Cryptic diversity among Western Palearctic tree frogs: Postglacial range expansion, range limits, and secondary contacts of three European tree frog lineages (*Hyla arborea* group)

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1. Introduction

For many European vertebrate species including amphibians, phylogeographic hypotheses have been established in the last decade (for review: Hewitt, 2011). While morphological or behavioral traits mark the boundaries of some species (e.g. Fijarczyk et al., 2011), the situation is less clear for sibling species and cryptic lineages, which are revealed only by the recent application of molecular markers (e.g. Stöck et al., 2006; Teacher et al., 2009; Hauswaldt et al., 2011; Recuero et al., 2012; Bisconti et al., 2011; Garcia-Porta et al., 2012). Western Palearctic tree frogs of the *Hyla arborea* group provide a good example (Faivovich et al., 2005; Smith et al., 2005; Wiens et al., 2005, 2010). Until recently, most European populations were considered to belong to a single species, *H. arborea* (e.g. Schneider and Grosse, 2009; http://www.iucnredlist.org/apps/redlist/details/10351/0), except for the Apennine Peninsula (plus Sardinia and Corsica), where *H. intermedia* (resp. *H. sarda*) had been assigned species status, confirmed by the lack of introgression at a contact zone with *H. arborea* (Verardi et al., 2009). A phylogenetic analysis based on 3200 bp of mitochondrial and 860 bp of coding nuclear DNA (Stöck et al., 2008a) revealed this former, wide-ranging *H. arborea* to comprise three highly diverged lineages: *H. arborea*, occurring from Greece to northeastern Europe, and *H. intermedia*, ranging from Asia Minor to northeastern Europe, and not previously distinguished from *H. arborea*. Phylogenies based on mtDNA show that *H. mulleri* and *H. orientalis* are as much diverged from *H. arborea* as is the recognized species *H. intermedia*, hence supporting a similar taxonomic status.

We characterize divergence times, intraspecific diversity and distributions for recently recognized lineages within the *Hyla arborea* species group, based on mitochondrial and nuclear sequences from 160 localities spanning its whole distribution. Lineages of *H. arborea*, *H. orientalis*, *H. mulleri* have at least Pliocene age, supporting species level divergence. The genetically uniform *H. mulleri*, although largely isolated by the Pyrenees, is parapatric to *H. arborea*, with evidence for successful hybridization in a small Aquitanian corridor (southwestern France), where the distribution also overlaps with *H. meridionalis*. The genetically uniform *H. arborea*, spread from Crete to Brittany, exhibits molecular signatures of a postglacial range expansion. It meets different mtDNA clades of *H. orientalis* in NE-Greece, along the Carpathians, and in Poland along the Vistula River (there including hybridization). The East-European *H. orientalis* is strongly structured genetically. Five geographic mitochondrial clades are recognized, with a molecular signature of postglacial range expansions for the clade that reached the most northern latitudes. Hybridization with *H. savignyi* is suggested in southwestern Turkey. Thus, cryptic diversity in these Pliocene *Hyla* lineages covers three extremes: a genetically poor, quasi-Iberian endemic (*H. mulleri*), a more uniform species distributed from the Balkans to Western Europe (*H. arborea*), and a well-structured Asia Minor-Eastern European species (*H. orientalis*).

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2. Methods

2.1. Amplification, cloning, and alignment of sequences

Samples of 462 frogs covering the whole European Hyla distribution (Fig. 1) were collected from living adults (buccal swabs), tadpoles (tail tips), or tissues from adult voucher individuals stored in scientific collections (Appendix S1). Buccal swabs were stored at −20 °C, tissue samples in 100% ethanol. DNA was extracted with Qiagen DNeasy Tissue Kit or the BioSprint robotic workstation (Qiagen), eluted in a 200 μl Qiagen Buffer AE and stored at −18 °C. The mitochondrial cytochrome b (ca. 1 kb) was amplified with primers Lo and H1046 as described (Stöck et al., 2008a). To amplify ca. 545 bp of intron 1 of Fibrinogen A, alpha-polypeptide, we used two primers (MVZ47: 5′_AGTGAAGATACTCACTGCTGCTAGG_39; MVZ48: 5′_GGAGGATATCT-AAGCACTCT-AAAAAG_39) and the following protocol: PCRs were performed in 12.5 μl reactions containing 7.55 μl H2O, 1.25 μl of PCR buffer including 1.5 mM MgCl2, 0.1 μl of dNTPs, 0.1 μl Taq QIAGEN, 0.75 μl of each primer having a concentration of 10 mM, and 2 μl of genomic DNA with a concentration of 20 ng/μl. For subsequent cloning, two such reactions from each individual were pooled to increase volume. The PCR protocol followed a “touch-up” approach with 10 cycles of annealing temperatures (55–60 °C) increasing by 0.5° each cycle (with 30 s at 95 °C, 30 s at annealing temperature, and 45 s at 72 °C), followed by 25 cycles with 30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C, and a final extension of 7 min at 72 °C. All PCR-products (each clone of Fibrinogen; direct sequencing of PCR products of cytochrome b) were sequenced in both directions, visualized on an ABI 3730 sequencer, and aligned with Sequencher 4.9, followed by the algorithms as implemented in Seaview (Gouy et al., 2010).

2.2. Phylogenetic analyses

In a first step, we reduced the total number of mtDNA sequences to the number of haplotypes found at each locality. Maximum likelihood (ML) phylogenies were generated with PhyML 3.0 (Guindon et al., 2010) using the GTR model for cytochrome b and HKY model for the Fibrinogen alpha nuclear marker. For each case, we chose a BioNJ tree as a starting tree and used the combined subtree pruning and regrafting (SPR) plus nearest neighbor interchange (NNI) options for tree improvement. All other parameters were set as default (http://atgc.lirmm.fr/phyml/). Bootstrap values were based on 1000 (mtDNA) or 100 (nuDNA) resampled datasets. Bayesian phylogenetic analysis using the reported marker-specific substitution models was performed in MrBayes v3.1.0 (Ronquist and Huelsenbeck, 2003), with the default heating values for three out of four chains, running 20 × 10^6 generations separately for the mtDNA and nDNA datasets, with tree sampling every 1000 generations. The “burnin”-value was selected by visualizing the log likelihoods associated with the posterior distribution of trees in the program Tracer (http://tree.bio.ed.ac.uk/software/tracer/).

Fig. 1. Map with approximate range limits of Western Palearctic tree frogs (range limits according to maps available through the Global Amphibian Assessment http://www.iucnredlist.org/initiatives/amphibians) with sampling localities (see Map IDs in Appendix S1). Hyla meridionalis and “new taxon 1” (acc. Stöck et al., 2008a) are united as “Hyla meridionalis s.l.”; H. intermedia and “new taxon 2” (Stöck et al., 2008a) as “Hyla intermedia s.l.”. Approximated range limits in interleaved colors indicate parapatric ranges or deficiency of knowledge.
All trees generated before the log likelihood curve flattened were discarded.

2.3. Demographic analyses and estimates of divergence time

We used DnaSP v.5 (Librado and Rozas, 2009) to calculate and visualize the distributions of observed and expected pairwise nucleotide site differences (‘mismatch distributions’), between all individuals within the mtDNA clades of Hyla arborea, H. molleri, and subclades within H. orientalis, as well as the respective expected values for growing populations (Librado and Rozas, 2009). We included only cytochrome b markers for which >904 bp 100% readable sequences were available (H. arborea: 86%, H. orientalis: 94% H. molleri: 100%, total: 92%).

Divergence times to the most recent common ancestors were estimated from the cytochrome b and Fibrinogen alpha markers independently, assuming an uncorrelated exponential relaxed molecular clock (BEAST v. 1.6; Drummond et al., 2006; http://beast.bio.ed.ac.uk/Main_Page). In the absence of appropriate fossils, we based our prior on results from previous work (Smith et al., 2005; Stöck et al., 2008a), assuming a normal distribution for the divergence time between H. meridionalis and other tree frogs, with a mean of 10 millions of years ago (Mya) and standard deviation of 1 My (thus effectively spanning a large range from 7.5 to 12.5 Mya).

We applied the marker specific models of sequence evolution as described for PhyML, and a Yule tree prior (constant speciation rate per lineage) as most appropriate for species-level divergences (Drummond et al., 2007). DNA cytochrome b data were analyzed both with and without codon partition, with different partitions for codons 1 + 2 and 3.

3. Results

Maximum likelihood and Bayesian phylogenetic analyses yielded mtDNA trees with congruent topologies (Figs. 2 and S1). The same clades were recovered for the nuDNA tree, but with markedly lower support (Fig. 3). Results turned out to be very robust regarding partitioning. In the following, we focus on each of the three species lineages of the Hyla arborea group.

3.1. European tree frog (Hyla arborea)

This species ranges from the Western Balkan Peninsula across Central into mainland Western Europe (Fig. 1), showing almost no genetic structure on the mitochondrial or the nuclear level (Figs. 2 and 3). Specifically, it occurs on Crete (locs. 94, 97–101), the Peloponnesus and mainland Greece (locs. 81–83, 89), along the eastern Adriatic coast (locs. 54–56, 58, 71, 77, 78), and throughout the Eastern Pannonian Basin (Hungary, NE-Romania, W-Ukraine: locs. 75, 85–87), where it is separated from the eastern tree frog (H. orientalis) by the Carpathian Arc. Hyla arborea is the only tree-frog taxon occurring from central Poland (west of the Vistula River: locs. 65–67, 70) throughout central (locs. 52 and 53) to northwestern (locs. 34–41, 43) and western Europe (locs. 28, 31, 32). The mtDNA mismatch distribution (Fig. 4a) shows significantly high matching of simulated and observed curves (Table 1), pointing to a recent and rapid expansion.

3.2. Eastern tree frog (Hyla orientalis)

Our data show that this lineage, whose old name was resurrected when molecular evidence showed its mitochondrial and nuclear divergence from H. arborea (Stöck et al., 2008a; Gvozdik et al., 2010), in fact represents a genetically very diverse and well-differentiated group of lineages based on both mtDNA (Figs. 2 and 5a) and nuclear DNA (Fig. 3). Using mtDNA, we found five well-supported subclades (Fig. 5): one in the Talysh Range (locs. 154–158), and a second well-structured one in the Caucasus and adjacent areas (locs. 134–139, 147–149, 153); for both, mismatch distribution analyses (Figs. 5b and c) failed to reach significance (Table 1). Another well-structured mtDNA-group with two subclades inhabits western Asia Minor (locs. 107, 118–120) and the western coast of the Black Sea (locs. 108, 111, 112, 114, 121), without signs of recent demographic changes (Table 1). Finally, a well-supported, widespread haplotype clade with almost no substructure inhabits the Crimea, the northwestern coast of the Black Sea and the entire northeastern European region including Ukraine, Belarus, Russia, and Poland, with the Vistula River as its approximate western border. For this latter group, demographic analyses revealed an almost perfect match of simulated to empirical data (Fig. 5e and Table 1), also pointing to a recent expansion.

The two groups of subclades based on mtDNA (i: Caucasus and Talysh vs. ii: Asia Minor and Black Sea, Eastern Europe and Crimea; Fig. 5a) are not entirely recovered based on nuDNA, where two weakly supported subclusters (Fig. 3) unite Eastern European and Talysh with western Asia Minor frogs.
Fig. 3. Maximum likelihood tree obtained with the program PhyML based on 545 bp of nuDNA fibrinogen alpha (intron 1). The number of identical clones obtained for each sequence is given after the sample ID (as in Appendix S1), and before the locality ID (as in Fig. 1 and Appendix S1). Bootstrap support values from 100 resampled data sets (normal font) for this tree are followed by Bayesian posterior support values (%) for major respective nodes in **bold italics** (after the “/”) from analysis using Mr. Bayes v3.1.0.
3.4. Secondary contact zones in Western Palearctic tree frogs

We newly localized five major contact zones: First, in northeastern Greece, we narrowed the potential contact between *H. arborea* and *H. orientalis* to less than 70 km (locs. 83a and 105a), without evidence of genetic interactions. Second, the Western Carpathians of Serbia (loc. 77a) show co-occurrence of *H. arborea* and *H. orientalis* mtDNAs. Thirdly, to the north of the Carpathian Arc, in the lowlands of central Poland, we have evidence for parapatric ranges of *H. arborea* and *H. orientalis* (locs. 68 and 69), with the Vistula River representing a reasonable approximation for range borders of both lineages. Fourth, near the Atlantic coast of SW-France (locs. 30–30a), we found range overlap and hybridization of *H. arborea* and *H. malleri*, as nuclear intron alleles from *H. arborea* were detected in two individuals with *H. malleri* mtDNA; one frog also possessed nuclear alleles from both *H. arborea* and *H. malleri* (Fig. 5). Fifth, we found a contact zone between *H. orientalis* and *H. savignyi* in SW-Anatolia (loc. 120), where we indentified an apparently re-combined nuclear allele, seemingly stemming from successful hybridization of both species that occur in geographic proximity. *Hyla orientalis* and *H. savignyi* have close geographic proximity in the south of the Great Caucasus (locs. 145–148), but no documented hybridization.

3.5. Divergence-time estimates

The posterior predictions for the divergence time between *Hyla meridionalis* and other Western Palearctic *Hyla* lineages were very close to the mode assumed for the prior, and very consistent between mtDNA and nuDNA (namely, 9.7 and 9.8 Mya for the cytochrome *b* and Alpha-Fibrinogen, respectively; Table 2). For the inner groups, the mtDNA and nuDNA markers also yielded similar and widely overlapping ranges of the divergence-time estimates (Table 2), with most lineages formed between late Miocene and lower Pliocene time periods (*H. sarda*, *H. savignyi*, *H. felixarabica*, *H. arborea*), while the remaining lineages (*H. malleri*, *H. orientalis*) are suggested to be of Pliocene age. The mean substitution rates predicted for cytochrome *b* and Alpha-Fibrinogen were 0.0161 and 0.00262 per lineage per million years respectively, similar to those found in other anurans (e.g. Mulcahy and Mendelson, 2000; Hoegg et al., 2004).

4. Discussion

4.1. Cryptic diversity

Throughout the European range of *H. arborea*, we show great mtDNA homogeneity (Figs. 2 and S1), and also nuDNA-uniformity. A fast postglacial range expansion of the *H. arborea* mtDNA haplotype group from a Balkanian refugium into its entire current range is very well documented by the mtDNA mismatch distribution (Fig. 4a and Table 1) and the corresponding haplotype network (Fig. S2), which shows the most frequently represented haplotype (Fig. S2: rectangle) ranging from western France to Western Ukraine (Fig. 1: locs. 28 and 88) with its closest relatives at the Adriatic coast (Albania: loc. 78; Croatia: e.g. locs. 71) and in Greece (e.g. loc. 83). The Balkanic region harbors a greater diversity of haplotypes than does the rest of Europe (Fig. S2). Our previous study based on the coding nuclear Rag-1 for a small subset of samples (Stöck et al., 2008a) also detected a larger amount of genetic diversity in the south of the range, interpreted as diversity in the proposed Pleistocene refugium, the Balkan Peninsula and Adriatic coast.

Despite limited sampling from some regions for *H. malleri*, we covered most geographic extremes of the range including its northern limits in southwestern France. As recently concluded by Barth et al. (2011), who had larger sample sizes for the western range, we find that the Iberian endemic *H. malleri* exhibits little mtDNA diversity throughout its range. As for many Iberian species, it could circumvent the Pyrenees only to the West but has spread to northern latitudes much less than has *H. arborea*.

In sharp contrast to *H. arborea* and *H. malleri*, we found substantial mtDNA but also nuDNA-based genetic structure within...
Fig. 5. Mitochondrial DNA-diversity within *Hyla orientalis*. (a) Unrooted maximum-likelihood tree for 905 bp of cytochrome *b* [for details: legend of Fig. 2]. (b–e) Mismatch distributions from 905 bp of mitochondrial cytochrome *b* for the corresponding haplotype clades as shown in (a). The dotted line shows the frequency distribution of the observed pairwise differences; the solid line shows the frequency distribution of the expected pairwise differences under the sudden expansion model, performed in DnaSP v.5. (f) Geographical representation of clades shown in (a–e).
the recently recognized Eastern tree frog *H. orientalis* (Figs. 3 and 5). Much of *H. orientalis’* diversity occurs in Asia Minor and suggests circum-Black Sea Pleistocene refugia. The clade that post-glacially colonized the northern latitudes shows high mtDNA-uniformity and significant signs of recent range expansion (Fig. 5e), similar to the signature across all of *H. arborea’s* mtDNA (Fig. 4a). The reason that Gvozdik et al. (2010) found all their *H. orientalis* samples (to) form a compact cluster with substantial genetic variation, although without any deep divergences appears to result from sampling only some Asia Minor and Caucasian regions.

### 4.2. Divergence times

The posterior predictions for the divergence time with *H. meridionalis* are extremely close to the mode assumed for the prior and very consistent between cytochrome b and Alpha-Fibrinogen. Furthermore, the associated mean substitution rates are similar to those found in other anurans (e.g. Mulcahy and Mendelson, 2000; Hoegg et al., 2004), providing support for our calibration of the phylogenies with *Hyla meridionalis*.

Despite previous estimates of divergence time for some Western Palearctic tree frog species (Canestrelli et al., 2007; *H. intermedia*; Recuero et al., 2007: *H. meridionalis*, Gvozdik et al., 2010; *H. orientalis*, *H. savignyi*, *H. felixarabica*, *H. meridionalis*), our study is the first that includes all extant species, and that uses mitochondrial and nuclear sequence markers. As far as comparable (divergence of *H. orientalis* vs. *H. savignyi* + *H. felixarabica*) our estimates are compatible, given the highest posterior density interval spanning “the period from the Early Pliocene through the Miocene, between 4.9 and 23.0 My” (Gvozdik et al., 2010).

Some of the discrepancies between our mtDNA and nuDNA-based estimates (Table 2) may be explicable by fewer data on the nuclear than on the mtDNA level for several clades. We confirm considerable divergences between two subclades of both “*H. meridionalis* s.l.” and “*H. intermedia* s.l.” (Fig. 2), as previously shown by other authors and markers (Recuero et al., 2007; Canestrelli et al., 2007), and temporarily called “new taxa1 and 2” (Stöck et al., 2008a). More work is needed to understand potential taxonomic implications for these lineages but is beyond the scope of this paper.

### 4.3. Contact and hybrid zones

The Eastern Mediterranean contains several major Pleistocene refugia, with the territory of Greece representing a meeting zone of faunal elements of Asia Minor and of Balkan Peninsular (plus African) origin (Lymbéras and Poulaikakis, 2010). Western Greece, Crete and some western Aegean islands are colonized by the mitochondrial lineage that also occurs on the western Balkan Peninsula and stretches into Central and even Western Europe (*H. arborea*), while the eastern Greek provinces of Macedonia, Thrace and the eastern Aegean islands are phylogenetically close to the clade of Asia Minor origin (*H. orientalis*). Although we narrowed the potential contact to ca. 70 km (locs. 83a and 105a), our data are not sufficient yet to reveal potential contacts of tree-frog lineages in northeastern Greece and the Aegean islands.

To the north of Greece, the Carpathians represent a major barrier for tree frogs. West of this mountain range occurs the *H. arborea* haplotype group, and to the east of the Carpathian Arc that of *H. orientalis*, which also inhabits the entire rest of the Eastern European *Hyla* range. Large Carpathian river valleys provide rare opportunities for secondary contacts, with so far one locality of co-occurrence of both mtDNAs (loc. 77a). To the north of the Carpathian Arc, secondary contact and hybridization between *H. arborea* and *H. orientalis* are documented by mtDNA and microsatellite data from the lowlands of Poland (Borżée, 2010, in prep.). Interestingly, the mtDNA subclade of *H. orientalis* that meets the uniform *H. arborea* in Poland (Fig. 1 and 5) differs from the subclade (Figs. 1 and 4d: triangles) that is in potential contact in Serbia and northeastern Greece. This offers interesting comparative research opportunities on secondary contacts of differently, but quite closely related populations.

Since the splitting of *H. mulleri* from *H. arborea* by Stöck et al. (2008a), occurrence and range limits at the Atlantic coast of SW-France, in the Aquitaine region, have been ambiguous with respect to species (see also Barth et al., 2011). Our new data (locs. 29–30a) not only revealed the only Western Palearctic region with three co-occuring tree frog taxa but also (at least) F₁-hybridization between *H. mulleri* and *H. arborea*. As in the overlapping distributions of *H. meridionalis* and *H. mulleri*, in the Spanish Sistema Central Mountains, few hybridization events have been reported (Oliveira et al., 1991; Barbadillo and Lapena, 2003); even genetic interactions between three species appear possible, but more research is required.

In addition to the three newly localized contact zones of *H. arborea* with *H. orientalis* (NE-Greece, Poland), and with *H. mulleri* (SW-France), a well-known contact zone with *H. intermedia* exists in NE-Italy (Verardi et al., 2009), where neither hybrids nor backcrosses were identified, indicating a lack of current gene exchange between the two species. However, introgressed alleles appeared in both species, indicating past introgressive hybridization. Using bioacoustic inference, pending genetic confirmation, Schneider (2001) narrowed the contact between *H. orientalis* (as “*H. arborea*”) and *H. savignyi* to less than 10 km in the Anamur plain of south-west Anatolia. Parapatry with one documented locality of hybridization (Karkom, Israel) has been shown between *H. savignyi* and *H. felixarabica* (Gvozdik et al., 2010).

### 4.4. Comparisons with phylogeographic patterns of other terrestrial groups

Our data contribute to knowledge of the evolutionary history of Western Palearctic tree frogs as well as the comparative phylogeography of Europe, and should improve conservation measures. As recently noted by Riesser and Smith (2010) for North America: “Identifying congruence in the geographical position of lineage breaks and species range limits across multiple taxa is a focus (…) of comparative phylogeography. These regions are biogeographical hotspots for investigations into the processes driving divergence at multiple phylogenetic levels”. Indeed, the postglacial colonization routes and resulting

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secondary hybrid zones of tree frogs in the Western Palearctic coincide with several of those known from other terrestrial species. Namely, the postglacial colonization route of *H. arborea* resembles that of the grasshopper *Chorthippus parallelus*, and the advance of beech (*Fagus sylvatica*) and black alder (*Alnus glutinosa*) from their Balkanian refugia (*King and Ferris, 1998; Hewitt, 1999, 2004; Magri, 2008*), with the broad-leaf forest providing direct summer habitats for tree frogs, suggesting partial co-colonization. As do *H. arborea* and *H. mulleri*, these three species meet Iberian counterparts in the Pyrenees (*Hewitt, 1999*) and form hybrid zones in their vicinity. Postglacial colonization of northeastern Europe to the east of the Carpathians by *H. orientalis* resembles that by the green toad *Bufo variabilis* (*Stöck et al., 2006, 2008b*).

### 4.5. Implications for conservation of European tree frogs

Amphibians are undergoing a massive and extensive crisis (*Wade and Vredenburg, 2008; Hoffmann et al., 2010*), with complex causes that include land-use changes (*Hof et al., 2011*). The remaining amphibian biodiversity should thus be especially assessed and protected in regions with industrial agriculture and intense land use and fragmentation (such as Western Europe) or currently facing major land-use changes due to political and economic transformations (such as Eastern Europe). While most *Hyla* species are still common in parts of their Western Palearctic range, habitats are fragmented, and these frogs are in significant decline over much of their Western European distribution (http://www.iucnredlist.org/apps/redlist/details/10351/0), mainly by “loss of breeding habitats, habitat isolation, fragmentation, and pollution”. Tree frogs are considered less threatened in Eastern Europe (*www.amphibiaweb.org*, incl. refs.). However, land-use changes caused by ongoing political and economic transformation pose upcoming threats also for the latter regions. Our data therefore support conservation efforts by fine-tuning measured locations of refugia harboring great genetic diversity (*e.g. Moritz, 2002*), which are “essential refuges for Earth’s many small-ranged species” (*Sandel et al., 2011*). The localized areas of secondary contact should be considered “natural arenas to investigate processes driving speciation” (*Rissler and Smith, 2010*), which require special conservation efforts.

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