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Mitochondrial uniformity in populations of the treefrog *Hyla molleri* across the Iberian Peninsula

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Abstract. Based on DNA sequences of fragments of the mitochondrial COI and 16S rRNA genes we provide a first assessment of genetic variability of Iberian treefrog populations that have been historically allocated to *Hyla arborea*, partly as subspecies *H. a. molleri.* Our data from 147 specimens and 33 populations confirm strong divergence between these frogs and Central European *H. arborea* but relatively low differentiation across their range, supporting their status as a separate species, *H. molleri.* Preliminary phylogeographic data indicate a possible weak genetic differentiation of populations from the northern coast of the region of Galicia. We suggest inclusion of nuclear markers and an extension of the sampling into the coastal regions of Asturias and Cantabria, as well as the identification of the contact zone between *H. molleri* and *H. arborea* in either the Spanish Basque country or in France, as priorities for future research on this species.

Keywords: Amphibia, Anura, cryptic species, Galicia, *Hyla arborea*, *Hyla molleri*, Hylidae, phylogeography, Spain.

Treefrogs of the family Hylidae are part of the Nobleobatrachia, a major clade of neobatrachian frogs with a clear center of origin and diversity in the Neotropics (Frost et al., 2006). Only a few hylids have succeeded in dispersing to the Palearctic, and a single subclade of the genus *Hyla*, the *Hyla arborea* group, has reached Europe (Hua et al., 2009). Compared to the impressive diversity of hylids in Central and South America, the Palearctic *Hyla* are characterized by high morphological and ecological similarity. Species delimitation until recently was based on coloration and bioacoustics, with some species additionally distinguished on the basis of their genetic differentiation. Recently, Stöck et al. (2008) and Gvoždík et al. (2010) have provided molecular data that challenged the classical taxonomy of treefrogs in the western Palearctic and led to the revalidation or description of several additional species. Following these authors, at present the following *Hyla* are recognized from this area: *Hyla arborea*, *H. felixarabica*, *H. intermedia*, *H. meridionalis*, *H. molleri*, *H. orientalis*, *H. sarda*, and *H. savygni*.

Despite these recent studies, taxonomic and distributional knowledge for several of these species is still incomplete (Speybroeck et al., 2010). This is particularly true for *H. molleri* from Iberia. In Spain and Portugal two *Hyla* species, clearly divergent in advertisement call and color pattern, and with strong divergences in nuclear and mitochondrial DNA sequences, are known to occur. *Hyla meridionalis* is found mainly along the Mediterranean coast and in the southern half of the Iberian Peninsula (Recuero et al., 2007) whereas the second species is distributed in its northern and central part and has traditionally been assigned to *H. arborea* (Márquez, 2002; Stöck et al., 2008), sometimes as subspecies *H. arborea molleri* (Rosa & Oliveira, 1994; Márquez, 2002; Recuero et al., 2007; Oliveira & Par-

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gana, 2008; Stöck et al., 2008). This latter taxon has been described by Bedriaga (1890) from Coimbra in northern Portugal, but no convincing and constant differences to *H. a. arborea* have been identified since. According to Bedriaga (1890), *H. a. molleri* is characterized by smaller body size and longer hind limbs than *H. a. arborea*, but Boulenger (1898) did not find sufficient morphological characters to distinguish *H. a. molleri* from typical *H. a. arborea.* Similarly, Schneider et al. (1974) could not detect any advertisement call differences between Iberian and Central European *Hyla* populations and concluded that they are conspecific. Typically it has been assumed (e.g., Rosa & Oliveira, 1994; Márquez, 2002; Oliveira & Pargana, 2008) that the subspecies *molleri* inhabits north-western Spain and northern Portugal while the populations of central Spain are assigned to *H. a. arborea.* Molecular data are equally scarce. Stöck et al. (2008) included only two samples of this northern-central Iberian form, one from Spain and one from Portugal. Based on very high genetic differences to *H. arborea* in mitochondrial and nuclear genes these authors concluded they should be separated at the species level, and resurrected the nomen *molleri* as full species, *Hyla molleri.* The species status of this taxon was further supported by its phylogenetic position based on mitochondrial DNA, sister to *H. orientalis* rather than the geographically adjacent *H. arborea.* The extent of the distribution of *H. molleri* and its intraspecific genetic structure remained unknown (Speybroeck et al., 2010). Stöck et al. (2011) have recently broadened their mtDNA sampling to include 20 samples from 16 localities from Spain and Portugal, all of which had very similar DNA sequences for cytochrome *b*. Here we provide an extended assessment of molecular variation of populations from most of the presumed range of *Hyla molleri* based on sequences of two mitochondrial genes. Our sampling is particularly intensive in the northwestern region of Galicia which is characterized by a bioclimatic transition between dryer, Mediterranean conditions in its southern part and very humid conditions in the north. We further test whether this ecotone may coincide with phylogeographic breaks in *H. molleri.*

DNA was obtained from tissue samples of tadpoles collected in the field (taking care to collect tadpoles from different ponds, or from different parts of large ponds, in order to avoid including siblings). In addition, buccal swabs were taken from encountered adult specimens. Our sampling was complemented with samples available from the tissue collection of the Museo Nacional de Ciencias Naturales in Madrid. Altogether, 147 samples of *Hyla molleri* from 33 localities in Portugal and Spain (fig. 1) and three outgroup samples of *Hyla arborea* from Germany and *Hyla savignyi* from Turkey were used. We extracted total genomic DNA using a salt extraction protocol (Bruford et al., 1992) and amplified fragments of 537 bp of mitochondrial 16S and 539 bp of cytochrome oxidase subunit I gene via polymerase chain reaction using primers COIVertF1 (TTC TCA ACC AAC CAC AAA GAC ATT GG) and COIVertR1 (TAG ACT TCT GGG TGG CCA AAG AAT CA) (courtesy of P. Hebert) and 16SIschF1 (CTY GTA CCT TTY GCA TCA TGR TTT A) and 16SIschR1 (CCT GAT CCA ACA TCG AGG TCG T) (newly designed; courtesy of M. Gehara). The thermocycling profile for COI comprised an initial denaturation at 94°C for 2:20 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (45-49°C for 45 s) and elongation (72°C for 1:30 s), and a final elongation step at 72°C for 10 min. The profile for 16S comprised an initial denaturation at 94°C for 1:30 min, followed by 35 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s) and elongation (72°C for 1:30 s). The final elongation step was the same as for COI. PCR products were run on an ABI 3130 automated sequencer. Sequences were checked for errors and aligned using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). All newly determined sequences were deposited in Genbank (accession numbers JN800770-JN801026).

Neighbor-Joining trees for preliminary analysis were generated using MEGA 4.0.2 software (Tamura et al., 2007). Genealogical relationships among sequences were estimated in the software TCS 1.21 (Clement et al., 2000) by calculating haplotype networks according to the method of Templeton et al. (1992). Alignment gaps in the 16S fragment were included in the analysis because they could be unambiguously scored from the chromatograms. We checked for evidence of isolation by distance (IBD) in a subset of the data by including only populations for which at least 5 individuals were sampled. This limited the IBD analysis to 11 populations (fig. 2), ten of which were from Galicia, the other from central Spain (population 25). We performed two IBD analyses, one for only the Galician samples, and another incorporating all 11 populations (including the geographical outlier, population 25). The geographic distances among these collecting localities were calculated using Geographic Distance Matrix Generation version 1.2.3 (Ersts, 2011). The geographical distance matrix was then log transformed. To test for IBD we performed

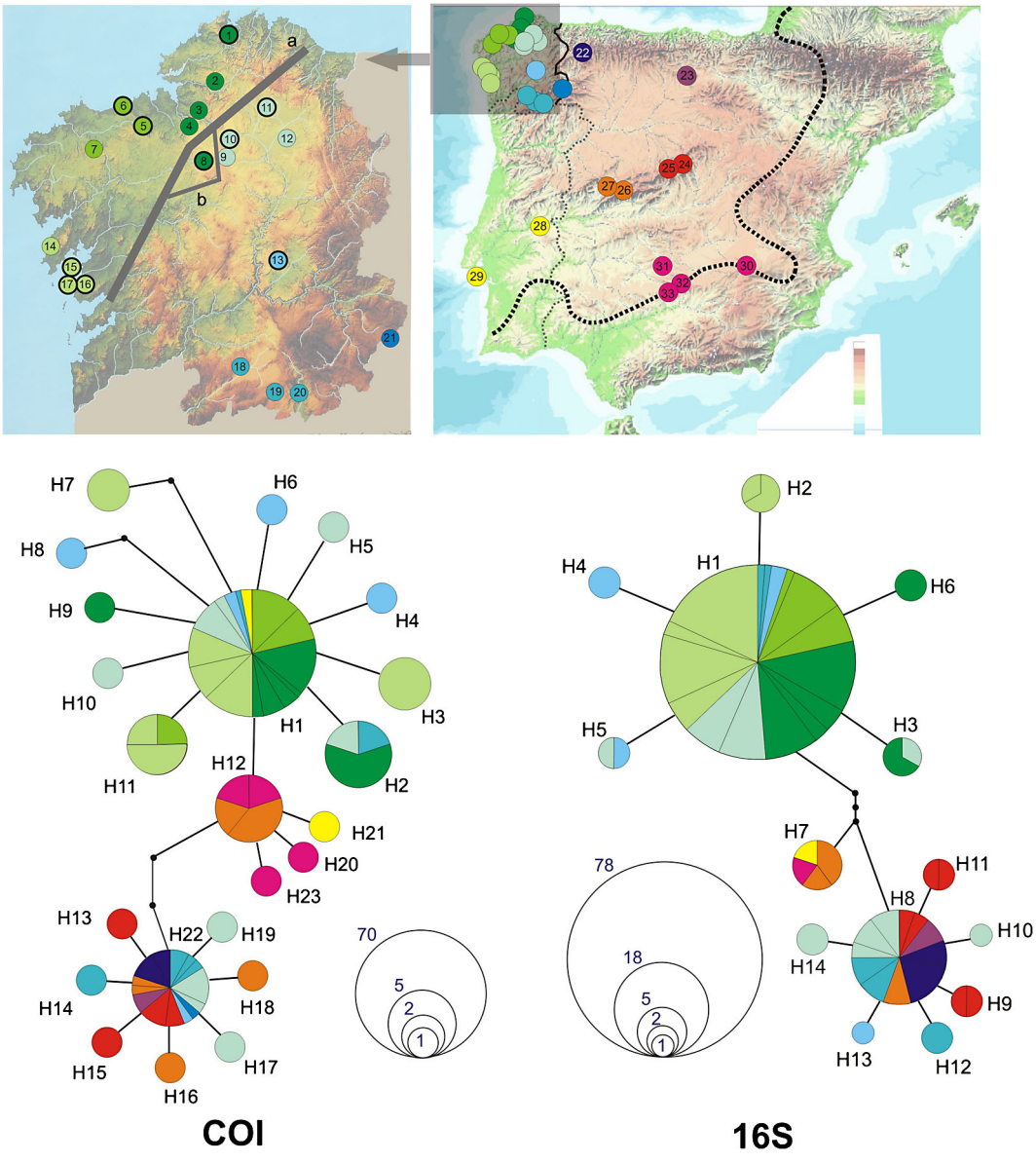

Figure 1. Map of Galicia, northwestern Spain, and of the Iberian Peninsula, with sampling localities (numbered as in table 1). Haplotype networks with independently numbered haplotypes of *Hyla molleri* based on sequences of the COI and 16S gene, respectively. Colors in haplotypes correspond to those of the localities on the maps. For a list of haplotypes in each population, see table 1. Size of circles in the haplotype network is proportional to the number of individuals sharing a given haplotype. The dashed line indicates the approximate southern and eastern border of the known distribution of the species. Grey lines in the Galicia map indicate the first two barriers to gene flow (marked a and b) as reconstructed by Barrier software between 10 Galician populations for which sufficient samples were available (marked by a stronger outline of dots): the primary barrier a separates populations 1, 5, 6, 15, 16, and 17 from populations 8, 10, 11, and 13; the secondary barrier b separates population 8 from 10, 11, and 13. This figure is published in colour in the online version.

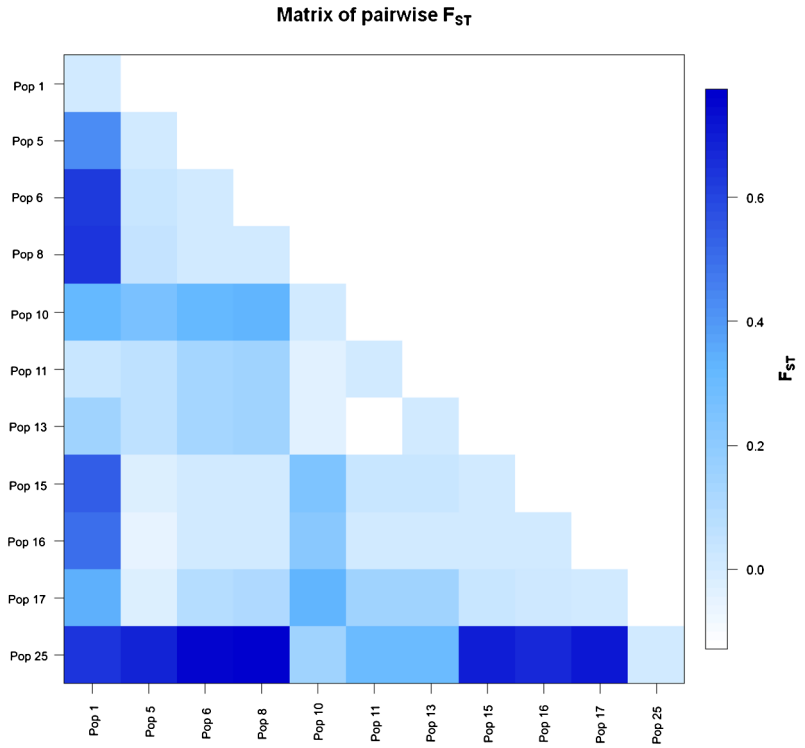

Figure 2. Matrix of linearized *F*st values (Reynolds et al., 1983) computed in an R extension for Arlequin (Excoffier and Lischer, 2010) for 10 populations of *H. molleri* from Galicia and one (Pop 25) from central Spain (Madrid area). Based on variation in a 539 bp fragment of the mitochondrial CO1 gene. Negative *F*st values should be interpreted as zero. This figure is published in colour in the online version.

a Matrix Correlation Analysis (Mantel Test) in the software Arlequin ver. 3.5 (Excoffier and Lischer, 2010) using pairwise *F*st values as proxies for genetic distances (DNA distance method: pairwise differences among sequences). *F*st values were linearized using Reynolds transformation (Reynolds et al., 1983) for short divergence time; 1000 permutations were used for significance testing. We also performed an AMOVA analysis with among population and within population variance components, with significance of the components tested by 1000 permutations. The locations of possible barriers for gene flow were analyzed with the program Barrier, version 2.2 (Manni et al., 2004). This method uses Monmonier's (1973) maximum difference algorithm on a Delaunay triangulation to identify zones of change in the genetic landscape, based on *F*st values as specified above.

Because quality sequences of either the COI or the 16S fragment could not be obtained for some specimens, we refrained from concatenating these two mitochondrial fragments and analyzed them separately. Out of 123 sequences of the 16S fragment we detected 13 segregating sites and 3 indel positions resulting in 14 different haplotypes in *H. molleri*. We found 25 segregating sites among 129 partial CO1 sequences leading to 23 haplotypes in the ingroup. A georeferenced list of mitochondrial haplotypes detected at each locality along with sample sizes is shown in table 1. The resulting networks (fig. 1) are highly concordant, as expected for such genealogically linked mitochondrial markers. The data indicate rather low mitochondrial differentiation of *H. molleri* populations across the Iberian Peninsula. In the 16S fragment, pairwise uncorrected divergences (p-distances) between *H. molleri* sequences range between 0.0-0.9% whereas their differentiation from *H. arborea* and *H. savignyi* amounts to 4.1-4.5% and 6.4-7.1%, respectively. In the COI fragment, distances of 0.0-1.4% are observed within *H. molleri* whereas distances to *H. arborea* and *H. savignyi* are around 14%. In the respective haplotype networks, the maximum number of muta-

Table 1. Localities sampled, locality number (as in map in fig. 1), number of individuals sampled per locality (N), and haplotypes encountered at each locality (haplotype number as in fig. 1).

Locality number	Locality	Province	Latitude	Longitude	N (COI)	Haplotypes (COI)	N (16S)	Haplotypes (16S)
1	Ortigueira	A Coruña	43.7014	−7.8575	5	H1, H2	7	H1
2	Goente, A Capela	A Coruña	43.4394	−7.9894	3	H1	4	H1, H3
3	Irixoa	A Coruña	43.2789	−8.0222	1	H1	–	–
4	Coirós	A Coruña	43.2381	−8.1508	4	H1	4	H1, H6
5	Monte Xalo, Cerceda	A Coruña	43.2242	−8.4219	7	H1, H11	7	H1
6	Barrañán, Arteixo	A Coruña	43.3094	−8.5481	9	H1	5	H1
7	Lagoa de Alcaián, Coristanco	A Coruña	43.1261	−8.7425	1	H5	1	H1
8	Lagoa de Sobrado	A Coruña	43.0381	−8.0081	10	H1	9	H1
9	A Freiría, Friol	Lugo	43.0217	−7.8931	–	–	1	H5
10	Mariz, Guitiriz	Lugo	43.1561	−7.9033	10	H1, H22	10	H1, H8, H14
11	Lagoa de Cospeito	Lugo	43.2442	−7.5514	5	H1, H2, H10, H17	7	H1, H8, H10
12	Lagoa de Caque	Lugo	43.1600	−7.4758	3	H4, H22, H19	3	H3, H8
13	A Estrada, Monforte	Lugo	42.5281	−7.4311	5	H1, H6, H8, H22	6	H1, H4, H5, H13
14	Lagoa de Muro, Porto de Son	A Coruña	42.6253	−9.0417	2	H7	2	H1
15	O Carreirón, Illa de Arousa	Pontevedra	42.5286	−8.8689	6	H1	7	H1
16	Rouxique, Sanxenxo	Pontevedra	42.4544	−8.8450	5	H1	5	H1, H2
17	Lagoa Bodeira, O Grove	Pontevedra	42.4761	−8.9044	15	H1, H3, H11	16	H1, H2
18	Veiga de Ponteliñares, A Limia	Ourense	42.0542	−7.8239	1	H22	–	–
19	A Saceda, Cualedro	Ourense	41.9278	−7.6306	3	H2, H14, H22	3	H1, H8
20	Vilaza, Verín, Monterrei	Ourense	41.9375	−7.4728	3	H1, H22,	3	H1, H12
21	Turbera Puente Porto, Sanabria	Zamora	42.1264	−6.8508	4	H22	4	H8
22	Santa María del Puerto	León	43.0278	−6.2258	3	H22	3	H8
23	Laguna de Pilavieja, Cernégula	Burgos	42.6625	−3.6486	2	H22	3	H8
24	El Berrueco	Madrid	40.8906	−3.5536	3	H22	2	H9, H11
25	Charcas de Miraflores, Peñalara	Madrid	40.9031	−3.9003	5	H1, H22, H13, H15	3	H8, H9, H11
26	Valle del Tiétar	Ávila	40.2753	−4.6669	4	H12, H16, H18, H22	3	H1, H7, H8
27	Candeleda-Navalcán	Ávila	40.0933	−5.2128	3	H12, H22	3	H7, H8
28	Charco Crespo, Castelo da Vide. São Mamede	Alentejo, Portugal	39.4131	−7.4583	2	H1	–	–
29	Verdizela near Lisboa	Portugal	38.6037	−9.1531	1	H21	1	H7
30	Laguna Ojos Villaverde – Ballesteros	Albacete	38.8352	−2.4585	1	H20	1	H7
31	Río Arenoso	Córdoba	42.38442	−3.84239	1	H12	–	–
32	Ventillas	Ciudad Real	42.61596	−3.87063	1	H12	–	–
33	Cardeña	Córdoba	42.34765	−3.84126	1	H23	–	–

tional steps separating haplotypes of *H. molleri* is 6 and 7 for 16S and COI, respectively.

To facilitate summarizing the geographical pattern observed in our data set, we used different color codes for localities in five general geographical areas (fig. 1): GC (green, localities 1-8 and 14-17), Galicia, coastal populations of A Coruña and Pontevedra provinces; GI (blue; 9-13 and 18-22), Galicia inland populations plus inland populations of adjacent areas of León province; P (yellow; 28-29), Portugal; CS (red-purple; 23-27 and 30-33), localities in central and southern Spain including populations 30-33 at the south-eastern border of the distribution area of *H. molleri*.

Haplotype sharing between localities and regions was extensive. Two major haplotypes are present in both networks (H1 and H22 in the COI network; H1 and H8 in the 16S network; see fig. 1). One of these (H1 in the two networks) is the central haplotype present mainly in GC but also in GI and one P population, but not in CS. The second major haplotype (H22 and H8, respectively) is mostly present in the CS populations. This haplotype is also present in many GI populations but not in GC.

There was an insignificant positive correlation ($r = 0.163$, $P = 0.153$) between genetic and geographic distance for 10 populations from Galicia. Pairwise Fst values calculated for COI among Galician populations were relatively low (<0.2) with the exception of populations 1 and 10 which were more strongly differentiated from others (fig. 2). Most of the variation in these Galician samples could be accounted for by differences within populations (82%), whereas among population variation explained the rest (18%); both variance components were highly significant ($P < 0.0001$). If we added the distant population 25 into the IBD analysis, the correlation was much stronger and significant ($r = 0.547$, $P = 0.009$; see also fig. 2). Accordingly, variation among populations climbed to 32%, while the within population variance component was lower at 68%, both were highly significant ($P < 0.0001$). The Barrier analysis restricted to the Galician populations suggested a major barrier separating most GC populations from GI populations + population 8 (fig. 1), and a secondary barrier separating population 8 from GI populations.

Our study provides the most comprehensive analysis thus far on the differentiation among northern and central Iberian populations of *Hyla*. Based on the molecular data we conclude that (i) the mitochondrial differentiation between *Hyla molleri* and *H. arborea* is constant and Iberian populations of *H. molleri* show only limited genetic variation and low phylogeographic structure; (ii) populations from north-western and central Iberia which in the past were designated as different subspecies (*arborea* and *molleri*) are likely conspecific and should all be assigned to the Iberian endemic species, *H. molleri*; (iii) haplotype sharing between populations of this species is generally high across Iberia, suggesting a considerable amount of gene flow or a rapid population expansion, similar to the situation in *Pelobates cultripes*, another widespread pond-breeding anuran in Iberia (Crottini et al., 2010).

Coastal populations in Galicia appear to be genetically slightly differentiated from populations in central Spain, with a broad area of admixture that encompasses much of inland Galicia and probably other areas of northwestern Spain and northern Portugal. The differentiation found between these two haplotype groups is, however, very low and unlikely to reflect any taxonomic differences. Moreover, we found two disjunct geographical areas with relatively high haplotype diversity: 2 populations (11 and 13) in the province of Lugo and two further sites (25 and 26) in central Spain. Each of these populations contained 4 different COI haplotypes despite low sample sizes (4-5 individuals per population). In contrast, we only found 6 COI haplotypes from 68 treefrogs from the well sampled coastal localities of Galicia. In many Iberian amphibian and reptile species the highest genetic diversity is localized in the southern part of their distribution area, close to their

putative refugia (e.g., Martínez-Solano et al., 2006; Sequeira et al., 2008; Gonçalves et al., 2009), which appears to differ from the situation in *H. molleri.* However, inference on refugia or sanctuaries (cf. Recuero and García-Paris, 2011) and possible range expansions in *H. molleri* are hampered by several limitations to our data set. Although improbable, we cannot rule out that a pattern of wide mitochondrial introgression masks the true relationships among populations, and even the presence of populations within the study area that indeed belong to *H. arborea* cannot yet be fully excluded; such a pattern is, however, known from only a few amphibians such as the newt *Lissotriton montandoni* (Babik et al., 2003) and both western and eastern Palaearctic waterfrogs (Plötner et al., 2008; Liu et al., 2010). Second, our geographic sampling is relatively uneven with many populations and often multiple specimens per population sampled in Galicia, and a coarser sampling in central Spain. This may mislead interpretation of population differentiation and thus isolation-by-distance. Moreover, Barrier analyses were only possible for a subset of populations from a geographically limited region. It is therefore necessary to validate our results by applying independent genetic markers and a wider geographic sampling. Based on our preliminary data, such forthcoming studies can be focused more precisely to test a number of specific questions. (i) It is of high priority to identify the contact zone between *Hyla arborea* and *H. molleri.* This zone can either be located in the Spanish Basque country which was not sampled in this study, or in France where the closest *H. arborea* populations known so far (Stöck et al., 2008, 2011) are from the Bretagne region and near Lyon. Future sampling should therefore be extended into northeastern Spain and France. (ii) We found indications for limited differentiation of coastal Galician populations of *H. molleri* relative to those of other regions of Spain and Portugal, and evidence for higher haplotype diversity in some inland localities. Future sampling should include coastal regions of Asturias and Cantabria to better understand the geographic range of this coastal haplotype group.

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References

Babik, W., Szymura, J.M., Rafinski, J. (2003): Nuclear markers, mitochondrial DNA, and male secondary sexual traits variation in a newt hybrid zone (*Triturus vulgaris* × *T. montandoni*). Mol. Ecol. **12**: 1913-1930.

Bedriaga, J.v. (1890 "1889"): Die Lurchfauna Europas. I. Anura. Froschlurche. Bull. Soc. Imp. Natural. Moscou. N.S. **3**: 466-622.

Boulenger, G.A. (1898): The Tailles Batrachians of Europe, Part II. Ray Society, London.

Bruford, M.W., Hanotte, O., Brookfield, J.F.Y., Burke, T. (1992): Single-locus and multilocus DNA fingerprinting. In: Molecular Genetic Analysis of Populations: A Practical Approach, p. 225-270. Hoelzel, A.R., Ed., IRL Press, Oxford.

Clement, X., Posada, D., Crandall, K. (2000): TCS: a computer program to estimate gene genealogies. Mol. Ecol. **9**: 1657-1659.

Crottini, A., Galán, P., Vences, M. (2010): Mitochondrial diversity of western spadefoot toads, *Pelobates cultripes*, in northwestern Spain. Amphibia-Reptilia **31**: 443-448.

Ersts, P.J. (2011): Geographic Distance Matrix Generator (version 1.2.3). American Museum of Natural History, Center for Biodiversity and Conservation. Available from http://biodiversityinformatics.amnh.org/open_source/gdmg. Accessed on 20/05/11.

Excoffier, L., Lischer, L.E.H. (2010): Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Res. **10**: 564-567.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., de Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R., Lynch, J.D., Green, D.M., Wheeler, W.C. (2006): The amphibian tree of life. Bull. Am. Mus. Nat. Hist. **297**: 1-370.

Gonçalves, H., Martínez-Solano, I., Pereira, R.J., Carvalho, B., García-Paris, M., Ferrand, N. (2009): High levels of population subdivision in a morphologically conserved Mediterranean toad (*Alytes cisternasii*) result from recent, multiple refugia: evidence from mtDNA, microsatellites and nuclear genealogies. Mol. Ecol. **18**: 5143-5160.

Gvoždík, V., Moravec, J., Klütsch, C., Kotlík, P. (2010): 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. Mol. Phylogenet. Evol. **55**: 1146-1166.

Hua, X., Fu, C., Li, J., Nieto Montes de Oca, A., Wiens, J.J. (2009): A revised phylogeny of Holarctic treefrogs (genus *Hyla*) based on nuclear and mitochondrial DNA sequences. Herpetologica **65**: 246-259.

Liu, K., Wang, F., Chen, W., Tu, L., Min, M.S., Bi, K., Fu, J. (2010): Rampant historical mitochondrial genome introgression between two species of green pond frogs, *Pelophylax nigromaculatus* and *P. plancyi*. BMC Evol. Biol. **10**: article 201.

Manni, F., Guérard, E., Heyer, E. (2004): Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by "Monmonier's algorithm". Human Biol. **76**: 173-190.

Márquez, R. (2002): *Hyla arborea.* In: Atlas y Libro Rojo de los Anfibios y Reptiles de España, p. 114-116. Pleguezuelos, J.M., Márquez, J.M., Lizana, M., Eds, Dirección General de Conservación de la Naturaleza-Asociación Herpetológica Española, Madrid.

Martínez-Solano, I., Teixeira, J., Buckley, D., García-París, M. (2006): Mitochondrial DNA phylogeography of *Lissotriton boscai* (Caudata, Salamandridae): evidence for old, multiple refugia in an Iberian endemic. Mol. Ecol. **15**: 3375-3388.

Monmonier, M. (1973): Maximum-difference barriers: an alternative numerical regionalization method. Geograph. Anal. **3**: 245-261.

Oliveira, M.L., Pargana, J.M. (2008): *Hyla arborea* Linnaeus, 1758. In: Atlas dos Anfibios e Répteis de Portugal, p. 120-121. Loureiro, A., Ferrand de Almeida, N., Carretero, M.A., Paulo, O.S., Eds, Instituto de Conservaçao da Natureza e da Biodiversidade, Lisboa.

Plötner, J., Uzzell, T., Beerli, P., Spolsky, C., Ohst, T., Litvinchuk, S.N., Guex, G.D., Reyer, H.U., Hotz, H. (2008): Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. J. Evol. Biol. **21**: 668-681.

Recuero, E., García-Paris, M. (2011): Evolutionary history of *Lissotriton helveticus*: Multilocus assessment of ancestral vs. recent colonization of the Iberian Peninsula. Mol. Phylogenet. Evol. **60**: 170-182.

Recuero, E., Iraola, A., Rubio, X., Machordom, A., García-París, M. (2007): Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. J. Biogeogr. **34**: 1207-1219.

Reynolds, J., Weir, B.S., Cockerham, C.C. (1983): Estimation for the coancestry coefficient: basis for a short-term genetic distance. Genetics **105**: 767-779.

Rosa, H.D., Oliveira, E. (1994): Genetic differentiation of the Iberian tree frogs *Hyla arborea molleri* and *Hyla meridionalis* (Amphibia: Anura). Z. Zool. Syst. Evol. Forsch. **32**: 117-128.

Schneider, H. (1974): Structure of the mating calls and relationships of the European tree frogs (Hylidae, Anura). Oecologia **14**: 99-110.

Sequeira, F., Alexandrino, J., Weiss, S., Ferrand, N. (2008): Documenting the advantages and limitations of different classes of molecular markers in a well-established phylogeographic context: lessons from the Iberian endemic Golden-striped salamander, *Chioglossa lusitanica* (Caudata: Salamandridae). Biol. J. Linn. Soc. **95**: 371-387.

Speybroeck, J., Beukema, W., Crochet, P.-A. (2010): A tentative species list of the European herpetofauna (Amphibia and Reptilia) – an update. Zootaxa **2492**: 1-27.

Stöck, M., Dubey, S., Klütsch, C., Litvinchuk, S., Scheidt, U., Periin, N. (2008): Mitochondrial and nuclear phylogeny of circum-Mediterranean tree frogs from the *Hyla arborea* group. Mol. Phylogenet. Evol. **49**: 1019-1024.

Stöck, M., Horn, A., Grossen, C., Lindtke, D., Sermier, R., Betto-Colliard, C., Dufresnes, C., Bonjour, E., Dumas, Z., Luquet, E., Maddalena, T., Sousa, H.C., Martinez-Solano, I., Perrin, N. (2011): Ever-young sex chromosomes in European tree frogs. PLoS Biol. **9**: e1001062.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**: 1596-1599.

Templeton, A.R., Crandall, K.A., Sing, C.F. (1992): A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics **132**: 619-633.

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