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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

Václav Gvoždík [a,b,c,*], Jiří Moravec [b], Cornelya Klütsch [d,e], Petr Kotlík [a]

[a] Department of Vertebrate Evolutionary Biology and Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, Czech Republic
[b] Department of Zoology, National Museum, 115 79 Prague, Czech Republic
[c] Faculty of Science, Charles University, Department of Zoology, Viničná 7, 128 44 Prague, Czech Republic
[d] Zoologisches Forschungsinstitut und Museum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany
[e] KTH-Royal Institute of Technology, Gene Technology, Roslagstullsbacken 21, 10691 Stockholm, Sweden

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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, *H. felixarabica* 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. savignyi*. Major genetic breaks revealed within species (*H. felixarabica*: 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. *Hyla 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. *Hyla savignyi* and *H. orientalis* carry signals of population expansions dated to the middle to late Pleistocene, while populations of *H. felixarabica* seem to have rather been constant in size, which might indicate more stable climatic conditions in the southern regions during the Quaternary.

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. The first group consists of taxa that are endemic to some smaller part of the Middle East (usually Anatolia), although they possess 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)]. Another group includes widespread taxa that 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

* Corresponding author at: Department of Vertebrate Evolutionary Biology and Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, Czech Republic. Fax: +420 315 639 510.
E-mail address: vgvozdik@email.cz (V. Gvoždík).

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(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 typically 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), shrews (Dubey et al., 2007a), or even skinks (Kornilios et al., 2010).

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, in particular 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. One species, *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 the south-western Arabian Peninsula (Schneider, 2009). The species was traditionally considered as monotypic but recent studies suggested that on the basis of preliminary molecular data *H. savignyi* might include two distinct evolutionary lineages, presumably at the species level (Gvoždík et al., 2007a; Stöck et al., 2008).

Another species, *Hyla orientalis* Bedriaga, 1890 [“1889”], is distributed parapatrically north- and westward from the range of *H. savignyi* in Asia Minor and presumably in the Caucasus. This species was previously not distinguished from *H. arborea* (Linnaeus, 1758) but Stöck et al. (2008) demonstrated that south-eastern European and western Anatolian *H. arborea* should be considered a separate species, *H. orientalis*. Furthermore, *H. arborea gumilevskii* Litvinchuk, Borkin, Rosanov, Skorinov, 2006, was recently described from the Talysh Mts. (Azerbaijan), and considered as possibly occurring also in northern Iran (Litvinchuk et al., 2006), but it was also subsequently synonymized with *H. orientalis* (Stöck et al., 2008). Yet another subspecies, *H. arborea schelkownikowi* Chernov, 1926, was recognized by some authors in the Caucasus area (Kuzmin, 1999; Tarkhnishvili and Gokhelashvili, 1999), though its validity was disputed by others (Litvinchuk et al., 2006; Schneider, 2004; Terentjev, 1960).

One more species, *Hyla heinzsteinitzi* Grach, Plesser, Werner, 2007, was recently described 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 already be extinct. Stöck et al. (2008) suggested according to mitochondrial DNA of a sample from the type locality that this taxon might be based on an introduced population of *H. japonica* Günther, 1859 [“1858”].

In contrast to most previous studies, which were almost exclusively based on mitochondrial DNA and limited sampling, we used here sequences of multiple mitochondrial (12S rRNA and 16S rRNA) and nuclear genes (rhodopsin and tyrosinase) as well as a dense sampling covering the distributions of tree frogs in the Middle East (Supplementary data; Fig. S1, Table S1). Although our study builds on earlier works (Gvoždík et al., 2007a; Stöck et al., 2008), only with our new approach, based on a combination of genetic, bioacoustic and morphological analyses, the data set becomes adequate to provide a compelling description of the evolutionary history and molecular systematics of tree frogs in the Middle East. Our goals are to (i) reveal the number, phylogenetic relationships and geographic distribution of evolutionary lineages; (ii) propose historical biogeography and demography scenarios to accommodate the genetic variation observed among and within lineages and (iii) evaluate the validity and revise boundaries of the currently recognized taxa.

2. Materials and methods

2.1. Samples for genetic study

Tissue samples (*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. 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 and 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. More details might be found in Supplementary data (Fig. S1, Table S1). Outgroup position of selected outgroup taxa was shown by Faivovich et al. (2005), Hua et al. (2009), 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′-CTGGGATTAGAT ACCCCACTA-3′) and 12Sbs (5′-TGAGGAGGGTGACGGGCGGT-3′), adapted from Kocher et al. (1989); the 16S segment by primers 16SL1 (5′-CGCCTGTTTAACAAAAACAT-3′) and 16SH1 (5′-CCGGTC TGAACTCAGATCACGT-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 performed 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 5×) 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 Collapse 1.2 (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 [AIC: TIM2 + G (Posada, 2003) for concatenated mitochondrial data and 12S rRNA; GTR + G (Tavaré, 1986) for 16S rRNA; TrN + G (Tamura and Nei, 1993) for rhodopsin; SYM + G (Zharkikh, 1994), for tyrosinase; BIC: the same results with an exception of tyrosinase, where K80 + G (Kimura, 1980) was selected].

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 neighbor interchanges) and SPR (subtree pruning and regrafting; Hordijk and Gascuel, 2005) algorithms to maximize tree likelihood, and using the best-fit substitution model for each data set (see above) 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 performed 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), which is the closest approximation of the best-fit substitution models selected by the AIC for each locus (see above) available in MrBayes. In mtDNA, 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 were discarded as a burn-in (log-likelihood score plots suggested that stationarity was achieved after sampling approximately 300 trees). 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 trees recovering any particular clade (Huelsenbeck and Ronquist, 2001). Each analysis was repeated four times with random starting trees and the results were compared to assess the 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 the equally 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 selected only the most common haplotype, usually basal, for 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 rate 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 20×10^6 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 4×10^6 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 and 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 and 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 squared 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 tests 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 statistic, which has more statistical power at 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. For all three tests, a significant departure from the null hypothesis indicates deviation from neutrality and/or population size expansion or decline (negative values of the Fu's F_S and Tajima's D).

Third, historical population demography of main phylogeographic lineages was 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 population grouping 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); measurements 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). ML analysis of the concatenated mtDNA data set with the TIM2 + $G_{0.054}$ model produced the most likely tree with log-likelihood ($\ln L$) = −2643.78 (Fig. 1). All independent BI runs produced essentially identical topologies and likelihood values (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 identical topologies with respect to the main clades.

Hyla savignyi s.l. with 54 haplotypes and *H. orientalis* with 22 haplotypes were mutually monophyletic for haplotype variation in all analyses (ML, BI, MP); however, a further deep divergence was found within *H. savignyi* s.l. (Figs. 1 and 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 species 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 includes specimens from the putative type locality (see Discussion), while the southern clade would represent a new species, hereafter provisionally called *Hyla* sp. nov.

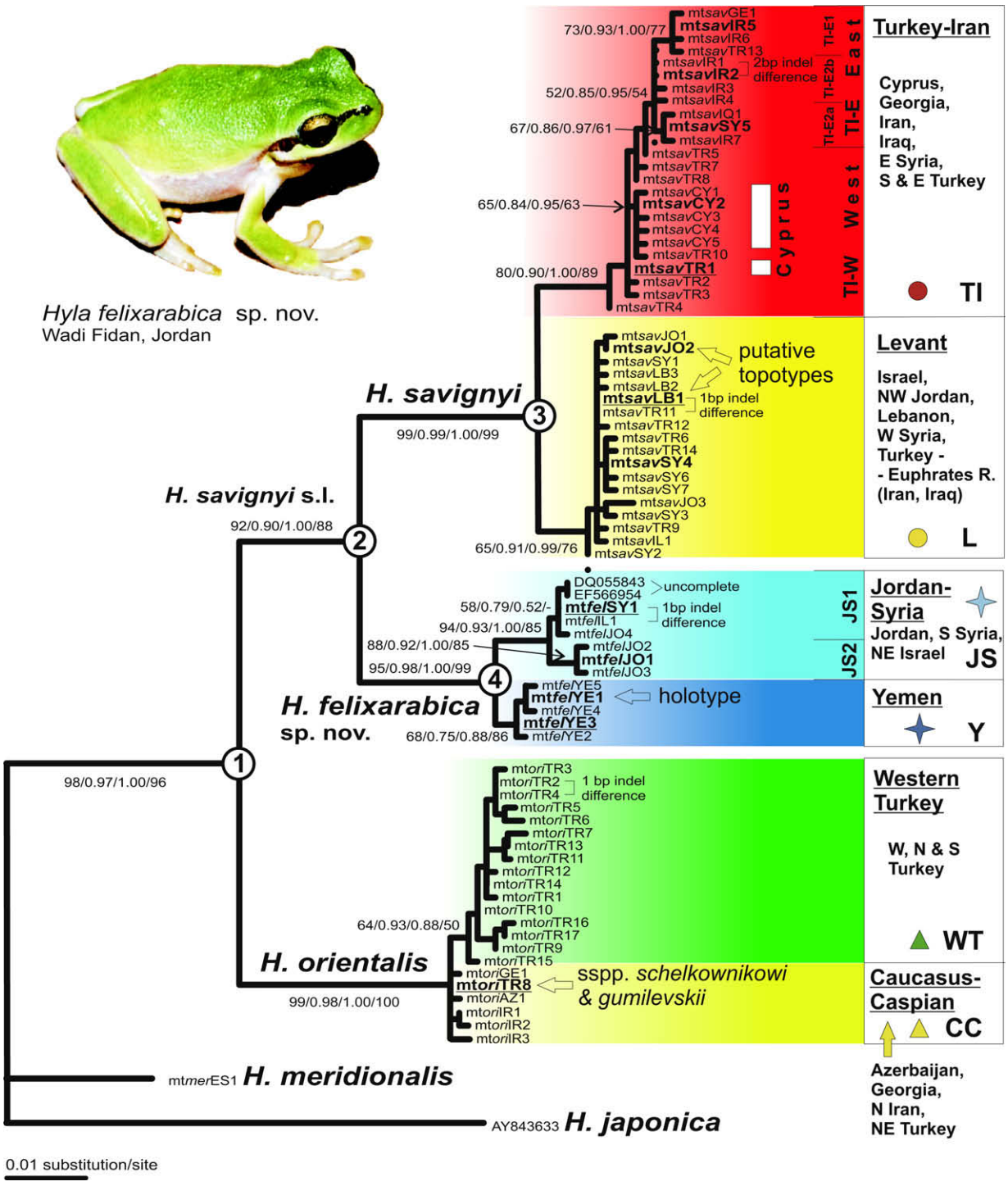

Fig. 1. Maximum likelihood phylogeny (TIM2 + G) of the Middle Eastern tree frogs (*Hyla*) based on mitochondrial data. For nodal support, maximum likelihood (ML) bootstrap (1000 pseudoreplicates), approximate likelihood-ratio test for branches for ML, Bayesian posterior probabilities (BPP < 0.50 not 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 molecular clock analysis.

3.1.1. Hyla savignyi

Hyla 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, loc. 50). The northern Euphrates population interrupts the geographical distribution of the second clade, hereafter called 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 derived

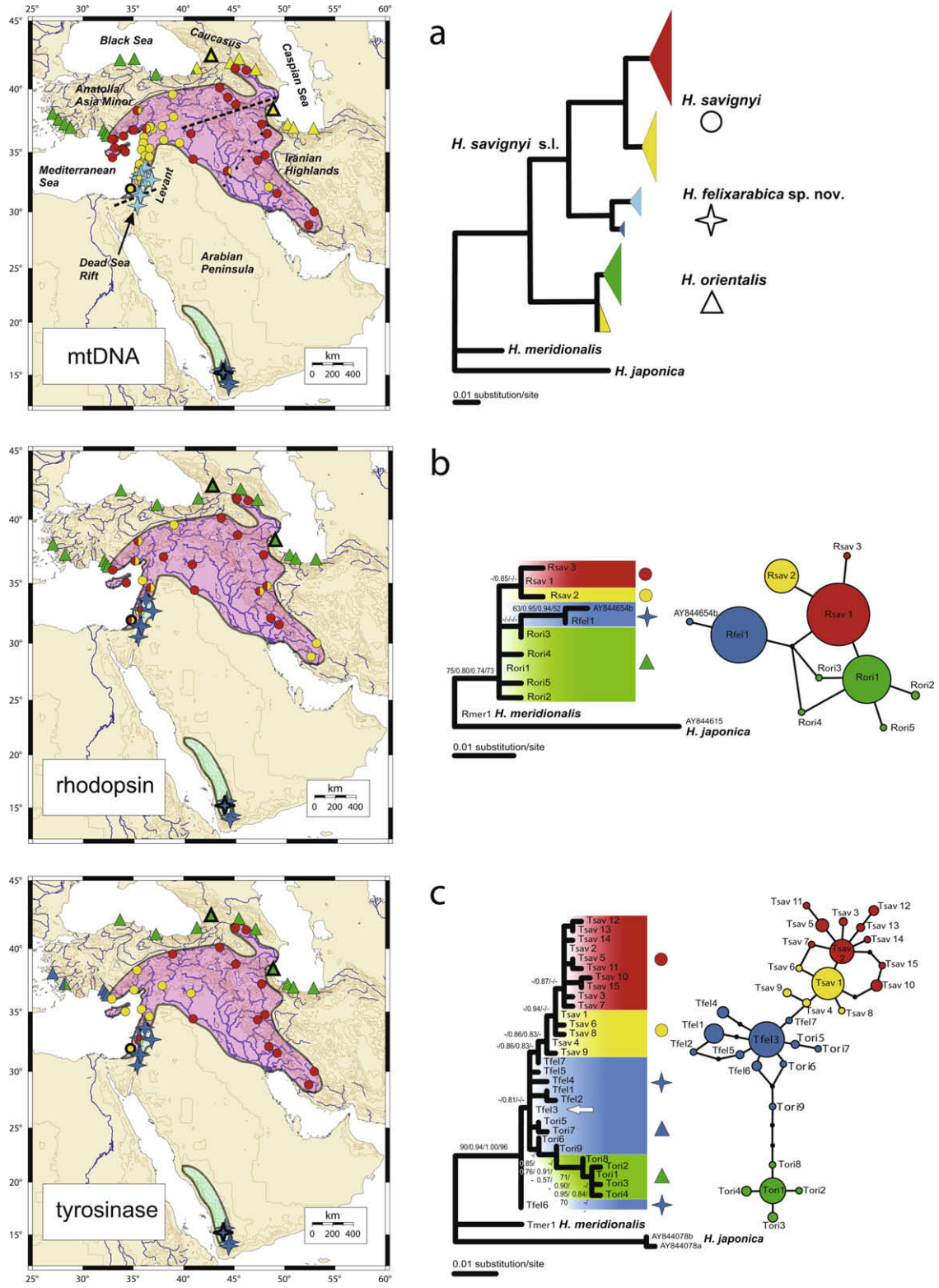

Fig. 2. Comparison of geographic patterns of variation in the mitochondrial (12S and 16S rRNA) and nuclear (rhodopsin, tyrosinase) DNA sequence data of the Middle Eastern tree frogs. (a) Mitochondrial DNA, map and schematic ML tree; dashed lines on the map indicate separation of the E1 (north) and E2 (south) groups of *H. savignyi*, and JS1 (north) and JS2 (south) of *H. felixarabica* 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 *H. felixarabica*, haplotype T*fel*3 (white arrow) was present in both species. Different symbols correspond to the species: *H. savignyi*, circles; *H. felixarabica*, 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 (*H. felixarabica* and *H. arborea schelkownikowi*), putative type locality (*H. savignyi*), or a locality close to the type locality (*H. arborea gumilevskii*, Iran). Geographic distributions of *H. savignyi* (pink) and *H. felixarabica* (blue) are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

subclade in the ML phylogram, although with rather low statistical support, while TI-W is basal and paraphyletic in ML (Fig. 1). Two individuals from the Levant lineage were found also in central Iraq (Bagdad, loc. 18) and south-western Iran (Choqa Zanbil, loc. 14). Two localities in Turkey from the contact zone of the two lineages (Ovaçiftliği, loc. 43; Payas, loc. 46) 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 previously expected (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, loc. 67) 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 geographic groups: Caucasian, which extends from the Caucasus along the southern Caspian coast as far as northern Iran (6 haplotypes; hereafter called the Caucasus–Caspian group, CC), and whose haplotypes form a paraphyletic group with respect to those of the Anatolian population (16 haplotypes; hereafter as the Western Turkish group, WT), which form a clade with only intermediate statistical support. One locality (Hopa, north-eastern Turkey, loc. 86) was found to represent a geographical contact between the two groups as it carried haplotypes from both of them ($n = 4$, CC vs. $n = 1$, 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 that showed more than one heterozygous position (one in rhodopsin, two in tyrosinase). For rhodopsin (Karkom, Israel, loc. 67) 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, loc. 14) 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, loc. 78) with the probability of 0.84 for one of the three heterozygous sites. Despite lower probabilities, the results were consistent across all five runs in each analysis, producing 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 and c). The SYM + G (AIC) tyrosinase ML phylogram had exactly the same topology as the K80 + G (BIC) ML tree (not shown). The Bayesian consensus tree had a 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). Thus, all phylogenetic methods produced similar tree topologies for tyrosinase, although with low statistical support for individual clades due to low sequence 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 and 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* were two widespread haplotypes 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, loc. 67), 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 (Supplementary data, Table S1).

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, loc. 67). This specimen was, at this locus, found to be an interspecific hybrid with *H. savignyi* (Supplementary data, Table S1). Tyrosinase haplotypes of *H. savignyi* (T*sav* in haplotype names) form a clade, which includes a subclade 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 clade clearly distinct from *Hyla* sp. nov. One individual from south-western Turkey (Marmaris, loc.78) was found to be a heterozygote carrying haplotypes (T*ori*8, T*ori*9) apparently derived from both groups, the northern Anatolian populations of *H. orientalis* (T*ori*1) and *Hyla* sp. nov (T*fel*3).

3.3. Comparison of mtDNA and nDNA pattern

The three evolutionarily independent markers, mtDNA and two nuclear genes were concordant in that they all diagnosed 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 for mitochondrial haplotype variation. Populations diagnosed by the major

mtDNA intraspecific clades were not found to be monophyletic at nuclear markers, although two geographic groups, of which one was 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 molecular 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, loc. 67) 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 occurs 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) (Supplementary data, Table S1).

3.4. Estimation of divergence times

The divergence dates of split events were estimated by a relaxed molecular clock approach (Drummond et al., 2006) based on the mitochondrial data set (Table 1). 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. occurred probably 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 2.

Table 1
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 population subsets. 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-felixarabica*	**11.100** (4.854–22.961)
2	*savignyi* vs. *felixarabica*	**8.408** (3.208–18.231)
3	*savignyi* L vs. TI	**2.753** (0.018–7.479)
4	*felixarabica* 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)
	H. felixarabica 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)

3.5. Phylogeographic structure and historical demography

3.5.1. Hyla savignyi

Hyla 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 3). Diagrams of the MMD (Fig. 3a and 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 performed for the TI-W and TI-E separately (Fig. 3c and 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-supported clade, 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 and 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 (three haplotypes from eastern Syria, Iraq and north-western Iran) and the paraphyletic TI-E2b from the east (four 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 clade with a haplotype from the Turkish coast (mt*sav*TR10, $n = 1$). One haplotype (mt*sav*TR1) outside the clade occurred 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 3). 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) indicated 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 the individual groups are shown in Table 1.

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 3). However, graphs showed unimodal distribution in the Y lineage only, while the distribution was strongly bi-

Table 2
Genetic distances. Maximum likelihood 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 average between groups net genetic distances.

Locus distances	Species/group	*H. savignyi*	*H. savignyi* L	*H. savignyi* TI	*H. felixarabica*	*H. felixarabica* Y	*H. felixarabica* 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**	–	–	–	–	–	–	–
	H. felixarabica	9.6	–	–	**1.1**	–	–	10.5	–	–	–
	H. felixarabica Y	–	–	–	–	**0.2**	1.4	–	–	–	–
	H. felixarabica 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**	–	–	–	–	–	–	–
	H. felixarabica	4.5	–	–	**0.8**	–	–	4.4	–	–	–
	H. felixarabica Y	–	–	–	–	**0.2**	1.0	–	–	–	–
	H. felixarabica 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
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**	–	–	–	–	–	–	–
	H. felixarabica	5.2/4.3	–	–	**0.7/1.0**	–	–	4.1/4.6	–	–	–
	H. felixarabica Y	–	–	–	–	**0.3/0.3**	0.6/1.1	–	–	–	–
	H. felixarabica 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

Table 3
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 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, loc. 67) was not included in evaluation of nuclear markers, as was not the inter-lineage hybrid population of *H. savignyi* s.s. (Ovaçiftliği, Turkey, loc. 43) 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	$L^{\dagger}$ (bp)	$k^{\ddagger}$	S	$\pi \pm$ SD (%)	$h \pm$ SD	$\theta_w \pm$ 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	
H. felixarabica		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	

x not significant if recombinations are considered.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; for Fu's F_S $p < 0.02$ was taken as a threshold at the 5% level (sensu Excoffier et al., 2006).
† 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.

modal in the JS lineage (Fig. 5a and 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 and d) and the intermatch distributions (Fig. 5e) had a peak that did not match the MMD peaks, indicating that each group 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 3). 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 and 0.3 Ma (Table 1).

3.5.3. *Hyla orientalis*

Hyla orientalis may be subdivided into two geographic groups, the Caucasus–Caspian (CC) and Western Turkish (WT). The CC group, however, contains a paraphyletic grouping of haplotypes; 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 clear unimodal distributions in the mismatches (Fig. 6b and 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 populations 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 the single exception of tyrosinase in *H. orientalis* as a single unit). The only other significant output was 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

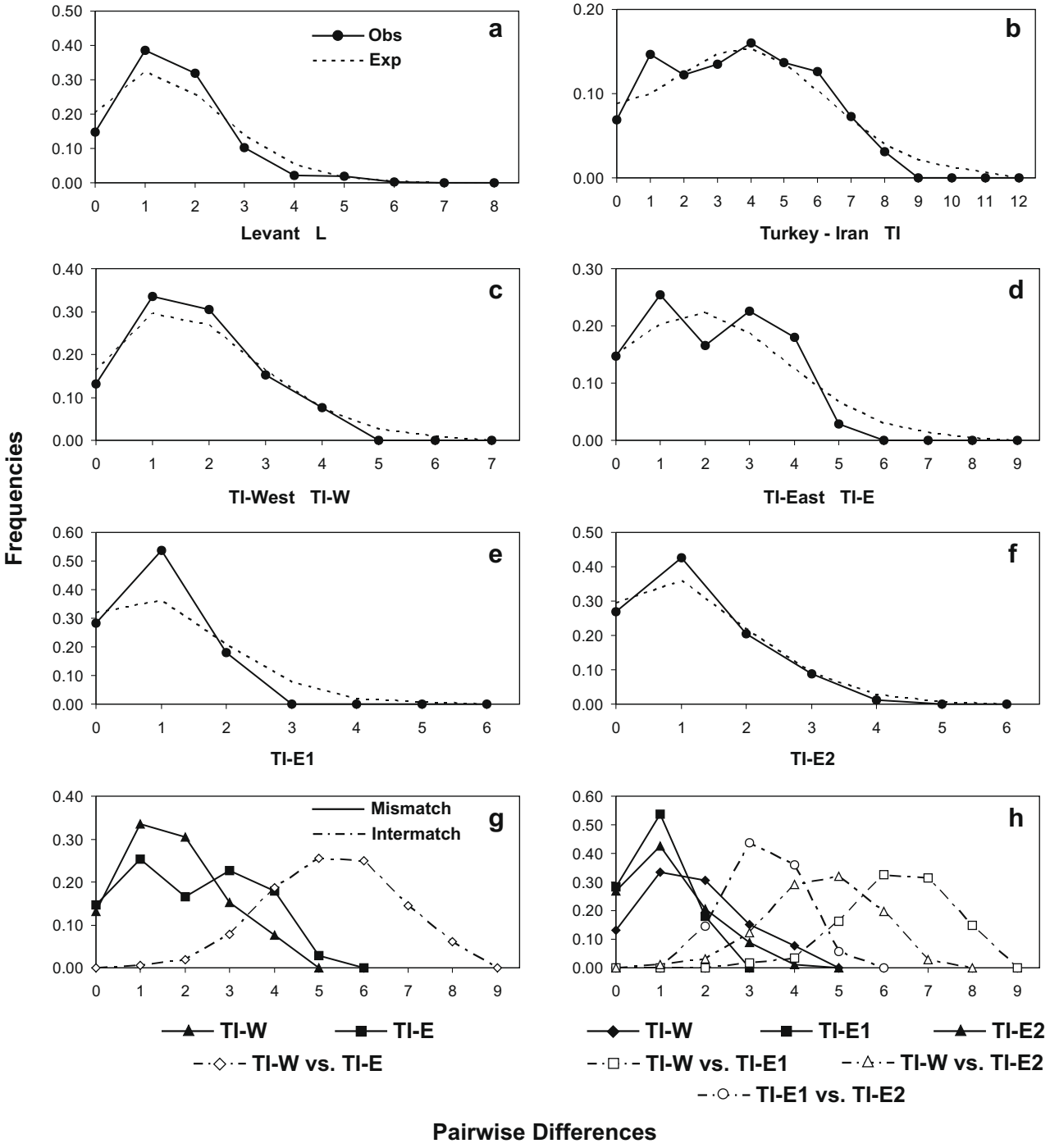

Fig. 3. Mismatch distributions for different population subsets of *H. savignyi* compared to the expected frequencies under the demographic expansion model. Intermatch distributions between different population subsets are also shown (g and h).

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 1).

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 lineages reciprocally monophyletic for mitochondrial DNA haplotypes with 5.7% uncorrected *p*-distances (12S + 16S rRNA) separating them. 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 re-

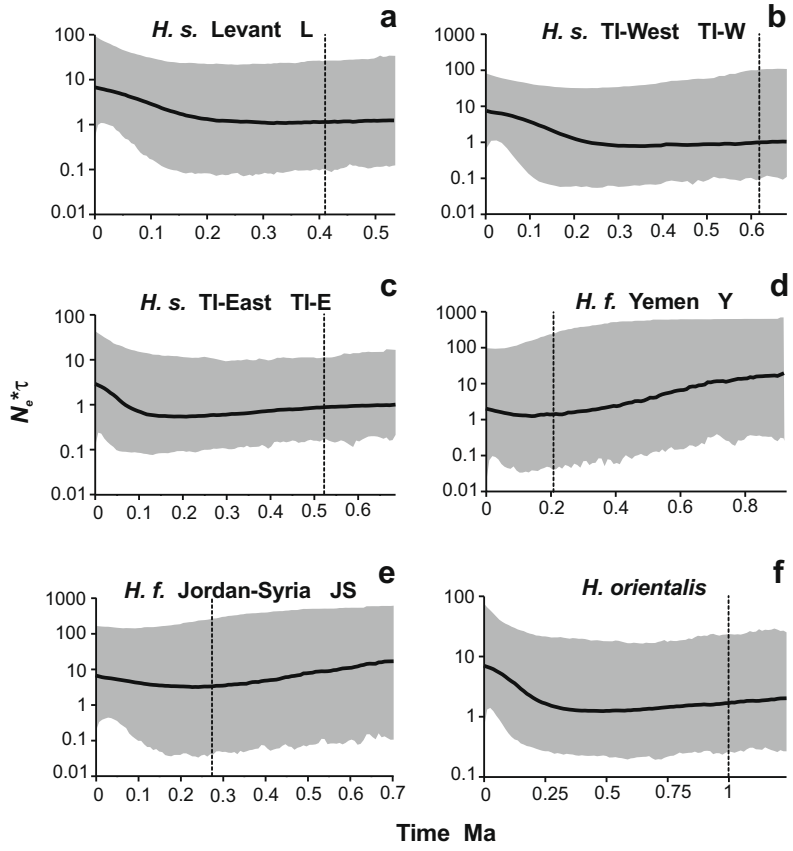

Fig. 4. Demographic history of main groups of *H. savignyi* (a–c), *H. felixarabica* sp. nov. (d and e) and the Middle Eastern populations of *H. orientalis* (f) as estimated with Bayesian skyline plots. Thick line shows the median value for the population size ($N_e \times \tau$; τ = generation length in units of time) on logarithmic scale; the shaded area represents the 95% highest posterior density and dashed lines are pointing to median estimates of the time to the most recent common ancestor (t_{MRCA}).

sults 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 taxon, occurred probably during the late Miocene, 11.1 and 8.4 Ma, respectively. The associated 95% HPD intervals were wide, but they clearly indicated that the splits occurred at minimum several million years ago (4.9 and 3.2 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 relationship between the new species and *H. orientalis*, neither genetic nor geographic [mtDNA, rhodopsin (present study); RAG1 (Stöck et al., 2008); nearest 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 (see Avise and Robinson, 2008; Degnan and Rosenberg, 2009). In accordance with genetic species concept (Baker and Bradley, 2006), 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 that 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 of their morphological characters (Gvoždík et al., 2008). Thus, we suggest that 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

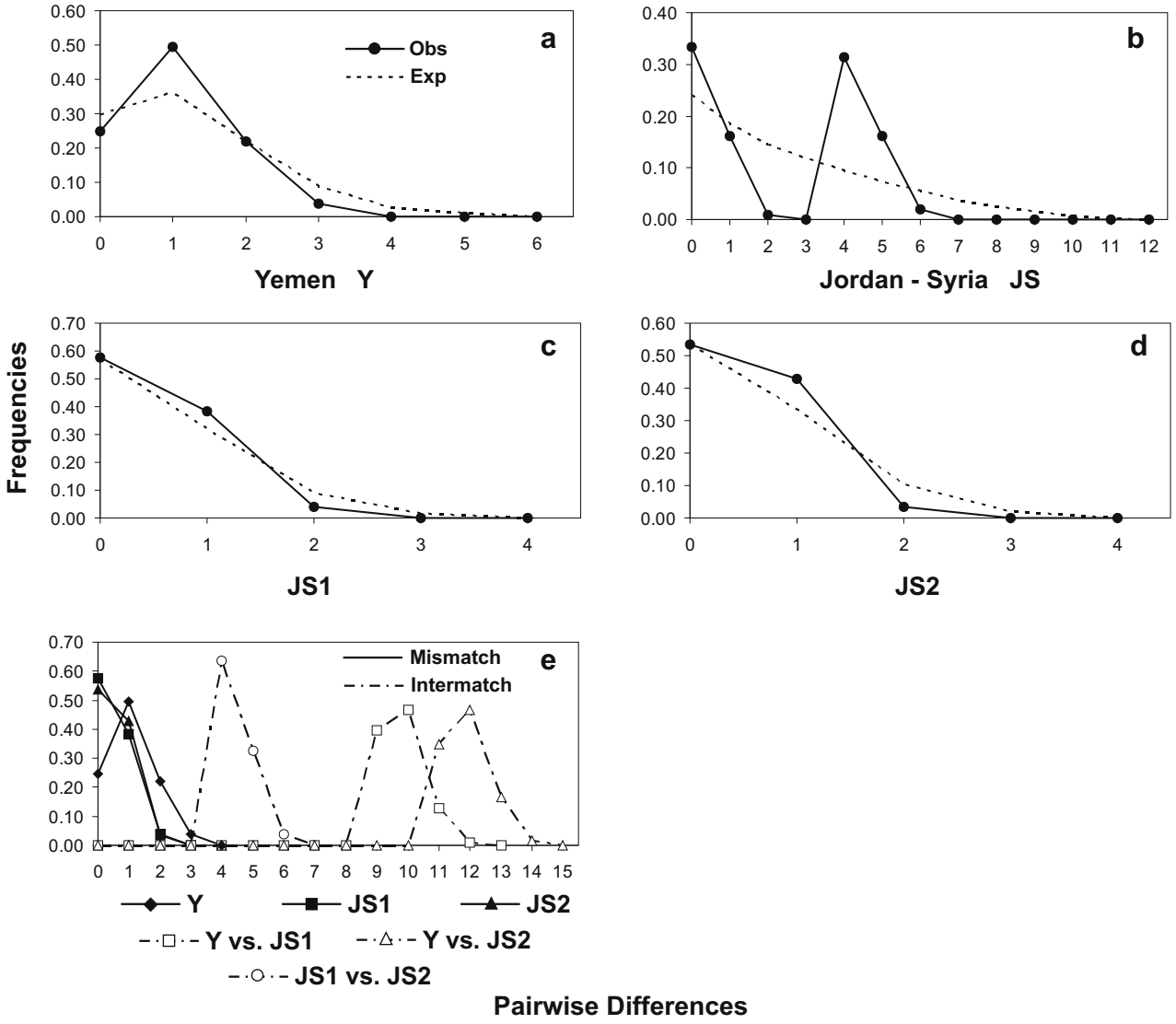

Fig. 5. Mismatch distributions for observed frequencies of different population subsets of *H. felixarabica* sp. nov. compared to the expected frequencies under the demographic expansion model of population size. Intermatch distributions between different population subsets are also provided (e).

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. *Hyla* sp. nov. is not known to occur in western Israel, or in the Judean Hills (V. Gvoždík et al., new additional unpubl. data), which is the distribution area of morphologically, acoustically and genetically distinct *H. heinzsteinitzi* (Grach et al., 2007; Stöck et al., 2008), and we therefore provide its formal description.

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

Synonymy. See Supplementary data.

Holotype. NMP6V 72076/1 (Fig. 7a and 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 and 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 felixarabica 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 felixarabica* 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

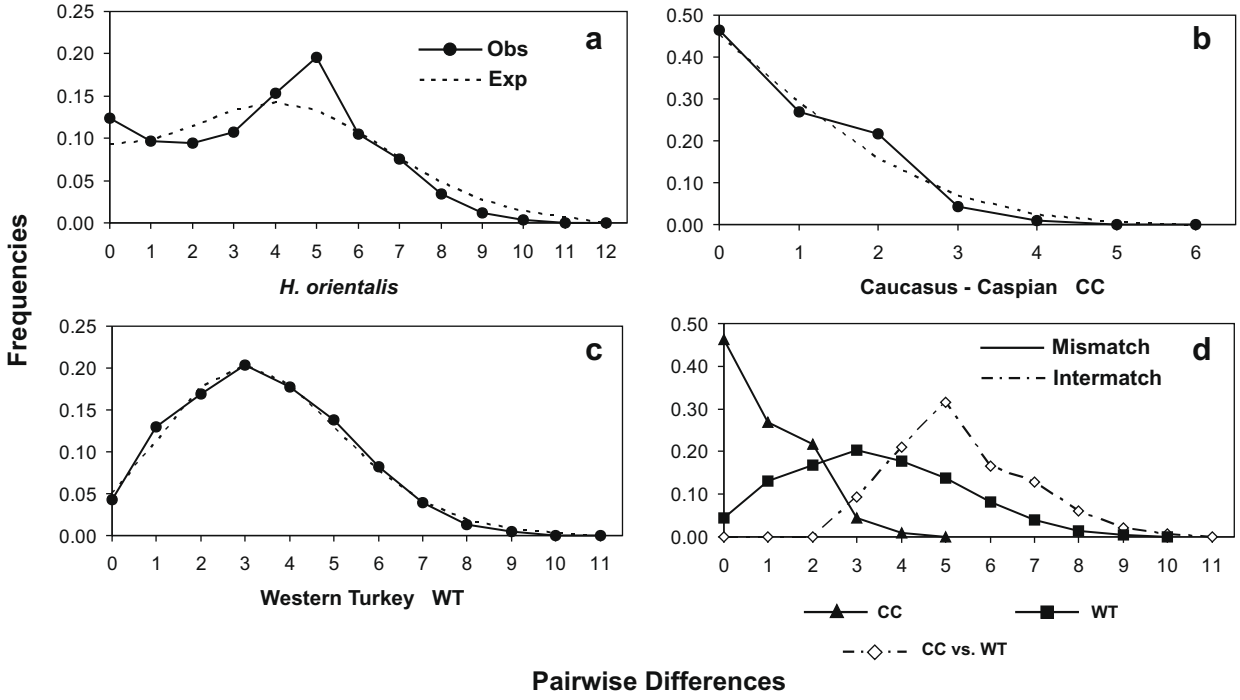

Fig. 6. Mismatch distributions for observed frequencies of *H. orientalis* populations under study (a) and their two main groups (b and c) compared to the expected frequencies under the demographic expansion model of population size. Intermatch distribution between the two groups is provided (d).

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 are further supported by data from 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 *H. felixarabica* 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* (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 *H. felixarabica* 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 was provided by the authors of the original description; however, pulses seem to be rather blurred and indistinct according to the published oscillograms.

(3) Morphologically, *H. felixarabica* 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; HL 14.2; HLt 12.0; ES 5.0; EN 3.3; NL 3.3; IND 3.5; EAD 6.8; IOD 3.0; EPD 12.0; ELW 3.7; 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 *H. felixarabica* from the Arabian Peninsula was described under the name *H. savignyi* in Balletto et al. (1985) and Gvoždík et al. (2008, Supplementary Material).

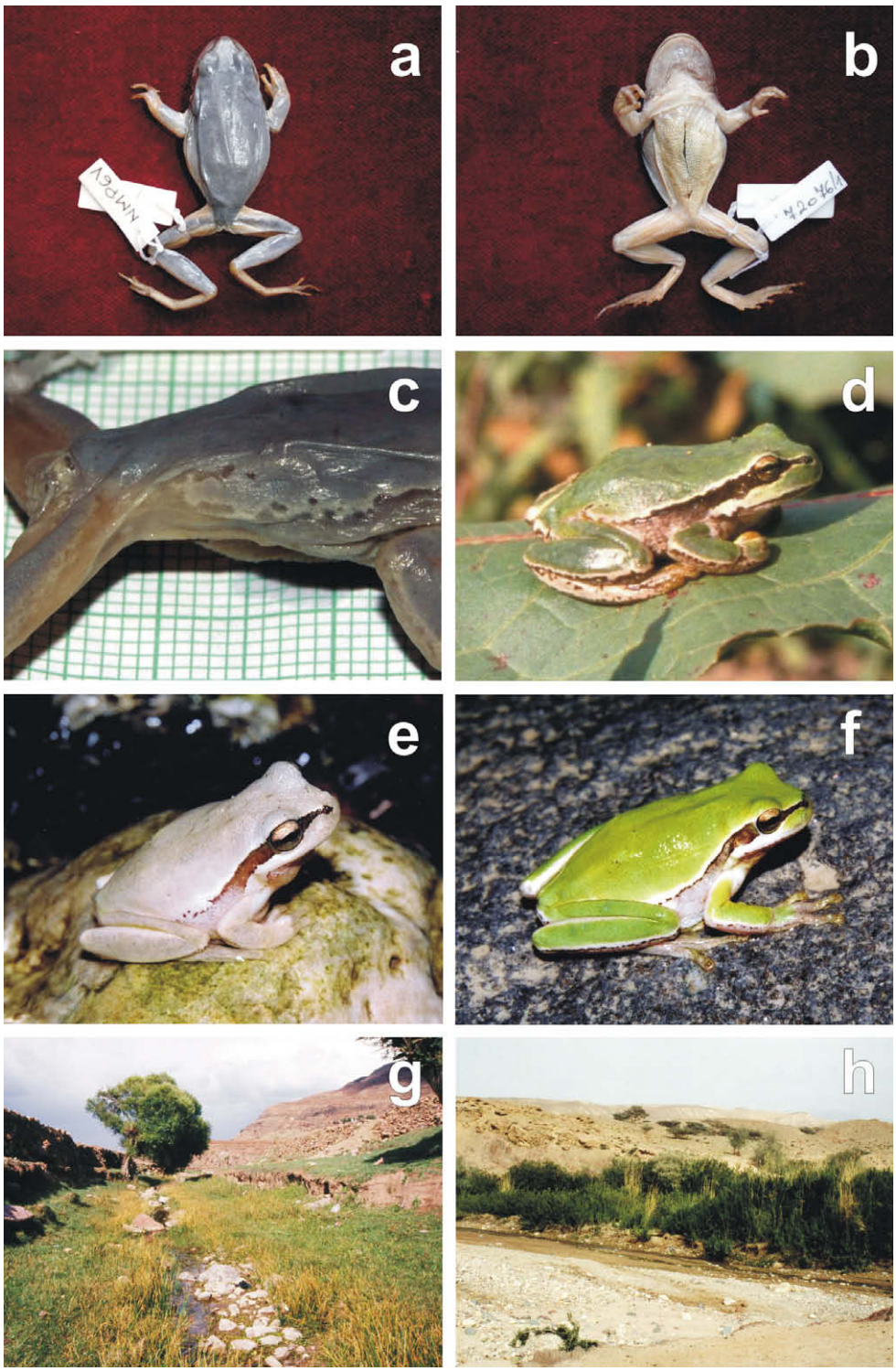

Fig. 7. *Hyla felixarabica* sp. nov. Holotype, NMP6V 72076/1, adult male, from (a) dorsal and (b) ventral views. (c) Photograph demonstrating irregular inguinal longitudinal loop-like streak on the paratype female, NMP6V 72076/4. (d) Paratype subadult female in life, NMP6V 72076/6. Adult males from the southern Levant in (e) pale beige night color phase, Wadi Fidan, Jordan, and (f) bright green night 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Karyotype. Al-Shehri and Al-Saleh (2005) described the karyotype of *H. felixarabica* 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 a detailed 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, *H. felixarabica* inhabits the regions above 1400 m a.s.l. from about

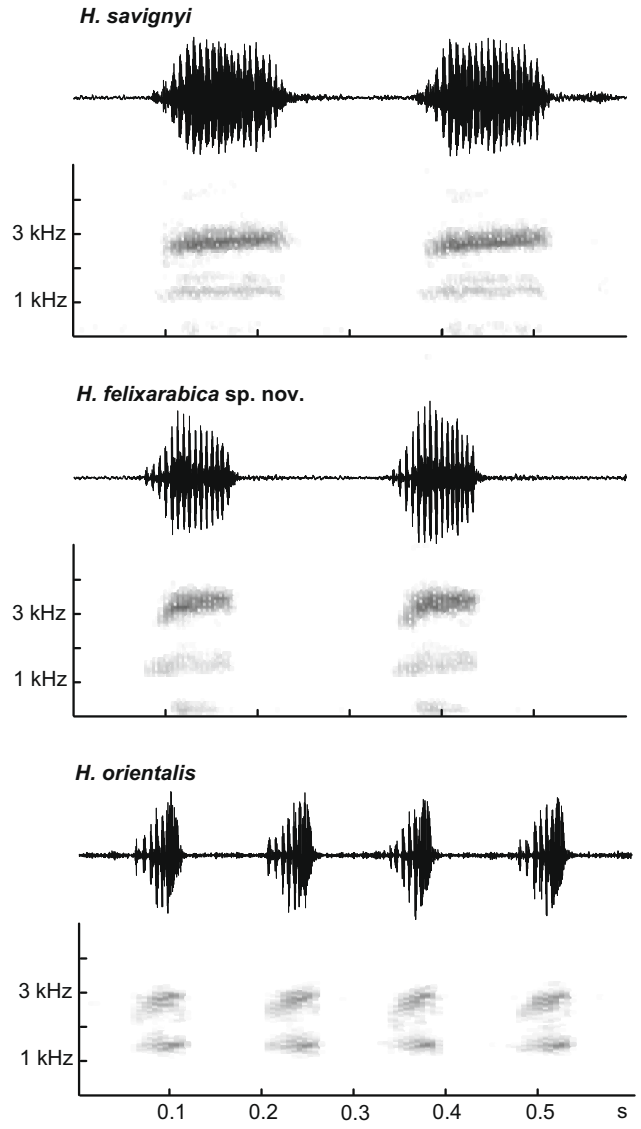

Fig. 8. Advertisement calls of *H. savignyi* (Ash Shuna, Jordan, loc. 20), *H. felixarabica* sp. nov. (Wadi Fidan, Jordan, loc. 55) and *H. orientalis* (Telavi, Georgia, loc. 71) as represented by oscillograms and respective spectrograms of the call segments (pulse groups) at the same time scale (section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

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, *H. felixarabica* seems to be distributed eastward from 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. *Hyla felixarabica* 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 *H. felixarabica*. Stöck et al. (2008) assumed that their 'new taxon 3' (= *H. felixarabica*) is sympatric in the Jerusalem region with *H. heinzsteinitzi*. However, our new data from Israel show that *H. savignyi* is the species present in that region (V. Gvoždík et al., unpubl. data). 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, loc. 67), where hybridization was documented. Occurrence of individuals with characteristic alleles of both species in the homozygous as well as heterozygous state (Supplementary data, Table S1) demonstrated that the hybrids were not F1 generation, which indicates that the population probably forms a part of 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 from 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 *H. felixarabica* 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 provisionally classify *H. felixarabica* as "Data Deficient" according to the IUCN (2008) Red List categories and 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.

4.2.3. Hyla orientalis

Stöck et al. (2008) assigned species rank to the south-eastern European, western Anatolian and Azerbaijani 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, loc. 69) 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 (loc. 72) 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 *H. felixarabica*. 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* (see Gvoždík et al., 2007b; Stöck et al., 2008), and therefore we cannot consider *H. orientalis* as the sister taxon to *H. savignyi–H. felixarabica*. Nevertheless, time to their common ancestor dated back probably 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 there making 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 *H. felixarabica*, dated back probably also to the late Miocene could be coincident with formation of the Dead Sea Rift (Garfunkel, 1988; Westaway, 2003; Zain Eldeen et al., 2002). *Hyla savignyi* has been restricted westward from the Rift while *H. felixarabica* occurs eastwards. 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 that within *H. felixarabica* separated 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 reflects the Pleistocene climatic 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 a phylogeographic pattern similar to *H. savignyi–H. felixarabica*. Within mice, genus *Mus*, some similarities might be seen in the Yemeni taxon *M.* (*musculus*) *gentilulus*, which forms a distinct clade with 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 a 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, *Bufo 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 *Bufo variabilis* occurs in the region as a single evolutionary lineage. *Hyla 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 makes contact with *H. savignyi* in Anatolia, in the Caucasus, and as we have shown also in Iran. Based on all molecular markers and advertisement calls studied (Gvoždík, 2010), tree frogs from the southern Caspian coast are clearly assignable to *H. orientalis*. This species occurs in Iran northward from the Alborz Mts' ridge, while *H. savignyi* is distributed southward from this mountain system. Whether they 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 that this region was a possible glacial refugium. The Caucasus–Caspian cluster mixes 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 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 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 suggested 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 suggested 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 probably had its refugia in the coastal plain of the eastern Mediterranean, and when it started to expand it dispersed along the Euphrates River valley northward, where it probably was separated from 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 felixarabica 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 approximately 10× 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 approximately 0.25 Ma as suggested 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 could be found in the most southerly distributed species, *H. felixarabica*. The few studies on demographic histories of vertebrates in the region usually found population growth as the most likely 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 with our results of *H. felixarabica*, 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 *H. felixarabica*. Our study, therefore, adds to a growing body of evidence that refugial and speciation peculiarities 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 comparative phylogeographic studies of a wide variety of species and fine-scale sampling, as we have described here, in connection with the use of various types 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, *H. felixarabica*, which is distributed in the Arabian Peninsula and southern Levant, eastward from 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 of *H. savignyi* consist of two main mitochondrial lineages in each species, which originated presumably during the Plio–Pleistocene boundary. However, persisting gene flow or incomplete lineage sorting caused discordant intraspecific phylogeographic patterns of the nuclear markers. The Anatolian and Caucasus–Caspian populations of *H. orientalis* demonstrated high genetic variation, suggesting that these regions were important Pleistocene refugia. However, it will be necessary also to study European populations to infer a complete evolutionary history of this species, which will be a subject of a forthcoming study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.03.015.

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