Speciation history and widespread introgression in the European short-call tree frogs (Hyla arborea sensu lato, H. intermedia and H. sarda)

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ABSTRACT

European tree frogs (Hyla) characterized by short temporal parameters of the advertisement call form six genetically differentiated but morphologically cryptic taxa, H. arborea sensu stricto, H. orientalis and H. molleri from across Europe to western Asia (together referred to as H. arborea sensu lato), two putative taxa within H. intermedia (Northern and Southern) from the Italian Peninsula and Sicily, and H. sarda from Sardinia and Corsica. Here, we assess species limits and phylogeographic relationships within these ‘short-call tree frogs’ based on mitochondrial DNA and nuclear protein-coding markers. The mitochondrial and nuclear genes show partly incongruent phylogeographic patterns, which point to a complex history of gene flow across taxa, particularly in the Balkans. To test the species limits in the short-call tree frogs and to infer the species tree, we used coalescent-based approaches. The monophyly of H. arborea sensu lato is supported by the mtDNA as well as by the all-gene species tree. The Northern and Southern lineages of H. intermedia have been connected by nuclear gene flow (despite their deep mtDNA divergence) and should be treated as conspecific. On the contrary, the parapatric taxa within H. arborea sensu lato should be considered distinct species (H. arborea, H. orientalis, H. molleri) based on the coalescent analysis, although signs of hybridization were detected between them (H. arborea × H. orientalis; H. arborea × H. molleri). A mitochondrial capture upon secondary contact appears to explain the close mtDNA relationship between the geographically remote Iberian H. molleri and H. orientalis from around the Black Sea. Introggressive hybridization occurred also between the Balkan H. arborea and northern Italian H. intermedia, and between the Minor Asiatic H. orientalis and Arabian H. felixarabica (the latter belonging to a different acoustic group/clade). Our results shed light on the species limits in the European short-call tree frogs and show that introgression played an important role in the evolutionary history of the short-call tree frogs and occurred even between taxa supported as distinct species.

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1. Introduction

Western Palearctic tree frogs were considered to represent a single species Hyla arborea until the end of the 1960s. Later on, two taxa, H. meridionalis and H. savignyi from the western and eastern Mediterranean, respectively, were elevated from subspecies level to full species based on differences in advertisement calls (Paillette, 1967; Schneider, 1968, 1974; Schneider and Nevo, 1972). Advertisement calls are an important behavioral feature in anurans with a critical role in maintaining reproductive barriers between species (Schneider, 1977; Schneider and Sinsch, 2007). In the Western Palearctic tree frogs, the advertisement calls have been thoroughly studied (reviews in Schneider, 1974, 2004a). The call segments are formed by pulse-groups (notes) and intervening quiet intervals. Advertisement calls of the Western Palearctic tree frogs might be divided into three categories according to the...
pulse-group properties (Fig. 1; grouping made according to the results of Grach et al., 2007; Gvoždík et al., 2010; Schneider, 1974, 2004a). The ‘long-call tree frogs’ are characterized by long pulse-groups composed of a high number of pulses (34–56 pulses; 210–610 ms), ‘short-call tree frogs’ by short pulse-groups with a low number of pulses (6–12 pulses; 50–110 ms), and ‘medium-call tree frogs’ by intermediate values (13–25 pulses; 85–190 ms).

The Tyrrenian tree frog, H. sarda from the islands of Sardinia, Corsica, Elba and surrounding islets, was distinguished from H. arborea on the basis of the differentiation at allozyme loci (Lanza, 1983; Nascetti et al., 1985), but the advertisement call of this species was not found to be substantially differentiated from H. arborea (Schneider, 1974, 2004a). This is also true for the Italian tree frog, H. intermedia (Schneider, 2004a,2004b), which also was distinguished from H. arborea on the basis of allozyme differences (Nascetti et al., 1995). With the expansion of DNA sequencing, new hypotheses on the taxonomy of the Western Palearctic tree frogs emerged (Gvoždík et al., 2010; Stöck et al., 2008, 2012). Besides the description of the new species H. felixarabica from the Arabian Peninsula (Gvoždík et al., 2010), deeply divergent lineages were uncovered within H. meridionalis (south-eastern part of the range) and in H. intermedia (northern part of the range) (Canestrelli et al., 2007a; Gvoždík, 2009; Gvoždík et al., 2007; Recuero et al., 2007; Stöck et al., 2008, 2012), which may each represent a separate (as yet unnamed) taxon (Stöck et al., 2008). Furthermore, H. arborea was suggested to represent three species according to the mitochondrial and single nuclear-gene phylogenies: H. orientalis in the east, H. molleri in the west, and H. arborea sensu stricto (s.s.) in the central part of the former H. arborea sensu lato (s.l.) range (Stöck et al., 2008).

For the Iberian (H. arborea) molleri, this new taxonomy corresponds to the former morphology-based intraspecific taxonomy of H. arborea. In contrast, the genetic break between the central (H. arborea s.s.) and eastern (H. orientalis) populations does not match the limits and distributions of the morphology-based sub-species, H. arborea kretensis (Crete, southern Balkans, Aegean, western Asia Minor; e.g. Stumpel, 1997), H. a. schelkownikowii (Caucasus; e.g. Kuzmin, 1999), H. a. gumiłevisoii (Talysh Mts. in Transcaucasia; Litvinchuk et al., 2006) and H. a. arborea in the rest of the range (except the Iberian Peninsula; e.g. Stumpel, 1997). [For historical overview of the taxonomy of the Western Palearctic tree frogs see Fig. 1.] This incongruence between molecular phylogenies and morphology-based taxonomy, which discouraged wider acceptance of the revised taxonomy (e.g. Grosse, 2009; Özdemir et al., 2012; Sillero et al., 2014; Speybroeck et al., 2010), is further fostered by the unexpected and surprisingly close sister-clade relationship between the eastern (near the Black Sea; H. orientalis) and western (on Iberian Peninsula; H. molleri) populations, currently geographically separated by the range of H. arborea s.s. (Gvoždík, 2009; Gvoždík et al., 2007; Stöck et al., 2008, 2012). As yet, no study has provided explanation for this biogeographic pattern.

The clade containing H. arborea s.l., H. intermedia and H. sarda (all considered H. arborea till the 1990s; e.g. Stumpel, 1997) has consistently received high support from molecular phylogenetic analyses (Gvoždík, 2009; Stöck et al., 2008, 2012), while relationships within the clade have remained ambiguous (Stöck et al., 2008, 2012). The exceptions are the sister-clade relationships of H. orientalis and H. molleri and of the Northern (N) and Southern (S) lineages of H. intermedia (Gvoždík, 2009; Stöck et al., 2008, 2012). However, using different mitochondrial markers (12S and 16S rRNA) than in Stöck et al. (2008, 2012), our preliminary data showed high support also to H. arborea s.l., i.e. H. orientalis, H. molleri and H. arborea s.s. forming a clade (Gvoždík, 2009). In addition to the unresolved internal topology of the clade, species limits in the short-call tree frogs are an intriguing issue, especially given the following evidence: (i) similar advertisement calls of H. arborea, H. orientalis, H. molleri, H. intermedia and H. sarda (Fig. 1; e.g. Castellano et al., 2002; Schneider, 1974, 2000, 2004a, 2004b); (ii) morphological similarity of H. arborea, H. orientalis (Gvoždík et al., 2008; both as H. arborea), H. molleri (Terentjev, 1960) and largely also H. intermedia (Nascetti et al., 1995; Rosso et al., 2004; Terentjev, 1960); (iii) incongruence in phyleogeographic patterns between different nuclear genes across the Balkans and northern Italy (H. orientalis/H. arborea/H. intermedia), especially along the western Balkan coast (Gvoždík et al., 2007; distinct Balkan Adriatic lineage was detected also by Stöck et al. 2008 and Dufresnes et al., 2013). The Western Palearctic short-call tree frogs thus seem to represent an assemblage of morphologically and acoustically cryptic taxa, and the extent of gene flow between them needs to be properly evaluated.

Current advances in molecular phylogenetics have introduced new methods, including multilocus coalescent-based species tree inference (Knowles and Kubatko, 2010) and coalescent-based species delimitation, which permit probabilistic tests with an assumption of no recent admixture between species and of bipartitions of individuals in gene trees that are shared across loci (Yang and Rannala, 2010). In this study, we focus on the short-call tree frogs (H. arborea, H. orientalis, H. molleri, two lineages of H. intermedia, H. sarda) using new molecular markers and a multilocus coalescent-based species-tree approach and a Bayesian species delimitation

![Fig. 1. Historical overview of the taxonomy of the Western Palearctic tree frogs and assignment of the taxa into groups according to the advertisement-call properties. Red box highlights ingroup species considered in this study. Example oscillograms of a 600 ms long portion of the advertisement call on right. Numbers in circles stand for the number of species recognized in the corresponding time period. Color dots correspond to the colors in Figs. 2 and 3. For the status of H. heinztei see discussion in Werner (2010) and Stöck et al. (2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
method to test (1) the monophyly of H. arborea s.l. (H. arborea, H. orientalis, H. molleri); (2) sister-clade relationship of the geographically disjunctive H. orientalis and H. molleri; and (3) species-level distinction of the taxa, including of the northern (N) and southern (S) lineages of H. intermedia, and of the western (W) Balkan H. arbo-rea/H. orientalis populations.

2. Materials and methods

2.1. Taxon sampling and laboratory procedures

We obtained sequence data for altogether 169 specimens from 95 localities distributed throughout the geographic range of H. arborea s.l., including one specimen from GenBank (DQ05835, loc. 36; Smith et al., 2005), 19 specimens from 8 localities of H. intermedia and 10 specimens from 6 localities of H. sarda, which totaled 198 ingroup samples from 109 localities (Fig. 2, Table S1). Due to the lack of reliable diagnostic morphological characters, the material of H. arborea s.l. could not be a priori sorted into the putative species, i.e. H. arborea s.s., H. orientalis and H. molleri (sensu Stöck et al., 2008). The H. orientalis material from Asia Minor and Caucasus-Caspian region used in a previous study (Gvozdiˇk et al., 2010) was reanalyzed in the context of the new data from the European part of the range. The outgroup species H. meridionalis data were taken from the previous study (Gvozdiˇk et al., 2010) and those for a second outgroup, H. japonica, were retrieved from GenBank (AY843633, AY844615, AY844078; Faivovich et al., 2005). The outgroup status of these two species relative to the short-call tree frogs was demonstrated by, e.g. Hua et al. (2009).

Genomic DNA was extracted from tissue samples using commercial kits following the manufacturers’ protocols. Two fragments of mitochondrial DNA (mtDNA), 12S rRNA (12S, 355 aligned bp) and 16S rRNA (16S, 546 aligned bp), and two nuclear genes (nDNA), tyrosinase precursor exon 1 (Tyr, 496 bp), and rhodopsin exon 1 (Rhod, 276 bp) were targeted. The nDNA markers were sequenced for a subset of samples (ca. 43%) representing all main mitochondrial groups and covering the entire geographic range (Table S1). Primers and PCR protocols followed the previous study (Gvozdiˇk et al., 2010). PCR amplicons were purified and directly sequenced with the PCR primers using a commercial sequencing service (Macrogen Inc.).

2.2. DNA alignment and phasing of autosomal genotypes

Alignments of nucleotide sequences were prepared with ClustalW (Thompson et al., 1994) as implemented in BioEdit 7.2 (Hall, 1999). Mitochondrial 12S and 16S fragments were concatenate-nated. Heterozygous sites in nDNA were identified according to electropherograms when two different nucleotides were present at the same position with the lower peak reaching at least a half of the higher peak. Genomic haplotype networks were inferred by the coalescent-based Bayesian algorithm of Phase 2.1 (Stephens et al., 2001; Stephens and Scheet, 2005) as implemented in DnaSP 5.10 (Librado and Rozas, 2009). The analyses were run without out-group species and were repeated five times for each nuclear-gene dataset with different seeds for the random number generator to check if the phase estimates are consistent across 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. Results with a probability > 0.70 were accepted. Two samples (H. intermedia HD03A, Tyr; H. arborea HJ55, Rhod) contained heterozygous positions whose phases were not resolved (mean probability 0.50), and one sample (H. intermedia HD04B, Tyr) had one position weakly resolved (mean probability 0.69). In these cases, both possible gametic phases and their phylogenetic positions were considered in haplotype networks, which were constructed using the parsimony approach under the 95% parsimony limit as implemented in TCS 1.21 (Clement et al., 2000). The alternative gametic phases did not change the networks substantially (see haplotypes Rrb3, Tint7, Tint8, Tint9, Tint10 in Fig. 2). No stop codons were detected in the haplotypes as checked by translation with the standard genetic code using BioEdit 7.2 (Hall, 1999). All new unique mitochondrial haplotypes and new nuclear alleles or nucleotide sequences with the standard IUPAC ambiguity codes, if heterogeneous positions remained unresolved, have been deposited in GenBank (KP109551–KP109674; Table S2). Genetic distances were calculated from 16S haplotypes (a marker commonly used in amphibian DNA barcoding; Vences et al., 2012) and estimated as p-distances averaged between and within taxa in MEGA 6.0 (Tamura et al., 2013). Incomplete mtDNA sequences covering only the 12S fragment (from localities 36 and 50) were used in the mitochondrial gene tree only.

2.3. Gene trees

For gene-tree estimation, mtDNA and phased Tyr and Rhod sequences were sorted into distinct haplotypes using Collapse 1.2 (Posada, 2006). Gene trees were reconstructed by Bayesian inference (BI) and maximum likelihood criterion (ML). For BI analyses performed by MrBayes v3.2.2 (Ronquist et al., 2012), best-fit partitioning schemes and nucleotide substitution models according to the Bayesian information criterion (BIC) were selected using PartitionFinder v1.1.0 (Lanfear et al., 2012): mtDNA, 12S and 16S treated as one partition (SYM+G); Tyr, 1st + 2nd/3rd codon position (K80+I/HK+Y+G); Rhod, 1st + 2nd/3rd codon position (JC/K80). Each dataset was analyzed with two runs of four Markov chains, which were run for 6 × 10^6 generations and samples saved every 100th generation. That the analyses reached stationarity was verified by examining the plots of log-likelihood scores using Tracer v1.5 [Rambaut and Drummond, 2009; all parameters had effective sample size (ESS) > 200], and the chain convergence was confirmed by the convergence diagnostics (average standard deviation of split frequencies, potential scale reduction factor) comparing the two simultaneous runs against each other. From the sampled trees, the first third was discarded as a burn-in and a 50% majority-rule consensus tree was produced from the remaining trees that were taken as representing the posterior distribution. For ML analyses performed by PhyML v3.0 (Guindon et al., 2010), best-fit models of sequence evolution were selected using jModelTest v2.1.4 (Darriba et al., 2012) according to the BIC: mtDNA, TIM2ef+I; Tyr, TrNef+I; Rhod, JC. The best option of a combination of the nearest neighbour interchange and the subtree pruning and regrafting algorithm of tree improvement and optimization of the topology and branch lengths were applied. The nodal support was assessed by 100 bootstrap pseudoreplicates. Clades supported with BI posterior probability (pp) values > 0.95 and ML bootstrap values > 70 were considered highly supported (Huelsenbeek and Rannala, 2004).

2.4. Data preparation for coalescent-based applications

Since the coalescent-based species-tree and species-delimitation analyses rely on, and are sensitive to, the assignment of each individual/allele to a taxonomic unit (Leaché et al., 2014; Olave et al., 2014), we carefully inspected distribution of haplotypes across different genes to identify specimens with inconsistent phylogenetic signal and thus identify potential hybrids (Table S1). Such potential hybrids or possible cases of allelic introgression (H. arbo-reus–orientalis; 10 individuals from the Balkans and Poland) were omitted from the species-tree analyses. Similarly, contrasting Tyr
alleles, such as in a sample from Brittany (loc. 4) or H. felixarabica-like alleles of six individuals of H. orientalis from south-western Turkey (haplotypes Tfel3, Tori5, Tori6, Tori7, Tori8; see Fig. 2 and Gvoždík et al., 2010), were also omitted from the analyses (see Section 3 for details). Remaining samples were allocated to the following taxonomic units according to their provenance and genetic identity: H. arborea s.s. (n = 64), H. orientalis (n = 74), H. moller (n = 14), H. intermedia South (n = 16), H. intermedia North (n = 3), and H. sarda (n = 10). Beside these 'standard' units (Stöck et al., 2012) we also tested a position of the western Balkan tree frogs (W Balkan H. arborea, loc. 33–35, 37, 38 in Slovenia, Croatia, Montenegro, Greece; n = 5), which carry inconsistent phylogenetic signal across the studied genes (Fig. 2), as noted earlier by Stöck et al. (2008). Coalescent-based model assumptions include an absence of recombination (Castillo-Ramírez et al., 2010; Lavretsky et al., 2014; but see Lanier and Knowles, 2012). Thus, the minimum numbers of recombination events (Hudson and Kaplan, 1985) in nuclear loci for different subsets of tree-frog species were estimated in DnaSP
5.10 (Librado and Rozas, 2009), with no recombination detected in Rhod, and a minimum of one (H. intermedia, H. sarda), seven (H. arborea s.l.) and ten (all ingroup taxa) recombination events detected in Tyr. Traces of recombination were removed from the Tyr dataset (not containing H. felixarabica-like alleles) using IMgc1 (Woerner et al., 2007), and a recombination-filtered dataset was produced by the approach of favoring the retention of individuals (α = 1.0; 423 bp and 86% samples kept; hereafter as TyrIMgc1 dataset; see also Table S1 and Fig. S1).

2.5. Species trees and divergence dating

A species tree was inferred using BEAST (Heled and Drummond, 2010) based on the dataset including all genes, mitochondrial 12S and 16S, the nuclear Rhod and the recombination-filtered Tyr (TyrIMgc1). Alignments of all individuals (without potential ‘hybrids’; see above) including one outgroup (H. meridionalis) for each gene were uploaded into BEAUti v1.8.0, a part of the BEAST package (Drummond et al., 2012), where they were assigned separate and unlinked substitution, clock and tree models. Hyla meridionalis was set as outgroup by enforcing monophyly of the remaining taxa. Best-fit partitioning schemes and sequence-evolution models were set according to the results from PartitionFinder v1.1.0 (Lanfear et al., 2012) following the BIC [mtDNA, 12S and 16S treated as one partition (HKY+G); TyrIMgc1 without partitions (K80); Rhod, 1st + 2nd/3rd codon position (K80/TrNef)]. Ploidy of mtDNA was set to mitochondrial (haploid), and the other two loci to autosomal nuclear (diploid). The Yule species-tree prior was set and UPGMA starting tree used for each gene and a strict molecular clock assumed. The clock rate for mtDNA was inferred from a calibration based on an assumption of H. sarda being a descendant of a radiation that had occurred during the Late Miocene (Stöck et al., 2012), particularly when the land bridge between the Sardo–Corsican block and continent was submerged at the end of the Mediterranean salinity crisis when the Mediterranean Basin was re-flooded at ca. 5.33 mya (Bisconti et al., 2011a; Hsü et al., 1973; Kriegsman et al., 1999; Rouchy and Caruso, 2006). Normal distribution prior was assigned with a mean of 5.33 (mya) and standard deviation of 0.16 in order to accommodate uncertainty in this estimate. Clock rates for Tyr and Rhod were estimated from mtDNA using 1/x prior distribution. Five independent BEAST runs were performed, each for 200 million generations, sampling every 20,000th generation to obtain a posterior sample of 10,000 trees. The likelihoods were inspected using Tracer v1.5 (Rambaut and Drummond, 2009) and convergence checked using ESS values (≥200) after discarding the 10% burn-in. The post-burn-in samples of the five runs were combined in the BEAST module LogCombiner v1.8.0. The output of 45,000 sampled trees was uploaded to another BEAST module, TreeAnnotator v1.8.0, to infer the final species tree as a maximum clade credibility tree ( MCC) with node ages represented by median heights and confidence intervals with 95% highest posterior densities (HPD). The set of all 45 thousand trees was superimposed and visualized as a ‘cladogram’ with DensiTree v2.01 (Bouckaert, 2010) to show the probability distribution of topologies, and the three most frequent ‘consensus trees’ (sensu Bouckaert, 2010) were inspected. To assess the influence of the mtDNA dataset on the species-tree topology, the nuclear dataset was also employed in a separate species-tree analysis with virtually the same settings but without molecular-clock calibration and run for 500 million generations.

2.6. Species delimitation

Coalescent-based Bayesian species delimitation (BSD) analyses were conducted in the program Bayesian Phylogeny and Phylogeography (BP&P v2.2; Rannala and Yang, 2003; Yang and Rannala, 2010) to test whether the operational taxonomic units fit the assumption of no post-divergence gene flow, which is inherent to the biological species concept (Yang and Rannala, 2010). This method accommodates the species phylogeny and accounts for lineage sorting due to the ancestral polymorphism using the reversible-jump Markov chain Monte Carlo (rjMCMC) algorithm that successively splits or joins nodes on the guide species tree, and employs population-size parameters θ and species divergence times τ in the coalescent model (Rannala and Yang, 2013; Yang and Rannala, 2010). Numerous possible species-tree topologies (guide trees) were tested using different datasets with individual alleles carefully assigned to putative species (see Olave et al., 2014); all markers (mtDNA and phased nDNA), nDNA only, Rhod only, and Tyr only. τ was used in both ways – including recombination and recombination-filtered (TyrIMgc1); all individuals (without H. felixarabica-like Tyr alleles), with or without ‘hybrids’, and considering W Balkan populations as a discrete unit or part of H. arbo-rea (Table S3). A gamma prior G(2,1000) was applied on the population-size parameters (θ). Also the age of the root in the species tree (τ0) was assigned the gamma prior G(2,1000), while the other divergence-time parameters were assigned the Dirichlet prior (Yang and Rannala, 2010). When both mtDNA and autosomal nuclear data were involved in an analysis, the parameters ‘heredity’ (allows θ to vary among loci) and ‘locusrate’ (allows variable mutation rates among loci) were estimated with a gamma G(4,4) and Dirichlet prior, respectively. The algorithm 0 was used with several values for the fine-tuning parameter τ to ensure that the rjMCMC mixed properly. BP&P analyses were run for 108 generations with first 50% of samples treated as a burn-in and discarded. If necessary, fine-tuning variables were adjusted to display proportion values close to 0.3–0.4 (within the interval 0.15–0.7) as recommended in the manual (Yang, 2013). Each analysis was run repeatedly to check consistency between runs. To control for a possible influence of the settings, the all-gene and nDNA MCC species-tree topologies were used as guide trees also for runs with alternative prior settings of θ and τ0 [G(1,10)/G(1,10); G(2,2000)/G(2,2000); G(1,10)/G(2,2000)], as recommended by Leaché and Fujita (2010), and runs employing the rjMCMC algorithm 1, with fine-tuning parameters τ = 2 and m = 1 (Yang, 2013).

3. Results

3.1. DNA polymorphism

The mitochondrial DNA dataset contained 198 ingroup sequences, which collapsed into 77 haplotypes (891 bp excluding insertion/deletion polymorphisms), contained the number of polymorphic sites S = 126 (89 parsimony informative), and showed the haplotype diversity h = 0.922 ± 0.014 (SD) and nucleotide diversity π = 1.91 ± 0.09%. The two nuclear genes were considerably less variable. Phased ingroup Tyr dataset (166 sequences, 496 bp) contained 47 haplotypes, with S = 38 (34), h = 0.900 ± 0.016, and π = 0.90 ± 0.04%, while the H. felixarabica-like recombination-filtered TyrIMgc1 (133 sequences, 423 bp) contained 22 haplotypes, with S = 26 (23), h = 0.820 ± 0.025, and π = 0.88 ± 0.05%. Gametic-phased ingroup Rhod dataset (174 sequences, 276 bp) contained 15 haplotypes, with S = 14 (7), h = 0.779 ± 0.022, and π = 0.60 ± 0.03%. The mitochondrial marker has a roughly two times higher polymorphism than Tyr and three times higher than Rhod.

3.2. Gene trees

The mitochondrial DNA phylogeny had an almost identical topology based on the BI and ML approaches (Fig. 3). Most of the
main clades are highly supported with \( H. \ sarda \) in a sister-clade position to all other taxa (Bayesian pp 1.00/ML bootstrap 63). These are grouped into two clades corresponding to \( H. \ intermedia \) and \( H. \ arborea \) s.l. The latter is highly supported (1.00/75), while the support for a clade containing two highly divergent lineages of \( H. \ intermedia \) (Northern/Southern) is weaker (0.94/56), reflecting

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**Fig. 3.** Haplotype gene trees of the mitochondrial DNA fragment (12S/16S) and nuclear \( Tyr \) and \( Rhod \) as represented by maximum-likelihood trees. Numbers along branches correspond to ML bootstrap support values and Bayesian posterior probabilities if higher than 50/0.50. Colors are in accordance to Fig. 2. Boxed haplotypes in \( H. \ arborea \) s.s. are present in individuals constituting the W Balkan operational unit as defined based on the incongruence in phylogenetic signal between the two studied nuclear markers (see also Fig. 2 and Table S1). Taxa of special interest due to their dual placements in nuclear phylogenies are highlighted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the deep mitochondrial differentiation of the two geographically close lineages. Despite the geographically remote distributions, the Iberian *H. molleri* and Eastern European/Western Asian *H. orientalis* together form a highly supported clade (1.00/88), while the support for each of the two taxa is rather low (*H. molleri* 0.85/60; *H. orientalis* 0.94/68). *Hyla arborea* s.s. is well differentiated (0.97/78) and is supported as the sister clade to *H. molleri + H. orientalis* (1.00/75). Mitochondrial DNA (16S) genetic distances among taxa are given in Table 1.

The two nuclear-gene phylogenies (Fig. 3) have generally low nodal support likely caused by the relatively low variation. However, both nuclear markers, *Tyr* and *Rhod*, possess variation sufficient to show clear structure (Figs. 2 and 3). The *Tyr* phylogeny differs from mtDNA in terms of the detection of major lineages/species in three main aspects: (i) *H. arborea* s.s. forms two main clusters. One contains predominantly samples from the western Balkans, while the second the remaining samples. (ii) *H. orientalis* also forms two main groups, which do not form a clade. One accommodates most samples, while the other is restricted only to south-western Asia Minor. In the latter group the most common haplotype of *H. felixarabica* (*Tf3*) was recorded (plus four haplotypes closely related to it), as documented earlier (Gvoždík et al., 2010). (iii) *H. intermedia*, Northern lineage is not clearly detectable and most alleles are in the Southern lineage. In the *Rhod* haplotype phylogenetic relationships, the most striking difference from the mtDNA phylogeny is the position of W Balkan *H. arborea* samples, which do not carry ‘typical’ *H. arborea Rhod* haplotypes, but have the most-common *H. orientalis* haplotype (*Rori1*) or a haplotype derived from it (*Rori6*) (Fig. 3). Similarly, the majority of samples (5/6) of the Northern lineage *H. intermedia* carries the most common *H. orientalis* haplotype (Fig. 3).

3.3. Geographic distribution of haplotypes and hybrid location

Intraspecific phylogeographic relationships based on mitochondrial DNA are presented in Fig. 4. The south-western endemics, *H. sarda* and *H. molleri* do not demonstrate clear phylogeographic pattern, with an exception of the presence of one relatively distant haplotype of *H. molleri* in Galicia. The Southern lineage of the Italian endemic *H. intermedia* forms two subgroups, southernmost and central. The most common *H. arborea* s.s. haplotype and its single-mutational (or indel) derivatives, arranged in a star-like pattern, have been recorded from over much of Europe, from Greece in the south to Denmark in the north (Fig. 4). On the contrary, in the southern Balkans a more diversified haplotype group is present, predominantly recorded from Greece but found also in northern Croatia and the Czech Republic. One relatively distant haplotype was detected on Pag, a Croatian island in the northern Adriatic Sea. The highest diversity was found in the eastern taxon, *H. orientalis*. Two main groups (weakly supported clades) are represented by the Caucasus-Caspian populations and by all the other populations, respectively. Besides the Caucasus-Caspian group, further structuring in the second group is also detectable. Most diversified is the Asia Minor population with haplotypes present in several different subgroups, but with less clear geographic structuring, which is true also for the remaining Eastern European populations, which have clear affinities to the north-western Asia Minor population.

For the nuclear *Tyr* and *Rhod* markers, the distribution of haplotypes generally agrees with the pattern in mtDNA, with the exception of the cases described above (Section 3.2). However, there have been also several striking findings at particular locations. Based on the combination of alleles across different loci, potential hybrids were detected in Brittany (*H. arborea × H. molleri*), Poland, and across the Balkans in Greece, Bulgaria and Romania (*H. arborea × H. orientalis*) (Fig. 2 and Table S1).

3.4. Species trees and divergence time estimate

The maximum clade credibility species tree based on the mitochondrial and nuclear loci inferred the same topology as the mtDNA gene tree: *H. sarda* being the sister lineage to all other short-call tree frogs, and *H. intermedia* being the sister lineage to *H. arborea* s.l., and with *H. orientalis* forming a clade with *H. molleri* and *H. arborea* s.s. closely related to the W Balkan *H. arborea* group (Fig. 5). However, high support is present only for the crown sister-clade relationships (*H. intermedia I – H. intermedia S; *H. orientalis – H. molleri; H. arborea – W Balkan*). Three most frequent topologies occurred with the posterior frequency of 38.7%, 33.2%, 11.6% – first with the same topology as the MCCT, second differing by the presence of a clade *H. intermedia (N+S) + H. sarda* in the sister-clade position to *H. arborea* s.l., and third interchangeing positions of *H. intermedia (N+S)* and *H. sarda* in comparison to the MCC. All the three topologies, accounting for almost 84% posterior trees, had the *H. arborea* s.l. taxa as a clade. On the contrary, if we consider only the two nuclear loci the MCCT (Fig. S2) as well as the three most frequent topologies (22.6%, 15.1%, 13.8%) form two main clades: (i) *H. arborea* s.s. (*W Balkan*) + *H. sarda*; (ii) all other taxa, i.e. *H. intermedia (N+S), H. molleri, H. orientalis*. The molecular clock based on all loci estimated that the main radiation within the short-call tree frogs occurred in the Pliocene (considering 95% highest posterior densities, HPD, 5.4–1.9 mya), with only the most recent splits occurring during the Pleistocene. The split between *H. orientalis* and *H. molleri* is dated close to that between the Southern and Northern *H. intermedia*, ca. 1.4 mya (HPD 2.8–0.4), while the structuring within *H. arborea* with respect to the W Balkan population dates to the Middle to Early Pleistocene ca. 200 kya (HPD 370–70 kya).

3.5. Species delimitation

Bayesian species delimitation supported all the taxa as species without a history of gene flow (speciation probability 1.00; Fig. 5), assuming various guide tree topologies, datasets, and con-

<table>
<thead>
<tr>
<th></th>
<th>H. arborea</th>
<th>H. orientalis</th>
<th>H. molleri</th>
<th>H. intermedia S</th>
<th>H. intermedia N</th>
<th>H. sarda</th>
<th>H. meridionalis</th>
<th>H. japonica</th>
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<td>5.8</td>
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</table>

Table 1
Genetic distances (uncorrected p; expressed as percentages) averaged among and within taxa based on the fragment of mitochondrial 16S rRNA gene.
ditions with respect to inclusion of potential ‘hybrids’, recombination in Tyr, or considering W Balkan populations as a discrete unit or as a part of H. arborea (see overview of results in Table S3). The alternative prior settings and type of rjMCMC algorithm also did not affect the results of high speciation probability for any node. Only when the Tyr dataset was evaluated independently was the
speciation probability always low (0.08–0.60) for the Northern and Southern lineage of *H. intermedia*, both for the dataset containing recombination and the recombination-filtered dataset. There was also low speciation probability for the node *H. arborea* – W Balkan in *Tyr* (0.32), but the probability increased substantially when recombination was filtered out (0.97). Interestingly, when the node W Balkan – *H. orientalis* was tested in *Rhod* where there is a high level of haplotype sharing (*R* ori; frequency 4/10 in W Balkan), the speciation probability was also high (0.99). This was likely a consequence of the *R* ori6 haplotype (frequency 5/10 in W Balkan) being common in the northern Adriatic.

4. Discussion

4.1. Species tree

The short-call tree frogs are phenotypically very uniform (*Grosse, 2009; Gvoždík, 2009; Gvoždík et al., 2008; Kaya, 2001; Pisanets and Matvye耶v, 2012; Rosso et al., 2004). *Hyla sarda* is the only species that is substantially differentiated in morphology, and it was the first taxon to be distinguished from *H. arborea* at the species level (*Lanza, 1983; Nascetti et al., 1985*), followed by *H. intermedia*, which was elevated to the species rank some ten years later (*Nascetti et al., 1995*). All the remaining taxa have been included under *H. arborea* until recently (*e.g. Grosse, 2009; Schneider and Grosse, 2009; Sillero et al., 2014; Speybroeck et al., 2010*). *Stöck et al. (2008, 2012)* found *H. arborea* s.l. paraphyletic with respect to *H. intermedia* (*Stöck et al., 2012* or to both *H. intermedia* and *H. sarda* (*Stöck et al., 2008*). However, the previous studies did not obtain high support for the inferred topologies except for the sister-clade relationship of the two *H. intermedia* lineages (Northern and Southern), and of *H. orientalis* and *H. molleri*. By contrast, in our analyses, *H. arborea* s.l. was highly supported in the mtDNA phylogeny (Fig. 3) and its monophyly was confirmed by the species tree analysis (Fig. 5) by the presence of the *H. arborea* s.l. clade in the three most common all-gene species-tree topologies and the overall frequency of 83.8% among the posterior trees.
This discrepancy in mtDNA phylogenies between the studies might be a result of different markers utilized by Stöck et al. (2008, 2012). On the other hand, our nuclear-only species tree does not correspond to the all-gene topology in supporting a sister-clade relationship between H. arborea and H. sarda (64.5% of the posterior trees), which has not been documented in any of the previously published phylogenies (Hua et al., 2009; Pyron and Wiens, 2011; Stöck et al., 2008, 2012). The H. arborea s.l. clade was present in the nDNA species tree with very low frequency of only 1.1%.

4.2. Mitochondrial DNA capture and autosomal gene introgression

We further assessed the striking sister-clade relationship in mtDNA of the geographically remote, eastern H. orientalis and the western/Iberian H. mulleri, which are currently separated by more than 1500 km, while their genetic distance (165) is only 0.9%. This clade was strongly supported in the all-gene species tree (Fig. 5a), including its presence in the three most common topologies from the posterior trees (Fig. 5b, left). The clade was present in 99.9% of the posterior trees. On the contrary, in the nuclear-only species tree (Fig. 5b, right), the sister-clade relationship of H. orientalis + H. mulleri was present only in one of the three most common topologies, with the overall frequency of only 23.2% in the posterior sample. In the nuclear-only species tree there was a clear tendency for a clade to show up that included the two taxa together with H. intermedia (59.3%), but besides the highly supported subclade H. intermedia N + H. intermedia S (99.8%), the other relationships within the clade were distributed among the posterior trees with relatively equal and lower frequencies. Thus, it seems that there are two plausible explanations for the observed pattern: (1) H. orientalis – H. mulleri split is relatively young (1.4 mya dated in our study), of the age when a substantial thermal drop is evidenced in the Mediterranean (Colleoni et al., 2012; Fig. 5), probably causing range restrictions and isolation of the two lineages. Or alternatively, and perhaps more likely, assuming the nDNA species tree is correct, then (2) H. orientalis – H. mulleri split occurred much earlier, and a mitochondrial introgression and capture (probably from an ancestral population of H. orientalis to H. mulleri) could have happened during a secondary contact in a climatically suitable/warm period of the Early Pleistocene, which allowed range extensions. This or similar event(s) could also be responsible for Rhod introgression from H. orientalis to W Balkan H. arborea and to the Northern lineage of H. intermedia (see also Verardi et al., 2009), for Tyr introgression from H. felixarabica to southern Asia Minor population of H. orientalis, and probably also for the genetic constitution of the Breton tree frogs (loc. 4), where H. mulleri alleles appear to have introgressed into H. arborea.

4.3. Species delimitation

Coalescent-based BSD supported species-level distinction of all taxa, including the W Balkan operational unit (Fig. 5), for a variety of guide trees and datasets (Table S3). Only Tyr does not present signatures of completed speciation within H. intermedia, implying the presence of some gene flow (detected previously by Canestrelli et al., 2007b). These results suggest that the two taxonomic units within H. intermedia should be treated as conspecific, despite the relatively high mtDNA distance (2.9% in 165). The H. intermedia N + H. intermedia S clade was present in 99.9% and 99.8% posterior species trees (all-gene and nDNA only, respectively). Similarly, signatures of speciation were not found in Tyr for the split between H. arborea and W Balkan (the clade was present in the posterior species-tree sample of the all-gene and nDNA approach with frequencies of 100% and 99.8%, respectively). Nonetheless, when recombination is removed from the Tyr dataset, the speciation probability becomes significant in the latter case, suggesting that the recombined alleles carry the signal of gene exchange among H. arborea populations. In addition, when recombination is filtered out the cluster of alleles originated from the western Balkans (not directly corresponding to the W Balkan group; Fig. 2) is no more obvious (TyrTmc1 dataset; Fig. 51). Gene flow followed by recombination between H. arborea and H. orientalis alleles thus probably account for the presence of the W Balkans allele cluster in the non-filtered Tyr data (Fig. 2), despite the fact that BSD analysis of the filtered data suggests species-level distinction of the W Balkan tree frogs. The lack of monophyly and of private alleles, together with the cryptic phenotype (Gvozˇdík et al., 2008), support retaining the W Balkans populations in H. arborea.

4.4. New insights into the phylogeographic history

The intraspecific genetic structuring was largely formed during the climatic oscillations in the Middle and Late Pleistocene as evidenced in the case of H. arborea–W Balkan split (Fig. 5), which corresponds well to the conclusion of Dufresnes et al. (2013). The phylogeographic patterns of mtDNA (which shows higher diversity and thus geographic resolution than nDNA; Fig. 4) recovered in our study extend the previous findings in several ways, but we provide several important novel insights.

4.4.1. Hyla arborea sensu stricto

We found three main haplogroups, similar to the study of Dufresnes et al. (2013). One of them is found in the northern Adriatic (W Balkans) and we detected it on Pag Island, located between Istria and the Croatian coast where it was found previously (Dufresnes et al., 2013). This highlights the Adriatic region as a potential glacial refugium for H. arborea s.s. Another refugium was probably located in the southern Balkans, which is predominantly occupied by the second haplogroup harboring a relatively high genetic variation. One haplotype of this south-Balkan haplogroup was found at some northerly-located places like the northeastern inland Croatia (see also Dufresnes et al., 2013) and surprisingly also as far north as the Czech Republic. This distribution suggests multiple routes of northward expansion from the Balkan Peninsula. The range of the third, most widespread haplogroup is centered in central and north-western Europe, but it partly overlaps with the other two haplogroups. Its star-like pattern suggests a recent expansion (see also Dufresnes et al., 2013).

4.4.2. Hyla orientalis

For this species we uncovered a somewhat different pattern compared to Stöck et al. (2012). First of all, the Caucasus-Caspian population forms only a single haplogroup, distributed from the western forelands of the Caucasus in the Krasnodar region to the south-eastern Caspian coast in Iran. The most common haplotype is widespread throughout the area, including regions of the type locality of H. arborea schelkownikowi and H. a. gumilinskii, which supports their status as synonyms, currently under the name H. orientalis (Gvozˇdík et al., 2010; Stöck et al., 2008). On the contrary, Stöck et al. (2012) found a population from the Talysh region in south-eastern Azerbaijan as a distinct clade in a sister-clade position to the Caucasian population. This discordance might be explained by differences in the level of variation in the mtDNA markers used in our study and that of Stöck et al. (2012). It might also be due to the fact that the Caspian population of Stöck et al. (2012) was represented solely by samples originating from a relatively small area of the Talysh region, while Iranian samples were missing from their study. Interestingly, despite the distribution gap in the steppes of northern Crimea, the southern Crimean population of H. orientalis is a part of the predominantly Eastern-European haplogroup, not of the geographically close Caucasian group. This
Eastern-European haplogroup is distributed to the north and east of the Carpathians from Poland, Ukraine, Romania and southwards to Bulgaria and Turkey, and it is characterized by a complex genetic variation. This includes further sub-structuring, which is well evident in Asia Minor.

4.4.3. Hyla mulleri, H. intermedia and H. sarda

Our sampling of the Iberian, Italian and Tyrrenian tree frogs was not designed as a detailed phylogeographic study, which was performed in those regions earlier (Barth et al., 2011; Bisconti et al., 2011a, b; Canestrelli et al., 2007a, b). However, we found a distinct haplotype of H. mulleri in Galicia, suggesting that this region could play a role as a glacial refugium, while the rest of the range of this species is inhabited by a widespread haplogroup (Barth et al., 2011). The Southern H. intermedia was split into two haplogroups, corresponding to the southernmost (Calabria, Sicily) and central part of the Italian Peninsula, highlighting these regions as presumed glacial refugia (Canestrelli et al., 2007). The Northern H. intermedia is highly diverged from the Southern lineage in mtDNA (2.9% in 16S), but its nuclear genes bear signatures of gene flow to/from the Southern H. intermedia (Tyr; see also Canestrelli et al., 2007b) and H. arborea/H. orientalis (Rhod; see also Verardi et al., 2009). Our limited sampling of H. sarda did not recover a clear phylogeographic pattern, which was addressed in detail elsewhere (Bisconti et al., 2011a, b).

4.5. Hybridization

A case of hybridization between H. orientalis and H. savignyi in southern Asia Minor was mentioned by Stöck et al. (2012). However, that might have been a misinterpretation of the signatures of introgression, not from H. savignyi but from the allopatric H. felixarabica. Such introgression characterizes the population of H. orientalis in south-western Asia Minor, as was discussed above and briefly mentioned by Gvoždík et al. (2010). The nearest localities of the Arabian tree frog, H. felixarabica, are known from southern Syria, about 800 km away (Gvoždík et al., 2010), but the species likely had a secondary contact with H. orientalis during Pleistocene periods favorable to range extension (Gvoždík et al., 2010). To date, there is no unequivocal evidence for ongoing hybridization between the parapatric short-call H. orientalis and the medium-call H. savignyi (cf. Gül et al., 2012; Gvoždík et al., 2010). However, the present study found hybrids in two different pairs of short-call species, i.e. H. orientalis × H. arborea s.s. in Greece, Bulgaria, Romania and Poland, and H. arborea s.s. × H. mulleri in Britain, France (see Table S1). It is not trivial to distinguish introgression from retention of ancestral polymorphism due to incomplete lineage sorting. However, clear hybrids, including putative F1 hybrid, were found in southern Poland near Krakow (loc. 48; Fig. 2 and Table S1). This is in line with Stöck et al. (2012) who found hybrids in northern Poland and postulated a hypothesis that the geographic border between H. arborea s.s. and H. orientalis approximately follows the course of the Vistula River.

5. Conclusion

We conclude that the phylogenetic signal of mtDNA had strong influence on the reconstructed species tree of the short-call tree frogs, to some extent overwhelming the signal of the nuclear genes. However, the results strongly suggest that the western Balkan H. arborea does not represent a distinct species, as do neither Northern nor Southern lineage of H. intermedia, which should be treated as conspecific despite their mtDNA divergence. However, all other taxa should be considered species as any gene flow was detected between them by coalescent, regardless of some phylogene-


