevhodný pro účely taxonomie. V **první práci** jsme se zaměřili na frekvenci výskytu a typu skvrnitého zbarvení dorzální strany těla u kyperské populace rosniček *H. savignyi* ve srovnání s přilehlými pevninskými populacemi a zjištěné rozdíly (vyšší frekvence skvrnitosti a dorzálních pruhů u kyperské populace) jsme považovali za možný signál taxonomické odlišnosti této izolované ostrovní populace. Toto zjištění bylo zpočátku také podporováno signifikantními statistickými rozdíly, i když nepříliš výraznými, v oznamovacích hlasech samců (V. Gvoždík, nepublikováno). Nicméně ani jedno z těchto zjištění nemůže být dáváno do souvislosti s fylogenetickou, potažmo taxonomickou odlišností, neboť jak bylo jasně doloženo genetickými metodami (IV. práce), kyperská populace vznikla relativně recentně kolonizací z jižního Turecka. Signifikantnost rozdílů v hlasech tak byla nejspíše způsobena statistickým artefaktem daným nízkým počtem srovnávaných hlasů nahraných navíc při rozdílných teplotách, i když vliv teploty byl statisticky odfiltrován. Vyloučit se nedá ani možnost jistého posunu znaků pod vlivem recentně přerušeného toku genů této ostrovní populace a s tím související genetický drift, který mohl takový posun znaků zapříčinit. To bude zřejmě také vysvětlení posunu frekvence výskytu rozdílného typu zbarvení na Kypru a pevnině. Svůj podíl ovšem může hrát také vliv odlišných vnějších podmínek, jak bylo dokladováno s ohledem na utváření tělesných proporcí v případu druhé studie.

V **druhé studii** byla řešena otázka morfometrické diferenciace různých populací druhů *H. savignyi* a *H. arborea* (dle současné systematiky také druhu *H. orientalis* a nového druhu, který byl popsán v rámci IV. kapitoly výsledků práce). I se zohledněním později zjištěných výsledků genetických a s ohledem na současný stav taxonomického poznání bylo

[1] Bioakustické přístupy byly v dizertační práci použity jen jako doplňující, neboť se ukázalo, že získání početného množství kvalitních nahrávek z velkého geografického areálu by bylo nad rámec této dizertační práce vzhledem k náročnosti jejího multidisciplinárního přístupu (morfologie, genetika).

hlavním výstupem této práce zjištění, že vnitrodruhová fenotypová plasticita převyšuje a smazává pod vlivem vnějších podmínek mezidruhové rozdíly, jak bylo dokumentováno u mediteránních populací *H. savignyi*, *H. orientalis* a *H. arborea*, které obývají podobné ekologické habitaty a klimatickou zónu. Naopak vliv vnějších podmínek pak může také zesílit rozdíl ve tvaru těla u populací žijících v odlišných ekologických podmínkách, jak bylo dokumentováno u kavkazských populací *H. orientalis* a *H. savignyi*, které v oblasti obývají odlišné ekologické niky s odlišnými klimatickými podmínkami. Tato hypotéza byla následně podpořena i výsledky studie kavkazských vs. kaspických populací *H. orientalis* (V. Gvoždík, nepublikováno), které si jsou geneticky velmi příbuzné, zatímco morfologicky výrazně odlišné, což platí i o klimatických faktorech. Podobná zjištění byla učiněna také při studiu nově popsaného druhu (IV. práce) u populací z Arabského poloostrova a jižní Levanty, kdy se tyto populace vzájemně morfometricky odlišují, zatímco levantská populace nového druhu se od levantských populací *H. savignyi* příliš v biometrických datech neliší (V. Gvoždík, nepublikováno).

Krátká **třetí práce** pojednává o vyžití akustických dat, oznamovacích hlasů samců, při identifikaci dosud přehlíženého druhu rosničky, *H. orientalis* (dříve *H. arborea*) na území Íránu a sice z oblasti nížin při Kaspickém pobřeží. Pravděpodobný je také výskyt na východních svazích pohoří Táleš a severních svazích pohoří Elburz, které pak zřejmě tvoří bariéru mezi tímto druhem a *H. savignyi*. Nahrávky oznamovacích hlasů samců obou druhů z území Íránu pak ukázaly jasné akustické rozdíly a tím i význam tohoto přístupu v systematice těchto morfologicky komplikovaných druhů žab.

Nejobsáhlejší **čtvrtá práce** vyústila za pomocí molekulárně-fylogeografického přístupu v podrobné poznání fylogeografie rosniček východního Mediteránu a Blízkého východu a v popis nového druhu rodu *Hyla*. Vznik tohoto druhu byl datován do pozdního miocénu, období, kdy se v regionu formoval tektonický zlom, který je dnes tvořen Wádím al-Araba, Mrtvým mořem, údolím Jordánu a Hula a v Libanonu údolím Bikáa. Nový druh je znám jen na východ od tohoto tektonického zlomu, zatímco *H. savignyi* na západ. V oblasti této propadliny se pak zřejmě oba druhy stýkají a příležitostně hybridizují, jak bylo doloženo z jedné lokality v severovýchodním Izraeli. Nový druh odlišený od *H. savignyi* se mimo genetická data liší také v oznamovacích hlasech samců a částečně také morfologicky. Tato práce sice tedy ukázala na taxonomickou odlišnost populace z Arabského poloostrova, nicméně ne ve smyslu její izolovanosti (severozápad Arabského poloostrova není rosničkami obývaný pro jeho nehostinné pouštní podmínky rozšířené v oblasti před 5000 – 6000 lety),

nýbrž vzniklou pod vlivem tektonických událostí v jižní Levantě. Naproti tomu druhá izolovaná populace z Kypru, od začátku studie pod zvláštním zájmem, se ukázala být výsledkem recentní kolonizace z jižního Turecka. Kolonizace, u které se nedá vyloučit její zapříčinění člověkem, neboť mezi Kyprem a pevninou je hluboký mořský příkop, který zajišťoval separaci Kypru od pevniny i v dobách poklesu mořské hladiny v obdobích pleistocénních glaciálů. Práce dále odkrývá genetickou strukturu populací jednotlivých studovaných druhů (*H. savignyi*, *Hyla* sp. nov. a blízkovýchodní populace *H. orientalis*), kdy hlavní divergence uvnitř druhů jsou datovány do období hranice mezi pliocénem a pleistocénem, pravděpodobně v souvislosti se změnou klimatických podmínek, zatímco detailnější fylogeografická strukturalizace je zřejmě odrazem klimatických oscilací v průběhu pleistocénu. Od pozdního pleistocénu pak byla u *H. savignyi* a *H. orientalis* detekována populační expanze, zatímco u nového druhu zůstala velikost populace spíše konstantní. Dle genetické strukturalizace byly jako hlavní glaciální refugia zjištěny u *H. savignyi* oblasti Levanty, jižního Turecka, Mezopotámie, Zakavkazska a pravděpodobně údolí či úpatí pohoří Zagros; u *Hyla* sp. nov. zřejmě wádí v jižní Levantě a pravděpodobně také samostatná refugia na Arabském poloostrově; u *H. orientalis* pak podél jihozápadního a severního tureckého pobřeží a v zakavkazské a jihokaspické oblasti.

Pátá práce popisuje nový druh amazonské rosničky rodu *Osteocephalus* na základě studia morfologie (nový druh např. postrádá vokální štěrbiny u samců), reprodukční strategie (nový druh se rozmnožuje v prázdných vodou naplněných tobolkách juvie ztepilé, *Bertholletia excelsa*) a genetiky (nejblíže příbuzným druhem je zřejmě *O. planiceps*). Práce se pokusila také o předběžné odvození fylogenetických vztahů uvnitř rodu na základě mitochondriální DNA. K tomu byly využity sekvence získané z vlastních dat, ale také sekvence získané z veřejné databáze GenBank. To se ukázalo být lehce problematické, neboť několik těchto sekvencí zjevně pocházelo od jedinců chybně determinovaných, což bylo v práci diskutováno. Dokonce se podařilo mezi „genbankovými“ sekvencemi odhalit vyloženě chybnou, chimérickou sekvenci složenou zřejmě omylem ze dvou odlišných druhů. Tato zjištění mimo jiné poukazují na nutnost zvýšené opatrnosti při používání těchto veřejně dostupných dat, kdy by se badatel vždy měl snažit co nejpřesněji dopátrat původu jedinců, popřípadě i vyšetřit dokladové exempláře, pokud existují.

Závěr a výhledy do budoucího výzkumu

Díky výzkumům v rámci dizertační práce bylo zjištěno, že morfologické parametry nejsou vhodnými znaky pro studium příbuzností populací a jejich použití v systematice

západopalearktických rosniček může být problematické. Z tohoto pohledu se jeví lépe znaky akustické, tj. oznamovací hlasy samců (*advertisement calls*), ačkoliv ani ty nemusí nutně nést výrazné rozdíly u alopatrických taxonů. Zcela nejvhodnější jsou pro systematiku genetická data, která nám při fylogeografickém zpracování umožní odhalit evoluční linie i u morfologicky uniformních či naopak příliš plastických taxonů, či u taxonů s totožnými akustickými projevy. Pro zodpovědné zhodnocení taxonomických výstupů je však třeba vyšetřit signál většího množství nezávislých genetických markerů a to nejlépe také v kontextu dalších příbuzných druhů.

Budoucí výzkum západopalearktických rosniček bude zaměřen na dořešení otázky fylogenetické (taxonomické) diferenciace a fylogeografie druhu *Hyla arborea* sensu lato a dále na otázku evoluce oznamovacích hlasů a jejich významu při speciaci a mezidruhovém rozpoznávání.

Z výsledků dizertační práce je také patrné, že ideálním řešením jakékoliv taxonomické revize by byla podrobná multilokusová fylogeografická studie založená na početných, přesně lokalizovaných a determinovaných vzorcích, včetně vzorků z typových lokalit. To může ovšem být v některých regionech složitější realizovat, zejména pak v oblasti tropů. Nicméně takový přístup by byl záslužný nejen pro obecné poznání, ale hlavně také pro správný přístup v ochraně stále ještě podhodnocené světové biodiverzity.

Supplementum

Slow Worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: Genetic structure reveals deep divergences.

Gvoždík V., Jandzik D., Lymberakis P., Jablonski D. & Moravec J.

Molecular Phylogenetics and Evolution: in press.

Následující práce zařazená formou supplementa se svým taxonomickým zaměřením sice tématu dizertační práce nedotýká, nicméně je s ním úzce spjata metodicky. Prezentuje výsledky molekulárně-systematické studie založené na fylogeografickém přístupu u morfologicky komplikovaného rodu plaza, slepýše (*Anguis*, Anguidae). Dle výsledků analýz sekvencí mitochondriální a jaderné DNA je navržena změna taxonomie uvnitř tohoto rodu vycházející z formálního stanovení dvou nových platných druhů slepýšů zahrnovaných dříve do druhu *A. fragilis* nebo považovaných za jeho poddruh. Genetická struktura populací jednotlivých druhů pak umožnila formulovat hypotézy o evoluční historii těchto druhů.

Anguis incerta, Komínky, Chřiby, Česká republika (Czech Republic)

Slow Worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: Genetic structure reveals deep divergences

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Abstract
Phylogenetic relationships of the Western Palearctic genus *Anguis* were inferred based on a fragment of mitochondrial DNA and two nuclear protein-coding loci, C-mos and PRLR. *A. cephallonica* from the Peloponnese was confirmed as a valid species, sister to all other evolutionary lineages, which were shown to represent three distinct species: (1) *A. fragilis* sensu stricto occurring in Western and Central Europe, the north-western Balkans, with possibly isolated populations in the eastern Balkans, and presumably also in the western Scandinavia and Italy; (2) *A. incerta* distributed from the eastern Czech Republic and the Baltic region eastward to northern Iran, presumably also in eastern Scandinavia, and the north-eastern Balkans; (3) *A. graeca* restricted to the southern Balkans, and partially sympatric with *A. cephallonica*. According to the more variable mitochondrial marker, *A. graeca* appears to be sister to *A. incerta*, and these taxa together form a sister clade to *A. fragilis*, whereas the less variable nuclear markers *A. incerta* show to be closer to *A. fragilis*. The C-mos gene has not provided substantial variation within this species complex, while the PRLR gene, which was used for the first time in phylogeographic study in a reptile, distinguished all species successfully. Intraspecific differentiation of *A. incerta* is discussed, and subspecific status of the Caucasian and Caspian populations is suggested. The uncovered genetic differences should be taken into account in all future biogeographical, morphological and ecological studies, as well as in conservation.

Keywords: *Anguis*; Phylogeny; Phylogeography; NADH dehydrogenase subunit 2 (ND2); tRNA; prolactin receptor (PRLR); oocyte maturation factor (C-mos); Systematics; Taxonomy.

1. Introduction

Two slow worm species, *Anguis* (Reptilia: Anguidae) are currently recognized: *A. cephallonica* Werner, 1894 and *A. fragilis* Linnaeus, 1758 (Arnold, 2002; Völkl and Alfermann, 2007). Whereas the first species is restricted to the Peloponnese and adjacent islands of Zakynthos, Kephallenia and Ithaca, the second one is widespread in the Western Palearctic region. Traditionally, two forms, regarded by some authors as different subspecies (e.g. Arnold, 2002; Musters and in den Bosch, 1982), or alternatively as morphotypes (e.g. Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990), have been distinguished within *A. fragilis* – western *A. f. fragilis* and eastern *A. f. colchica* (Nordmann, 1840). Morphological differentiation (e.g. prefrontal shield position, ear opening condition, number of scales around the midbody, blue dorsal spotting) and taxonomic status of these forms has been subject to several morphological and biogeographical studies (e.g. Beshkov, 1966; Lác, 1967; Musters and in den Bosch, 1982; Shcherban', 1976; Voipio, 1962; Wermuth, 1950). Also a long contact zone between both forms has been suggested to occur in the north-south direction from the west of Finland and the Baltic Sea coast, through Central Europe (along the border between the Czech Republic and Slovakia) to the north-western Balkans (Dely, 1981; Petzold, 1971; Völkl and Alfermann, 2007). A rather complex pattern of distribution of different morphotypes and their intermediates in the Balkan populations has been explained as sympatric occurrence of both forms (Arnold, 2002; Beshkov, 1966; Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990; Musters and in den Bosch, 1982; Stojanov, 2001), or as evidence for the existence of an intermediate form (Mayer et al., 1991). However, it is evident that external morphology is not fully concordant with extant intraspecific subdivision, and questions of the taxonomic status as well as interrelationships of the given forms have remained open until the present study.

With the aim to elucidate the phylogenetic relationships and taxonomic position of the populations forming the contact zone of particular slow worm forms in the Czech Republic and Slovakia (Bárta and Tyrner, 1972; Kminiak, 1992; Lác, 1967; Moravec, 1997; Rozínek et al., 2001), as well as the status of the "intermediate" Balkan populations, we focused our attention on genetic variation of *A. fragilis* using a phylogeographic approach based on mitochondrial and nuclear DNA sequence data.

2. Material and methods

2.1. Sampling

Tissue samples of individual *Anguis* specimens (n = 50; 43 localities) were obtained from museum voucher specimens (National Museum, Prague, Czech Rep., NMP; Natural History Museum of Crete, Irakleio, Greece, NHMC) and road-killed individuals. Oral swabs or miniature tail biopsy were occasionally taken from living animals. Three additional mitochondrial sequences and one nuclear (C-mos) of four individuals originating from three different regions were taken from GenBank, together with two sequences of two outgroup species (Table 1 and Fig. 1). We sampled populations of *A. fragilis* along a west – east transect through the Czech Republic and Slovakia. Another sample set covered the area of Greece and adjacent territories of the southern Balkans. Individuals of several populations from distant areas (Iberian Peninsula, British Isles, Baltic and Caucasus regions, and northern Iran) were used for comparison. Closely related *Anguis cephallonica*, as well as more distant *Hyalosaurus koellikeri* Günther, 1873 and *Pseudopus apodus* (Pallas, 1775) (generic affiliation sensu Macey et al., 1999) were employed as outgroup species. Both, the Caucasian (*P. a. apodus*) and the Balkan (*P. a. thracius* Obst, 1978) populations of *Pseudopus*, were included into the analyses to compare genetic distinctiveness between populations of these distant territories within the outgroup *Pseudopus*, as well as the ingroup *Anguis*.

2.2. Laboratory procedures

Total genomic DNA was extracted from tissue samples using different commercial kits following manufacturers' protocols. A fragment of mitochondrial DNA (mtDNA) and two nuclear genes (nDNA) were targeted for molecular phylogenetic analyses. Mitochondrial DNA comprised the complete NADH dehydrogenase subunit 2 gene (ND2), five complete transfer RNA genes – tryptophan, alanine, aspargine, cysteine, tyrosine (tRNA-Trp, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr) and the light strand replication origin which is located between tRNA-Asn and tRNA-Cys. PCR primers were taken (H5934) or modified (L4437n: 5'-AAGCTATTGGGCCCATACC-3') from Macey et al. (1997). Amplification of all sequences involved an initial cycle of denaturation at 94 °C for 2 min, and 35 subsequent cycles of 94 °C for 35 s, 50 °C for 35 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Sequencing was carried out using a combination of PCR primer (H5934) and newly designed internal primers AinND2F (5'-CCCAAGACYTAACAACA-3'), AND2inR2 (5'-ATGAAGCCGGATAGAGG-3') and AND2inRc (5'-ATGAAGCCGGATAGTGG-3'; specific for *A. cephallonica*). In one sample (from Montenegro) only partial ND2 sequence (744 bp) was obtained due to low quality of source DNA.

Two nuclear protein-coding loci were chosen as independent markers for comparison of their genetic pattern to the mitochondrial signal. Oocyte maturation factor (C-mos) gene was sequenced as this gene has been used in several squamate phylogeny studies before (e.g. Slowinski and Lawson, 2002). Secondly, prolactin receptor (PRLR) gene was used herein for the first time in phylogeny/phylogeography study of reptiles as Townsend et al. (2008) suggested this marker the most variable nuclear protein-coding locus in squamate reptiles tested in their comparative study. C-mos was amplified using primers S77 and S78 (Lawson et al., 2005) and PRLR with primers PRLR_f1 and PRLR_r3 (Townsend et al., 2008). Both genes were amplified according to the following PCR program: initial cycle of denaturation at 94 °C for 7 min, 40 subsequent cycles of 94 °C for 40 s, 48 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 7 min. Sequencing was carried out using the PCR primers. All sequencing was done by Macrogen Inc. (Seoul, S. Korea, http://www.macrogen.com). Sequences of all particular haplotypes have been deposited in GenBank (FJ666554 – FJ666589 for mtDNA fragment; GQ285104 - GQ285118 for PRLR; GQ285119 - GQ285123 for C-mos).

2.3. Phylogenetic analyses

All alignments were performed in BioEdit 7.0 (Hall, 1999) and tRNAs were aligned with respect to their secondary structures following Macey et al. (1999). The complete mtDNA alignment included a 1428 bp stretch, however, two positions within tRNA-Trp, and one within tRNA-Cys, respectively, were excluded from phylogenetic analyses because of unique insertions present only within the outgroup samples (the Albanian *P. apodus*, and *H. koellikeri*, respectively). The ND2 gene sequences were examined by translation with the vertebrate mitochondrial genetic code into amino acids using DnaSP 4.50 (Rozas et al., 2003). No stop codons were detected. The same program was used to estimate the average genetic distances between particular taxa or populations. This was done twice; i. e., for the entire mitochondrial fragment, and then solely for the ND2 gene. The whole fragment served also for estimation of average intra-specific and intra-population variation. The computed distances were based on uncorrected *p*-distances, and only distinct haplotypes were assessed. One haplotype was omitted from the calculations because of its incompleteness (the sample from Montenegro).

Alignment of nuclear genes was prepared by hand as the genes are protein-coding exons with no indels (C-mos segment = 555 bp; PRLR segment = 544 bp). In the PRLR fragment, three individuals showed more than one heterozygous positions. For haplotype inference of such cases, a coalescent-based Bayesian method of Phase 2.1 (Stephens and Scheet, 2005; Stephens et al., 2001) as implemented in DnaSP 5.00 (Librado and Rozas,

2009) was employed. The analyses were run multiple times (5x) with different seeds for the random number generator and checked if gametic phase estimation was consistent through the runs according to goodness-of-fit values. Each run was conducted under the parent-independent mutation model with a burn-in-period of 100 followed by 1000 iterations. No stop codons were detected in nuclear haplotypes as inferred by Phase 2.1 as checked by translation with the universal nuclear genetic code using BioEdit 7.0 (Hall, 1999). However, there were two samples phased with low statistical support (see Results), and only one of them (Czech sample from locality No. 3) was used for subsequent analyses and checked for both options of possible haplotype combinations. The other (Slovenian) sample was not directly included into the analyses because of its low probabilities of gametic phases inference. Haplotype networks for both the C-mos and PRLR phased data were constructed using the statistical parsimony algorithm implemented in TCS 1.21 (Clement et al., 2000) under the 95% limit of parsimony. For further phylogenetic computations all sequences, mt and phased nDNA, were sorted into distinct haplotype data sets using Collapse1.2 (Posada, 2006).

The best-fit model of sequence evolution was selected using jModelTest 0.1.1 (Posada, 2008). Likelihood scores for each particular model were computed through maximum likelihood (ML) optimized trees using the implemented PhyML algorithm (Guindon and Gascuel, 2003). As Posada and Buckley (2004) argued that the Akaike information criterion (AIC; Akaike, 1974) and the Bayesian information criterion (BIC; Schwarz, 1978) offer important advantages over the hierarchical likelihood-ratio tests, we used both information criteria. In the mtDNA data set, we compared the results of best-fit model selection for the whole mitochondrial fragment, and separately for the ND2 gene and the tRNAs, respectively. Finally, using the implemented Consense program from the PHYLIP package (Felsenstein, 2005), we obtained the model-averaged phylogenetic tree as inferred from the 50% majority rule consensus tree of 88 ML tree topologies, one for each model, weighted according to the BIC weights. This allowed us to estimate phylogenetic uncertainty due to model selection (Posada, 2008).

Phylogenies were constructed using maximum likelihood (ML), Bayesian inference (BI), maximum parsimony (MP), and neighbor-joining (NJ). For ML analysis, PhyML 3.0 (Guindon and Gascuel, 2003) was employed using the best-fit model according to the BIC [TrN+I+G, Tamura and Nei (1993) for mtDNA; HKY, Hasegawa et al. (1985) for PRLR]. We set an option of 10 random starting BioNJ trees. The best of the nearest neighbor interchange (NNI), and the "new" subtree pruning and regrafting algorithm (SPR; Hordijk and Gascuel, 2005) of branch swapping was used as a tree topology search, with options to optimize the topology and branch lengths. We computed bootstrap values based on 1000 resampled data sets (Felsenstein, 1985), as well as the approximate likelihood-ratio test for branches (aLRT; Anisimova and Gascuel, 2006) as branch supports. Bayesian analyses were carried out with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood settings corresponded to the general time-reversible model with a proportion of invariant sites and rate heterogeneity (GTR+I+G; Tavaré, 1986), which is the closest approximation of the TrN+I+G model (selected by the BIC) available in MrBayes, in the mtDNA data set, and to the HKY model (Hasegawa et al., 1985) in the PRLR data set. No partitions were applied as the same model was selected for all data sub-/sets of the mtDNA tested under the BIC. All MrBayes analyses were performed with two runs and four chains for each run for six million generations, and sampling every 100^{th} tree. First 300 trees (burn-in value) were discarded, as log-likelihood scores of sampled trees plotted against the generation time showed that stationarity was fully achieved after the first 30,000 generations in both mtDNA and PRLR. A majority rule consensus tree was then produced from the remaining trees after discarding the burn-in trees, and the posterior probabilities calculated as the frequency of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001). The BI analysis was run three more times in both data sets with random starting trees and the

results were compared to check for local optima. Within MP analyses, all characters were equally weighted, gaps (in mtDNA) were treated as a fifth state, and a heuristic search was conducted with 100 random taxon stepwise addition replicates using tree bisection and reconnection (TBR) branch swapping. The topology was reconstructed using 50% majority rule consensus of most parsimonious trees, and support values were assessed using 1000 bootstrap pseudoreplicates (Felsenstein, 1985). MP and NJ analyses were performed with PAUP* 4.0b10 (Swofford, 2003). The NJ analyses were executed twice: first time with uncorrected *p*-distances, and second time with distances based on the best model according to the BIC (TrN+I+G and HKY for mtDNA and PRLR, respectively). The branch support was evaluated by bootstrap analysis (Felsenstein, 1985) with 10,000 pseudoreplicates. MP and NJ were employed also on the ND2-translated and PRLR-translated amino acid data sets. The settings were the same as for the DNA analyses, gaps were treated as a 21st amino acid in MP, and mean character difference was used as a distance measure in NJ. All distinct haplotypes were used in the amino acid analyses as well, and checked for synonymous and non-synonymous mutations.

3. Results

3.1. Mitochondrial DNA sequence diversity

Among the 1425 base pair positions examined, 409 were variable, 296 of which were parsimony informative. Several indels have occurred in all tRNAs, except for tRNA-Ala and tRNA-Asn, and in the light strand replication origin. One codon deletion in the ND2 gene was detected even in one ingroup clade (see below). For phylogenetic analyses, a data set of 39 distinct haplotypes, including outgroups, was used. Within the procedure of substitution model selection, the AIC selected the TrN+I+G model (Tamura and Nei, 1993) as the best-fit model for both the ND2 and tRNAs data sets. When combined as one fragment, TIM1+I+G (transitional model; Posada, 2003) was recommended. The BIC selected the TrN+I+G model in all cases, thus, this model was finally used for ML analysis, which resulted in the most likely tree with log likelihood (ln*L*) = -5696.45 (Fig. 2). Model-averaged phylogeny (Posada and Buckley, 2004; Posada, 2008) showed exactly the same topology of the main clades as the most likely tree (not shown). Moreover, all main clades were supported by all most important models as weighted by the BIC suggesting that the different models support the same topology of the main lineages in our data set. All independent BI runs resulted in essentially identical topologies and likelihood estimates (mean ln*L* = -5612.38). MP analysis produced six most-parsimonious trees with a length of 747 steps (consistency index, CI = 0.656; retention index, RI = 0.869). All trees had exactly identical topologies with respect to the main clades. Also both NJ trees, generated with uncorrected *p*-distances and the TrN+I+G (Tamura and Nei, 1993) distances, were consistent in their general topologies, and similar in the bootstrap support values.

The ND2-translated amino acid data set consisted of 345 characters, 78 were variable, of which 54 were parsimony informative. MP produced 357 most-parsimonious trees with a length of 137 steps (CI = 0.715; RI = 0.889). All MP trees were congruent in the topologies of the main clades, although, the bootstrap support for branching patterns was low, resulting in a polytomy of most of the main clades in the bootstrap majority-rule consensus tree. A similar polytomy was obtained by the NJ algorithm after bootstrap analysis. The NJ tree with branch lengths is depicted in Fig. 3.

In all mtDNA nucleotide analyses, *A. cephallonica* from Peloponnese was shown a sister lineage to all other lineages within the radiation (Fig. 2). However, deep divergences were uncovered within the cluster of *A. fragilis* sensu lato (s. l.). This cluster (80/91/79/0.90 = ML/MP/NJ bootstrap values/BI posterior probability) is divided into three main clades. Clade A (100/100/100/1.00) consists of haplotypes from the Iberian Peninsula, the British Isles, Central Europe, Slovenia as far as north-eastern Greece in the case of two isolated samples.

Clade B (83/88/96/1.00) was found in four regions: (a) the Carpathians, (b) the Baltic region, (c) Caucasus, and (d) the Caspian region. These four regions clustered into three distinct subclades: B1 (100/100/100/1.00) consisting of haplotypes from the Carpathians and the Baltic region, B2 (100/100/99/1.00) corresponding to the Caucasus area and B3 (99/98/99/1.00) with two samples from the southern Caspian region. However, the mutual relationships between these subclades remain unresolved. Clade C (100/100/100/1.00) was found geographically located in the southern Balkan Peninsula (Greece, Albania, southern Montenegro, western Serbia), and is genetically very diverse, although without deeply divergent subclades. Clade B and C are likely sister clades (72/86/93/0.97).

Analyses of the translated ND2 data set also showed several well supported distinct lineages within *Anguis*, congruent with the DNA lineages (Fig. 3), i.e. a high number of nucleotide substitutions were non-synonymous. However, topologies of the lineages within the ND2-translated trees are rather unsupported, forming a polytomy, including particular subclades of the mitochondrial clade B, which are considered distinct within the bootstrap analyses, but without clear relationships to each other and to the other clades (see the bootstrap support, or rather non-support in Fig. 3). Contrary to the nucleotide-based topology, *A. cephallonica* seems to be a sister lineage to the clade A, however, with only low support (59/60 = MP/NJ bootstrap values). In contrast to the deep divergences between the clades in the ND2-translated data set, the geographically distant individuals from Spain, Slovenia, some Czech individuals, a Slovak sample, and even one individual from Greece (clade A) had only synonymous mutations.

From the mitochondrial analyses following results we may highlight: (1) Topologies of all mt phylograms and the fact that the mitochondrial divergences between *A. cephallonica* and any main mt clade of *A. fragilis* s. l. (7.0-7.8%) are similar to those among the main clades within *A. fragilis* s. l. (5.8-8.1%) argue for specific level status of these main lineages (Table 2, Fig. 2, Fig. 3). (2) Based on all examined mt haplotypes, the tRNA-Ala gene sequences seem to be discriminative for all main mt clades within *Anguis* (Fig. 4). (3) Presence of the unique deletion of one codon within the ND2 gene in the clade A is an unusual type of mutation, even between different genera within the family Anguidae (Macey et al., 1999), possibly with high evolutionary importance.

3.2. Nuclear DNA sequence diversity

Only parsimony haplotype network was applied to the C-mos data set, as very low level of variation was present within *Anguis* (5 distinct haplotypes; Fig. 5). The only heterozygous site in the only heterozygous sample (locality No. 15, Albania) was uncovered, thus there was no problem with the inference of gametic phases within this diploid marker. Haplotype of *A. cephallonica* was the most distant within the genus, seven mutational steps away off the most common haplotype (Cfi1), which was shared by the mitochondrial clades A and B with the exception of one sample from Iran (mt clade B, locality No. 45). Six of the seven mutation steps were, however, synonymous. Samples from the mt clade C were distinguished from the most common haplotype Cfi1 by one unique mutation step.

The PRLR data set contained higher variation than the C-mos, although still not very high. Seven slow worm individuals were heterozygous, three of which in more than one site (2, 3, 4 sites). One of the three individuals (sample from the locality No. 9, Greece) was phased with high probabilities above 0.95, the second one with low probability in one from three heterozygous positions (probability 0.51; locality No. 3, Czech Rep.), and the third one with low probabilities in three from four hetero- sites (probabilities around 0.70; locality No. 12, Slovenia). The latter was not further used in analyses, while the second one was employed and both haplotype possibilities as inferred from the ambiguous site were checked. Parsimony haplotype network was applied for 13 distinct haplotypes within *Anguis* (Fig. 6B). The Czech sample with one ambiguous position did not change the network substantially, when the other haplotype combination was applied (not shown). One haplotype stayed common with one

homozygous sample (Pf3, instead of previous Pf2), while the other one stayed unique on the tip of the network. Among 544 base pairs in total within the PRLR fragment 23 base pair positions were variable, ten of which were parsimony informative, including the outgroup genus *Pseudopus* (18 variable, 9 parsimony informative in *Anguis*). The HKY substitution model (Hasegawa et al., 1985), as selected by the BIC, was used for ML and BI analyses. We preferred this model to the 3-parameter model with gamma rate heterogeneity (TPM3uf+G; Kimura, 1981), which was selected by the AIC, to avoid over-parameterization of not so variable data sets. ML analysis resulted in the most likely tree with ln*L* = -927.53 (Fig. 6A). All independent BI runs resulted in essentially identical topologies and likelihood estimates (mean ln*L* = -946.85). MP analysis produced four most-parsimonious trees with a length of 25 steps (CI = 0.960; RI = 0.955). Majority-rule consensus tree resulted in the same topology as ML and BI phylograms. NJ trees computed based on uncorrected *p*-distances and the HKY distances showed also the same topologies and were similar in the bootstrap support values. The PRLR-translated amino acid sequence data set consisted of 181 characters, 18 of which were variable, and eight of these variable characters were parsimony informative. MP produced four most-parsimonious trees with a length of 21 steps (CI = 0.952; RI = 0.938). MP majority-rule consensus tree and NJ tree (both not shown) were very similar to those of the nucleotide sequence data set (Fig. 6A). The most important difference was that nucleotide haplotypes Pf1 and Pf2, differing only in one synonymous mutation, resulted in no difference between them in the amino acid data set.

A. cephallonica had the most distant haplotype within *Anguis* haplotypes in the nDNA analyses. Samples from the mt clade C from the southern Balkans were shown consistently distinctive in both nuclear genes from the other nuclear haplotypes of *A. fragilis*. However, variation of the C-mos segment was very low within *Anguis* and not practical for a phylogeographic approach. The PRLR phylogram showed samples from the mt clade A (Western Europe) monophyletic. Samples from the mt clades B2 and B3 (Caucasus – Caspian region) also formed a monophylum. Samples from the mt clade B1 (Eastern Europe) appeared as basal in respect to the two monophyla. Statistical supports of all clades were rather low, although it was clearly caused by general low number of variable sites.

3.3. Estimation of divergence times

Rate of molecular evolution for the mitochondrial region we used was estimated to 0.6 – 0.7% change per lineage per million years in ectotherm vertebrates based on uncorrected distances (Bermingham et al., 1997; Macey et al., 1998a,b). We found 12.7% of average uncorrected genetic distance between *Anguis* and *Pseudopus*. Applying mean rate of 0.65%, the calculated divergence date 9.8 Mya coincides with the result of Macey et al. (1999). Based on this rate, the basal radiation within *Anguis* could have occurred approx. 5.7 Mya in the late Miocene, followed by further rapid diversification. The lastly diverged lineages according to the mitochondrial gene tree, clade B and C, could have separated approx. 4.5 Mya in the early Pliocene. The three lineages of the clade B could then have started their own evolutionary history during the Pliocene/Pleistocene boundary approx. 2.5 – 2.8 Mya. At that time they apparently segregated into three different refugia – the southern Caspian, Caucasian, and presumably the Carpathian. However, one has to have in mind that all these calculations are rough, based on uncorrected genetic distances, and thus might be underestimated in the case some substitutional saturation has occurred.

4. Discussion

4.1. Genetic structure and relationships within Anguis

The results of this study reveal that there are not two (*A. cephallonica*, *A. fragilis*), but rather four comparable divergent evolutionary lineages representing separate species in *Anguis*. The observed levels of the mitochondrial sequence divergences within *A. fragilis* s. l.

are comparable to those observed between *A. cephallonica* and any of the main mt clades of *A. fragilis* s. l. The mean genetic distance between *Anguis* and its sister genus, *Pseudopus* (Macey et al., 1999), is only 1.8 times greater (12.7%) than average "intraspecific" variation within *A. fragilis* s. l. (7.0%). Furthermore, if we compare the genetic distance between *P. a. apodus* from the Caucasus region and *P. a. thracius* from the Balkans (2.9%) with the respective Caucasian and Balkan populations of *Anguis*, the distance is more than double in the latter (5.9%). Also nuclear protein-coding locus PRLR shows genetic structure, which is concordant with the mitochondrial genetic structure in the sense of main mt clades A, B, C, and thus, concordant also with geography. Only mt clade A (Western Europe) forms a monophylum in the PRLR phylogram, although all samples from the mt clade B (Eastern Europe) form a compact cluster as well, in which each haplotype differs from the neighboring one just in one mutation step. Haplotype Pi1 corresponds to the mt subclade B1 and seems to be basal in respect to the samples from the mt (sub)clades A, B2 and B3. This pattern may be caused by incomplete lineage sorting as autosomal loci are known to have fourfold slower rate of lineage sorting (Avise, 2000). On the other hand, samples from the mt clade C are clearly distinguished from the other *A. fragilis* s. l. and *A. cephallonica* haplotypes. Moreover, the results of Mayer et al. (1991), who examined several proteins, i.e. exonic nuclear markers on a set of slow worm individuals, are in good agreement with ours regarding the general patterns recovered. The study focused on the question of taxonomic position of *A. cephallonica* (*A. fragilis peloponnesiacus* Štěpánek, 1937 at that time) also distinguished three lineages within the remaining populations of *A. fragilis*. *A. f. fragilis* specimens of Mayer et al. (1991) originated from Austria, i. e. from the region of our *A. fragilis* mt clade A distributed along the southern border of the Czech Republic eastwards to the south-western Slovakia, and into Slovenia. Similarly, *A. f. colchica* sensu Mayer et al. (1991) from Hopa (Turkey) is identical with our mt clade B (or B2, respectively), which includes samples from the same locality (map No. 44 in Fig. 1). Also their intermediate "*fragilis*/*colchica*" form from Feneos (Greece) corresponds well to our mt clade C. Thus, bringing the data of Mayer et al. (1991) into context with our results, it is evident that the "three species concept" within *A. fragilis* s. l. is supported by mitochondrial and nuclear DNA sequence data as well as with protein data.

4.2. Taxonomic implications and nomenclature

In the light of the obtained results, we propose full species ranks for the main clades of *A. fragilis* s. l. and following nomenclature:

Clade A = *Anguis fragilis* Linnaeus, 1758, restricted type locality (Mertens and Müller, 1928): "Schweden" [= Sweden]; proposed common name: Common European Slow Worm.

Clade B = *Anguis incerta* Krynicki, 1837, restricted type locality (Mertens and Wermuth, 1960): "Wilna, Litauen" [= Vilnius, Lithuania]; proposed common name: Eastern Slow Worm.

Clade C = *Anguis graeca* Bedriaga, 1881, type locality: "Parnaß-Gebirge, Griechenland" [= Parnas Mts., Greece]; proposed common name: Greek Slow Worm.

The trinomen *A. fragilis colchica* [or more usually, although incorrectly as masculine *colchicus*; Linnaeus (1758) used the name *Anguis* in feminine gender as obvious from his originally established names like e. g. *A. maculata*, *A. reticulata*, etc.; see Article 30.1.4.2 of the ICZN (1999)] had for a long time been applied for the populations from south-eastern Europe and the Caucasus region (Mertens and Wermuth, 1960). Later the name *colchica* has often been applied for the eastern slow worm populations including the northern ones (Arnold, 2002; Dely, 1981). Our findings indeed confirmed that clade B includes also the Baltic populations from Lithuania. This fact leads us to the resurrection of the name *Anguis incerta* Krynicki, 1837 (type locality: Vilnius, Lithuania) from the synonymy of *A. f. fragilis* (Mertens and Wermuth, 1960) as it has priority over *Otophis eryx* var. *colchica* Nordmann,

1840 (= *A. f. colchica*) (type locality: "Abasien" [= Kuban' region, southern Russia] and "Mingrelien" [= region in western Georgia]). This step is justified by the fact that application of the name *colchica* should be restricted to the Caucasian populations (subclade B2). Regarding the finding that the Caucasian populations are geographically separated north of the Caucasus from the eastern European populations (Völkl and Alfermann, 2007) and show distinct genetic differentiation (Fig. 2, 3), we propose to treat the Caucasian populations as a distinct subspecies *Anguis incerta colchica* (Nordmann, 1840) (new combination). The same approach we apply also for the similarly divergent and presumably geographically isolated Caspian populations from northern Iran and probably also south-eastern Azerbaijan (subclade B3), for which the subspecific name *Anguis incerta orientalis* Anderson, 1872 (new combination) (type locality: "Rehst, on the Caspian Sea" [= Rasht, Iran]) is available.

The species rank of the southern Balkan populations required resurrection of the name *Anguis fragilis* var. *graeca* Bedriaga, 1881 (type locality: Parnas Mts., Greece) from the synonymy of *A. f. fragilis*.

4.3. Distribution and biogeography of the species

The exact distribution pattern of all three species is still little known. The Common European Slow Worm, *A. fragilis* sensu stricto (s. s.), is distributed from the Iberian Peninsula (confirmed on the mitochondrial basis) eastwards to Central Europe (Czech Republic, south-western Slovakia; confirmed by mt and nDNA). The range of this species presumably continues to Hungary west of the Danube River (Musters and in den Bosch, 1982), northwards to western Scandinavia (Norway, Sweden = type locality), and south-eastwards to the Apennine Peninsula and the north-western Balkans (according to the data from Völkl and Alfermann, 2007). Isolated refugia have also been suggested to occur in the north-eastern Balkans (Lác, 1967; Petzold, 1971), in Romania (Musters and in den Bosch, 1982), and in Bulgaria (Beshkov, 1966; Musters and in den Bosch, 1982). The only sample that we analyzed from Romania (from the Transylvanian region) fell within *A. incerta* according to both markers mtDNA and nDNA. However, we identified two *A. fragilis* s. s. individuals based on both independent molecular markers in north-eastern Greece, which is consistent with the assumption of an isolated refugium in Bulgaria (e.g. in the Rhodope Mts.). The situation in the north-western Balkans is not known at the moment. According to the distribution map in Musters and in den Bosch (1982), it is possible that the Greek (and Bulgarian) *A. fragilis* s. s. populations are not isolated, but rather the species may be continuously distributed west- and southwards the Danube River. However, it is probable that all three species (*A. fragilis* s. s., *A. incerta* and *A. graeca*) meet somewhere in the northern Balkans. Pozzi (1966) mentioned the *fragilis* form to be present in Slovenia and Croatia, and the *colchica* form in Bosnia and Herzegovina, Serbia, Montenegro and F.Y.R.O. Macedonia. This pattern could correspond to *A. fragilis* s. s. (the "*fragilis*" form), whereas the Balkan "*colchica*" morphotype presumably comprised two species, *A. graeca* and *A. incerta*. However, the most part of *A. incerta* distribution probably corresponds to the range of the former "*colchica*" form as suggested by Arnold (2002), Dely (1981), Petzold (1971), and Völkl and Alfermann (2007), with the exception of the southern Balkans (Greece, Albania, southern Montenegro, western Serbia), where *A. graeca* is present. For the moment, we documented *A. incerta* in Lithuania, north-eastern Poland, eastern Czech Republic, Slovakia, Romania, and further lineages assigned to the subspecies *A. i. colchica* in Georgia, the Caucasian part of Russia, north-eastern Turkey and another subspecies *A. i. orientalis* in northern Iran.

All species are probably mutually parapatric, although partial sympatry is feasible as has already been shown in *A. cephallonica* and *A. graeca* (*A. fragilis* at that time) in northern Peloponnese (Grillitsch and Cabela, 1990; Mayer et al., 1991). Ecological vicariance can be anticipated in the contact zones, as was shown by Beshkov (1966) in Bulgaria. However, there is no information about hybridization of the species at the moment. There are four

potential figures of contact, or possibly hybrid zones (*A. cephallonica*/*A. graeca*, *A. graeca*/*A. fragilis*, *A. fragilis*/*A. incerta*, and *A. incerta*/*A. graeca*). Shedding light on this will be subject of further research.

4.4. Intraspecific genetic variation

Uncovered intraspecific genetic variation had a very different pattern within each particular species studied. The most diverse mtDNA variation was found within *A. graeca*. It is probably tied to the fact that we sampled most of the species' range, which is located in the zone of an important glacial refugium (Joger et al., 2007; Taberlet et al., 1998), thus different sublineages in several microrefugia are likely to have persisted. On the other hand, the genetic uniformity of *A. fragilis* s. s. is surprising. Though our sampling of the range of this species is not comprehensive, we included geographically very distant populations such as the Iberian versus Slovak, or even the Greek, to prevent a possible bias caused by under-sampling. Nevertheless, the average intraspecific mtDNA variation is very low (0.5%), and most of the mutations within the ND2 gene were found to be synonymous. It is possible that the Spanish as well as the Greek populations resulted from a recent colonization, and the refugium of *A. fragilis* s. s. was located elsewhere. Here, Apennine Peninsula, southern France, and/or the north-western Balkans, which we did not sample (with the exception of one Slovenian sample, which is the most basal among the mt haplotypes of *A. fragilis* s. s. examined) come into account. The second possible explanation, however less likely, is that there is a low substitution rate within this species. It would also mean that the origin of this species dated to the late Miocene based on mean mt genetic distance comparison is underestimated. As discussed above, *A. incerta* shows the most distinct intraspecific differentiation, which is probably related to the existence of several separate Pleistocene refugia. In comparison to the Caucasian (B2) and Caspian (B3) subclades, the European subclade (B1) shows relatively lower genetic variation. We could hypothesize that it is due to incomplete sampling, as we have virtually no individuals from prospective refugia of the subclade B1, which could be located in the Carpathian Basin, or in the north-eastern Balkans.

4.5. Current knowledge of morphological differentiation

The morphology of the newly recognized species is not known sufficiently, as many of available morphological studies (Lác, 1967; Shcherban', 1976; Voipio, 1962; Wermuth, 1950) dealt with samples containing mixtures of different species. Nonetheless, the general morphological data historically distinguishing two morphotypes, i. e. "*fragilis*" and "*colchica*", could be applied to *A. fragilis* s. s. and *A. incerta*, respectively. *A. graeca* remains morphologically the most enigmatic, as its populations are known to display intermediate or mosaic characters of the "*fragilis*" and "*colchica*" morphotypes (Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990). Therefore, more precise morphological data for all species, based on the genetic screening, are desirable.

4.6. Conservation

The slow worm, *A. fragilis* s. l., has been suggested as Least Concern under the IUCN criteria (Cox et al., 2006). It is believed to be a widely distributed and quite common species, however its cryptic ecology perplexes a proper evaluation of populations' densities and possible threat *in situ*. Beside these general complications for evaluation of conservation status, the genetic structure has totally been omitted so far. This should be changed now, considering the slow worm, *A. fragilis* s. l., to be composed of three species, *A. fragilis* s. s., *A. incerta* and *A. graeca*. The first two species are seemingly widespread across Western and Eastern Europe, respectively, while *A. graeca* seems to be more geographically limited species, which calls for further attention to the Mediterranean Basin as a global biodiversity hotspot (Myers et al., 2000) and to the importance of the Balkan Peninsula in particular.

The evolutionary differentiation within the genus *Anguis* presented in our study should be taken into account in all future conservation efforts, as well as in all biogeographical, morphological, ecological, and/or etological studies.

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Table 1. Specimens examined, localities, museum voucher numbers (NMP = National Museum, Prague, Czech Rep.; NHMC = Natural History Museum of Crete, Irakleio, Greece) or references, and haplotype names (and GenBank accession numbers; given only once for each haplotype for sequences obtained within this study) of one mitochondrial (ND2 and tRNAs) and two nuclear (C-mos, PRLR; slash indicates heterozygotes) markers. N = number of individuals sequenced for mtDNA fragment; nDNA was sequenced only in one individual per locality in each case.

Map	Species	Locality	Latitude	Longitude	N	Museum No. / Reference	Haplotypes (GenBank Acc. Nos.)		
							mt DNA fragment	C-mos	PRLR
	Anguis cephallonica	**Greece**							
1		Gialova, Peloponnese	36.95° N	21.70° E	1	-	c1 (FJ666586)	Cc1 (GQ285119)	Pc1 (GQ285104)
2		Stymfalia Lake, Peloponnese	37.88° N	22.48° E	1	NHMC 80.3.92.1	c2 (FJ666587)	Cc1	Pc1
	***Anguis fragilis* s. s.**	**Czech Rep.**							
3		Stráž nad Ohří	50.33° N	13.10° E	1	-	f1 (FJ666554)	Cfi1 (GQ285120)	Pf2/4 (GQ285106/ GQ285108)
4		Nové Údolí	48.83° N	13.80° E	1	-	f1	-	-
5		Malá Skála	50.63° N	15.18° E	1	-	f1	-	-
6		Rantířov	49.41° N	15.52° E	1	-	f2 (FJ666555)	Cfi1	Pf1 (GQ285105)
7		Nejdek	48.82° N	16.77° E	1	-	f1	-	-
8		Ondřejovice	50.25° N	17.35° E	1	-	f3 (FJ666556)	-	-
		Greece							
9		Mesoropi	40.89° N	24.06° E	1	-	f4 (FJ666557)	Cfi1	Pf2/3 (see above/ GQ285107)
10		Lepida - Megalo Livadi junction	41.37° N	24.63° E	1	NHMC 80.3.92.2	f5 (FJ666558)	Cfi1	Pf2
		Slovakia							
11		Bratislava	48.15° N	17.07° E	1	-	f1	Cfi1	Pf3
		Slovenia							
12		Bohinj Lake, Stara Fužina	46.29° N	13.90° E	1	NMP6V 72692	f6 (FJ666559)	Cfi1	(GQ285118)[b]
		Spain							
13		Vilarmiel, Galicia	42.48° N	07.12° W	1	Albert et al. (2009)	f7 (EU443256)	-	-
		UK							
14a		Kent, Kingsferry Bridge, England	51.25° N	00.75° E	1	Ast (2001)	f1 (AF407536)		-
14b		Kent, Isle of Sheppey, England	51.25° N	00.75° E	1	Slowinski and Lawson (2002)	-	Cfi1 (AY099972)	-

	Anguis graeca	**Albania**							
15		Diviakë	40.95° N	19.47° E	1	-	g13 (FJ666572)	Cg1/2 (GQ285122/ GQ285123)	Pg1 (GQ285109)
16		Himarë	40.68° N	19.66° E	1	-	g7 (FJ666566)	-	-
17		Dukat	40.21° N	19.58° E	1	-	g15 (FJ666574)	-	-
18		Korce	40.61° N	20.82° E	1	NMP6V 73232	g16 (FJ666575)	-	-
19		Ersekë, Shelegurë Lake	40.32° N	20.67° E	2	-	g4, g5 (FJ666563, FJ666564)	-	-
		Greece							
20		Kerkyra, Korfu	39.59° N	19.90° E	1	NHMC 80.3.92.22	g11 (FJ666570)	Cg1	Pg1/2 (see above/ GQ285110)
21		Gliki, Acherondas	39.33° N	20.55° E	2	-	g9, g12 (FJ666568, FJ666571)	-	-
22		Aoos River, near Konitsa	40.05° N	20.76° E	1	NHMC 80.3.92.17	g6 (FJ666565)	-	-
23		Ampelochori	39.53° N	21.03° E	1	NHMC 80.3.92.21	g10 (FJ666569)	Cg1	Pg1
24		Pertouli	39.54° N	21.47° E	1	NHMC 80.3.92.16	g2 (FJ666561)	-	-
25		Fylakti	39.30° N	21.68° E	1	NHMC 80.3.92.4	g2	-	-
26	region of type locality of *A. fragilis* var. *graeca*	Mornos River	38.49° N	22.06° E	3	-	g1 (FJ666560)	Cg1	Pg1
27		Stomio	39.89° N	22.62° E	1	-	g3 (FJ666562)	Cg1	Pg1
28		Pefki - Artemision, Evvoias	39.01° N	23.23° E	2	NHMC 80.3.92.18 -19	g2	-	-
29		Kryoneritis, Evvoia	38.93° N	23.28° E	1	NHMC 80.3.92.5	g2	Cg1	Pg3 (GQ285111)
		Montenegro							
30		Ulcinj	41.93° N	19.21° E	1	NMP6V 71272	g14[a] (FJ666573)	-	-
		Serbia							
31		Užice	43.86° N	19.84° E	1	-	g8 (FJ666567)	Cg1	Pg1
	Anguis i. incerta	**Czech Rep.**							
32		Hostětín	49.05° N	17.88° E	1	NMP6V 73238	i2 (FJ666577)	-	-

33		Štramberk	49.58° N	18.10° E	1	NMP6V 72822	i3 (FJ666578)	Cfi1	Pi1 (GQ285112)
		Lithuania							
34	region of type locality of *A. incerta*	Paluše	55.33° N	26.10° E	2	-	i6 (FJ666581)	Cfi1	Pi1
35	region of type locality of *A. incerta*	Marcinkonys	54.04° N	24.44° E	2	-	i6	Cfi1	Pi1
		Poland							
36		Bocki	52.65° N	23.05° E	1	-	i4 (FJ666579)	Cfi1	Pi1
		Romania							
37		Finatale Clujuluj	46.83° N	23.62° E	1	-	i5 (FJ666580)	Cfi1	Pi1
		Slovakia							
38		Rovné	48.92° N	18.95° E	1	-	i1 (FJ666576)	Cfi1	Pi1
39		Šuňava	49.03° N	20.08° E	1	-	i1	-	-
40		Chlmecká skalka	48.88° N	21.93° E	1	-	i1	Cfi1	Pi1
	Anguis i. colchica	**Georgia**							
41		Vardzia - Apnia road	41.37° N	43.27° E	1	-	i9 (FJ666584)	Cfi1	Pi2/3 (GQ285113/ GQ285114)
42		Telavi	41.92° N	45.49° E	1	-	i9	Cfi1	Pi3/5 (see above/ GQ285116)
		Russia							
43		Babukal, Krasnodarsky Territory	43.67° N	39.63° E	1	Macey et al. (1999)	i11 (AF085622)	-	-
		Turkey							
44		Hopa	41.40° N	41.44° E	1	NMP6V 73694	i10 (FJ666585)	Cfi1	Pi2
	Anguis i. orientalis	**Iran**							
45		Motalla Sara-ye Lemir	38.20° N	48.87° E	1	NMP6V 72678	i7 (FJ666582)	Cfi2 (GQ285121)	Pi2
46		Nowshar	36.65° N	51.50° E	1	NMP6V 72680	i8 (FJ666583)	Cfi1	Pi2/4 (see above/ GQ285115)
	Pseudopus a. apodus	Dedop'lis Tskaro, **Georgia**	41.43° N	46.10° E	1	-	Paa1 (FJ666588)	-	PPaa1 (GQ285117)
		Voskresenskaya, Chechenia, **Russia**	43.35° N	46.10° E	1	Macey et al. (1999)	Paa1 (AF085623)	-	-
	Pseudopus a. thracius	Diviakë, **Albania**	40.95° N	19.47° E	1	-	Pat1 (FJ666589)	-	-
	Hyalosaurus koellikeri	Kenitra, 10 km S, **Morocco**	34.27° N	06.60° W	1	Macey et al. (1999)	AF085621	-	-

[a] only a fragment of the ND2 gene

[b] unphased heterozygous sequence – not directly used in phylogenetic analyses

Table 2. Genetic distances in percentage between the taxa (populations) based on uncorrected *p*-distances below and above the diagonal. Average intraspecific (-population) variation at the diagonal. Distances based on the whole mtDNA fragment below and at the diagonal (in bold), on the ND2 gene solely above the diagonal.

	Uncorrected *p*-distances (%)	1	2	3	4	5	6	7	8	9	10	11
1	*H. koellikeri*	**-**	17.9	-	-	15.8	17.4	16.4	15.4	-	-	-
2	*P. apodus*	**16.0**	**2.9**	-	-	13.8	14.6	15.0	14.1	-	-	-
3	*P. a. apodus*	**-**	**-**	**-**	3.1	-	-	-	-	-	-	-
4	*P. a. thracius*	**-**	**-**	**2.9**	**-**	-	-	-	-	-	-	-
5	*A. cephallonica*	**14.1**	**12.1**	**-**	**-**	**0.5**	9.0	9.2	8.6	-	-	-
6	*A. fragilis*	**15.6**	**13.2**	**-**	**-**	**7.8**	**0.5**	9.2	8.1	-	-	-
7	*A. graeca*	**15.0**	**13.1**	**-**	**-**	**7.6**	**8.1**	**1.3**	7.0	-	6.9	-
8	*A. incerta*	**14.0**	**12.4**	**-**	**-**	**7.0**	**7.1**	**5.8**	**2.4**	-	-	-
9	*A. i. incerta* (Europe)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	4.0	3.8
10	*A. i. colchica* (Caucasus)	**-**	**-**	**-**	**-**	**-**	**-**	**5.9**	**-**	**3.5**	**1.2**	4.4
11	*A. i. orientalis* (Caspian)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**3.2**	**3.6**	**1.6**

Figure Legends

Figure 1. Map showing localities of specimens used for the molecular analyses. Numbers correspond to those in Table 1; numbers in black squares indicate samples which were sequenced for both mt and nDNA. Black line delimits the distribution of *Anguis* according to Völkl and Alfermann (2007). Rhomboids = *A. cephallonica*; stars = *A. fragilis*; triangles = *A. graeca*; circles = *A. incerta*.

Figure 2. Maximum likelihood haplotype tree showing the *Anguis* phylogeny as inferred from the ND2 and five tRNAs mtDNA sequences. Substitution model TrN+I+G with following values was used: substitution rate matrix AC = AT = CG = GT = 1.00, AG = 26.43, CT = 9.17; proportion of invariable sites P_{inv} = 0.091; gamma shape rate variation among sites α = 0.201; base frequencies A = 0.33, C = 0.32, G = 0.13, T = 0.22. Numbers above branches indicate bootstrap support values for maximum likelihood/maximum parsimony/neighbor-joining analyses. Numbers below branches indicate the PhyML (Guindon and Gascuel, 2003) approximate likelihood-ratio test for branches values/Bayesian posterior probability values/uncertainity due to model selection. Asterisk indicates full support (100 or 1.00) for particular clade. Haplotype names as presented in Table 1.

Figure 3. Neighbor-joining tree based on amino acid sequences of the translated ND2 gene. Numbers above and below branches indicate bootstrap support values for neighbor-joining and maximum parsimony analyses, respectively. Sample names correspond to the haplotype names as in Fig. 2 and Table 1.

Figure 4. tRNA-Ala gene sequences showing their species-specific discriminative importance.

Figure 5. Haplotype network of the C-mos gene based on the statistical parsimony algorithm. Circle sizes correlate to haplotype frequencies and color to the proper mt clade (species). *A. fragilis* and *A. incerta* share common main haplotype. Six from seven mutational steps (black dots) between *A. cephallonica* and the common *fragilis-incerta* haplotype are synonymous. Haplotype names as listed in Table 1.

Figure 6. (A) Maximum likelihood phylogram based on phased haplotypes of nuclear PRLR gene. Parameters for the HKY substitution model were as follows: transitions/transversions ratio = 2.118; base frequencies A = 0.34, C = 0.21, G = 0.23, T = 0.22. Numbers above branches are bootstrap support values for maximum likelihood/maximum parsimony, and numbers below branches Bayesian posterior probability values/neighbor-joining bootstrap support. "Mt clades" are not necessarily monophyletic in the PRLR pattern, but correspond to the monophyletic mitochondrial clades as shown in Fig. 2 and Fig. 3. (B) Statistical parsimony haplotype network of the same data set, PRLR, with circle sizes proportional to haplotype frequencies; small black dots = missing haplotypes. Haplotype names as listed in Table 1.

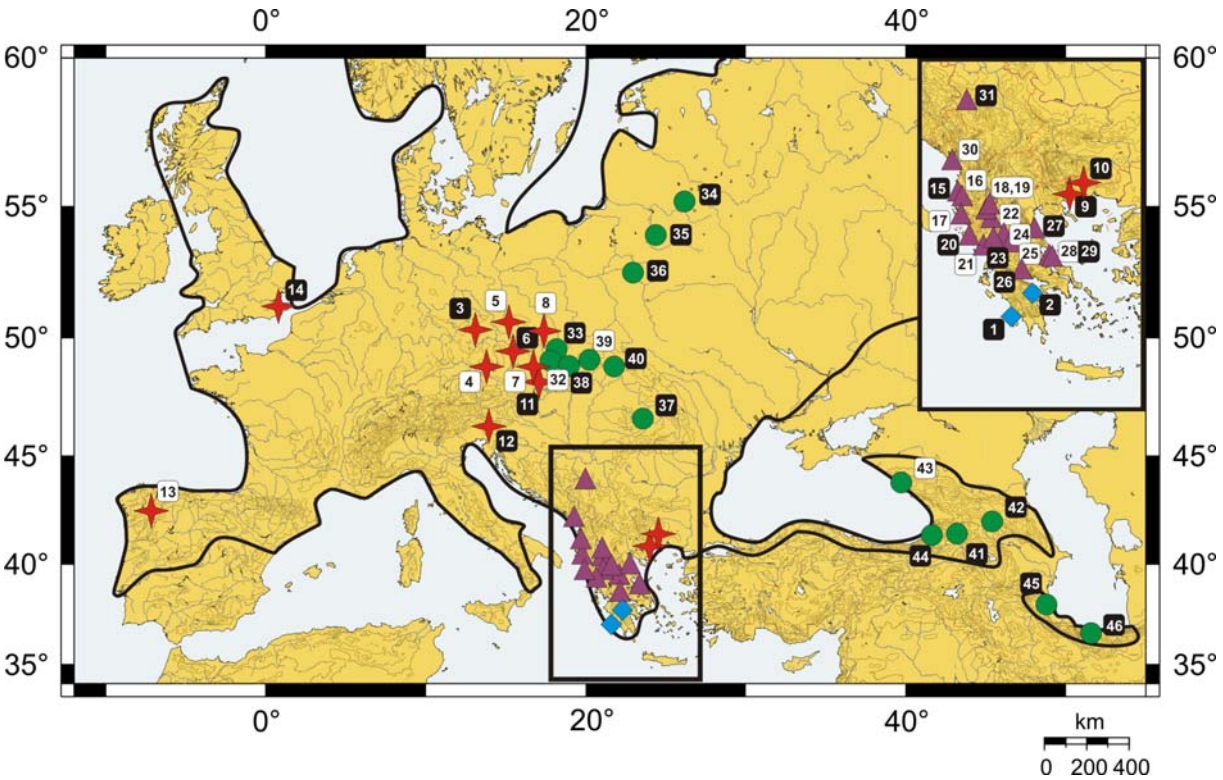

Figure 1.

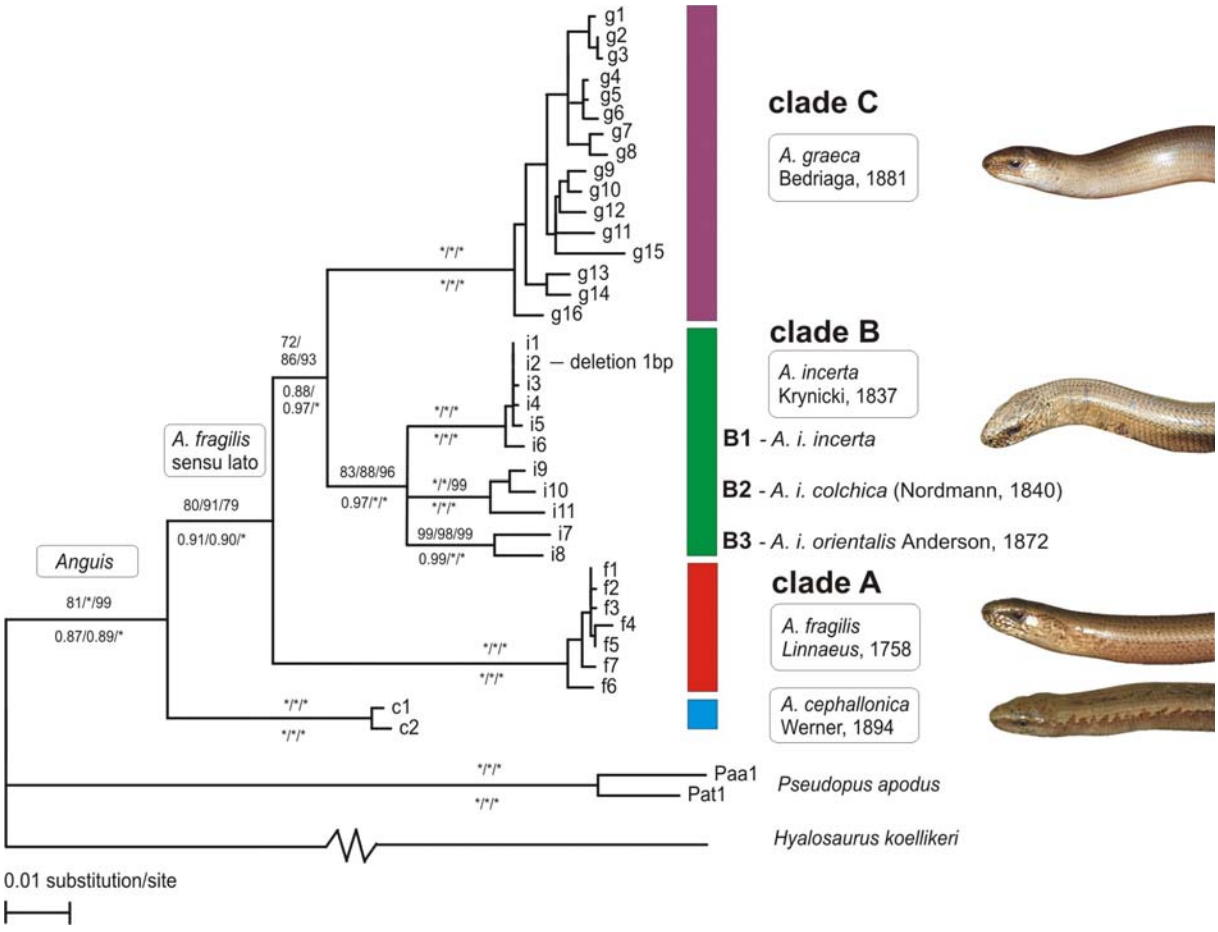

Figure 2.

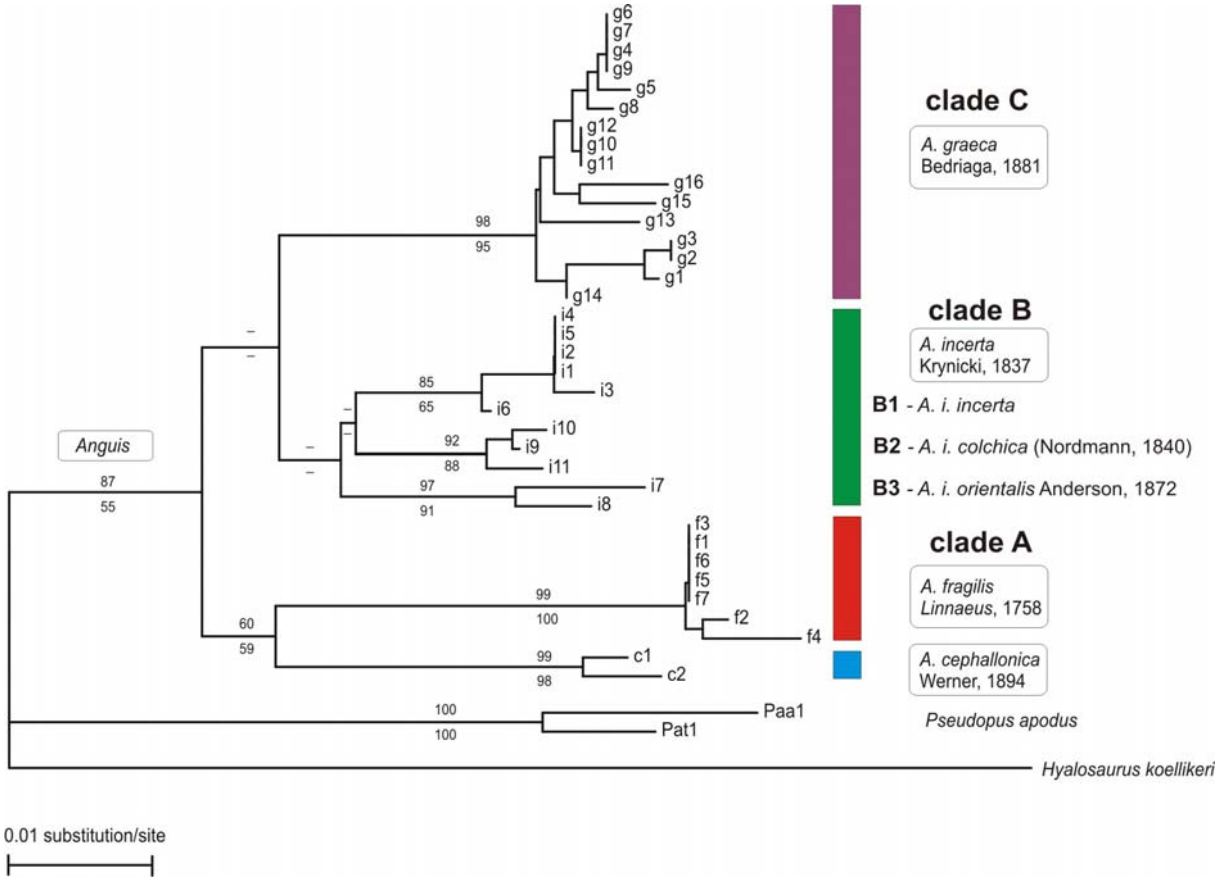

Figure 3.

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|....
A. cephallonica     TAAGACCTGTAAAACTCTAATCTACATCTTCTGACTGCAACCCAGATGCTTTAATTAAGCTAAGACCTT
A. fragilis         ..........G....T.........AC.....................................
A. graeca           ..........................AC............T.......................
A. graeca           ....G.....................AC............T.......................
A. graeca           ..........................AC............T.....................G....
A. incerta          ..........................AC.....................................
A. incerta          ..........................AC.....................................G....
```

Figure 4.

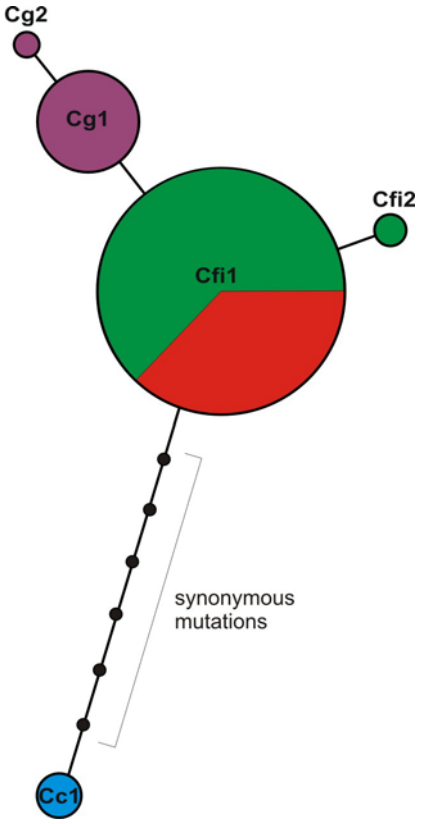

Figure 5.

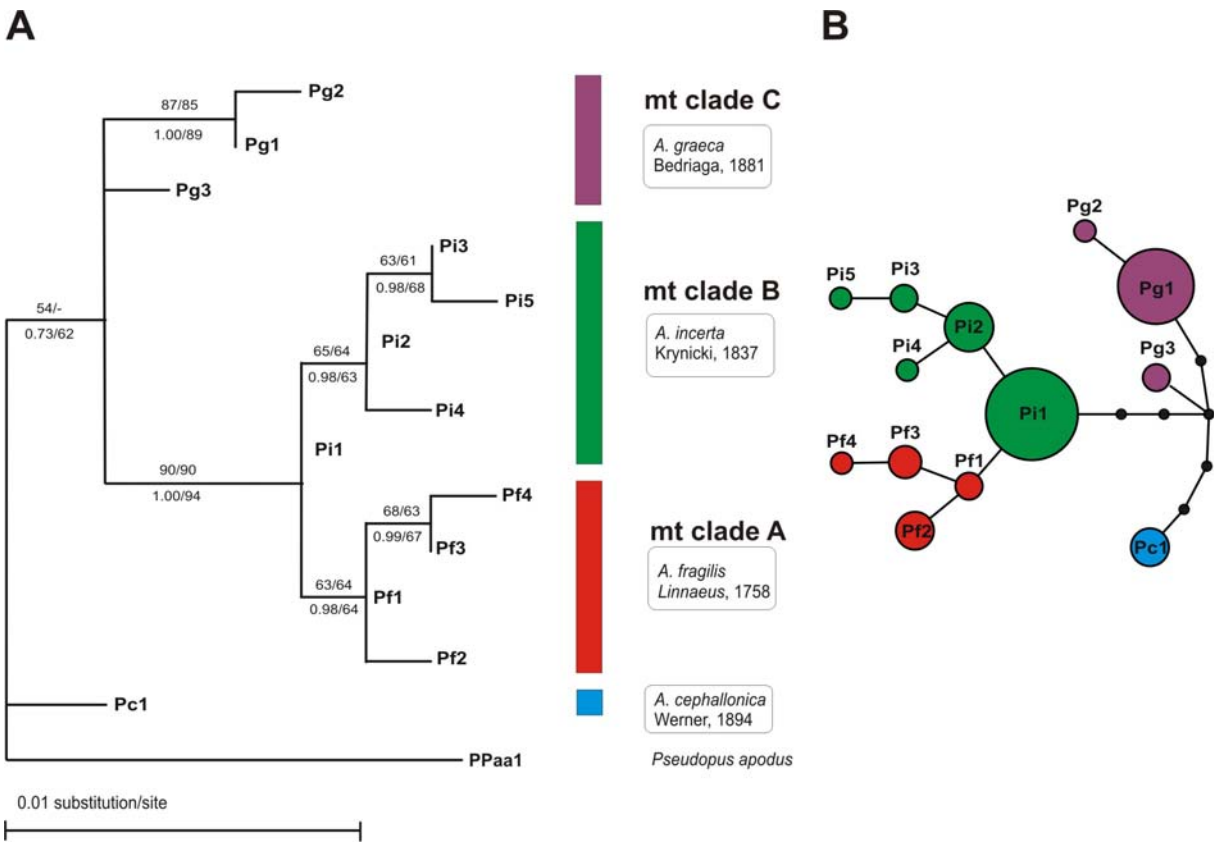

Figure 6.