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Diversity and distribution of deep mitochondrial lineages of the common frog, *Rana temporaria*, in northern Spain

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Abstract. We provide an update of the geographical distribution and phylogenetic relationships of mitochondrial haplotype groups of the European common frog, *Rana temporaria*, one of the most widespread amphibians on Earth. Our data set of 378 newly determined DNA sequences of the cytochrome b gene is combined with 640 previously published sequences, mainly filling a sampling gap in the Spanish Basque country. A haplotype network based on 331 bp provides evidence for a previously unknown, deeply divergent haplogroup in this region, which in the eastern part of its range occurs sympatrically with a widespread group of haplotypes from the Pyrenean mountains, and to the West contacts a haplogroup typically for the central Cantabrian mountains. In contrast, samples from the isolated Montseny massif in the Spanish region of Catalonia are not differentiated from Pyrenean populations. Samples from Scotland cluster in a widespread haplogroup with samples from northern France, western Germany, and Ireland. Our data furthermore suggest that previous records of haplotype sharing of British *R. temporaria* populations with those from northwestern Spain are probably erroneous. A phylogenetic analysis based on 4,413 bp of mitochondrial DNA confirms the strong divergence of the newly discovered Basque haplogroup and indicates that it might be the sister group of a clade containing all other *R. temporaria* haplogroups.

Key Words. Amphibia, Ranidae, *Rana temporaria*, cytochrome b, phylogeography, Iberia.

Introduction

The European common frog, *Rana temporaria* Linnaeus, 1758, is one of the most widespread amphibians on Earth, occupying a vast range of habitats and altitudinal levels across Europe with the exception of Mediterranean areas (Grossenbacher 1997, Sillero et al. 2014). Although a large number of studies have dealt with this species (see the summary by Grossenbacher et al. 1988 for older papers) and led to important insights into its genetic differentiation and evolution (Arano et al. 1993, Hitchings & Beebee 1997, Veith et al. 2002, 2003, Palo et al. 2004, Schmeller & Merilä 2007, Lesbarrères et al. 2007, Galán et al. 2009, Teacher et al. 2009, Phillimore et al. 2010, Zeisset & Beebee 2010, Stefani et al. 2012, Veith et al. 2012), comprehensive knowledge on the phylogeographic differentiation of the species across its range has only recently become available (Vences et al. 2013).

A leading result of these studies is that *Rana temporaria* is characterized by high genetic diversity. From a mitochondrial perspective, the largest part of its range (from the Pyrenees to Siberia) is occupied by two main haplogroups (Palo et al. 2004, Teacher et al. 2009, Vences et al. 2013), an eastern one ranging from Germany eastwards, and a western one ranging from the Pyrenees to central Germany, including Great Britain and Ireland, and reaching the Balkans and Greece to the South. While the eastern haplogroup is very uniform, the western group shows distinct substructures. In northern Spain, additional, deeply divergent haplogroups occur: the westernmost region, Galicia, and the western half of the adjacent region, Asturias, host the subspecies *R. t. parvipalmata* Seoane, 1885, which includes some morphologically divergent populations with small body size and reduced webbing (Galán Regalado & Fernández Arias 1993, Estéban & García-Paris 2002). This taxon forms its own mitochondrial haplogroup (Galán et al. 2009, Vences et al. 2013) and appears to be differentiated also in its nuclear DNA (Arano et al. 1993, Veith et al. 2003, Vences et al. 2013). Other deeply divergent haplogroups, however phenotypically not distinct, have been identified from eastern Asturias and the Benasque valley in the Spanish Pyrenees (Vences et

al. 2013). In contrast, morphologically divergent populations from specific sites in the Pyrenees for which taxonomic distinctness has been suspected (e.g., Dubois 1982, 1983) did not turn out to be divergent in their mitochondrial or nuclear markers (Veith et al. 2002, 2012). Within the Spanish range of *R. temporaria*, a substantial sampling gap still exists especially in the regions of Cantabria and the Basque country, an area that coincides with a nuclear DNA transition zone of Cantabrian and Pyrenean alleles (Veith et al. 2012), and with range boundaries of related frogs such as *R. dalmatina* (see Sarasola-Puente et al. 2012).

Besides this subdivision into major haplogroups, *R. temporaria* is also characterized by high genetic variation within populations. This genetic variation, in mitochondrial, but especially in nuclear markers, and across most of its range, is higher than in other congeneric species such as *R. dalmatina, R. italica, R. latastei*, including its inferred sister species *R. pyrenaica* (Ficetola et al. 2007, Canestrelli 2008, Carranza & Arribas 2008, Vences et al. 2013). This high genetic variation of *R. temporaria* is relevant to its biogeography and taxonomy, but might also hold the key to understanding the ecological and morphological variation of this species, which at least in traits such as breeding season (e.g., Phillimore et al. 2010) is characterized by a high adaptive potential.

Here we extend previous work (Vences et al. 2013) with newly determined mitochondrial DNA sequences of 378 samples, collected predominantly in northern Spain, but also including some previously unstudied populations from Great Britain and Italy. Our work led to the discovery of a previously undetected, deep mitochondrial lineage of the species in the Spanish Basque country and thereby confirms that Iberia is the centre of early diversification of *Rana temporaria*.

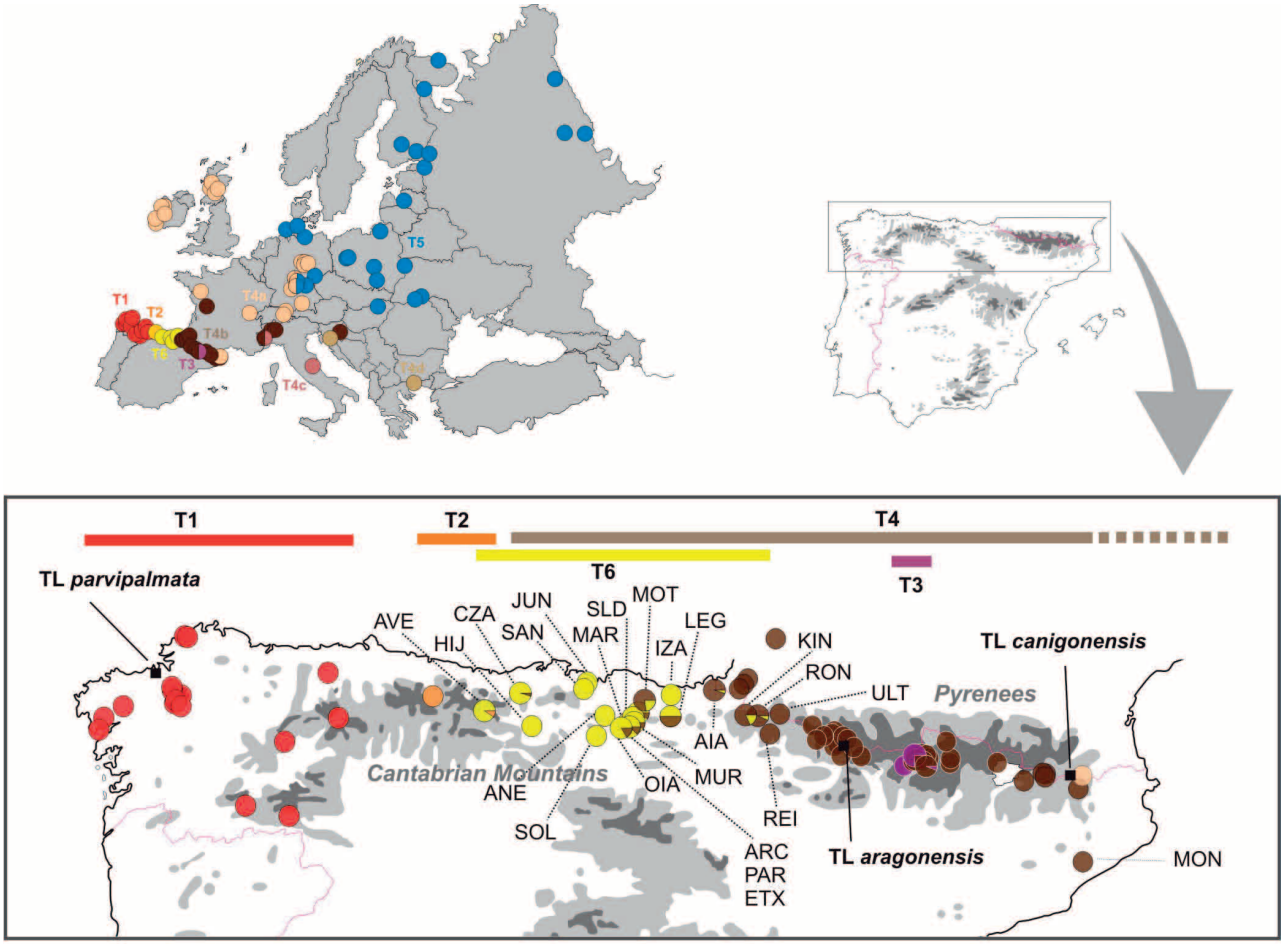

Figure 1. Maps of Europe (upper left) and northern Spain (below; showing the area indicated in the upper right map of Iberia), illustrating the distribution of the main haplogroups T1–T6 of *Rana temporaria*. In the map of Europe, not all locations are shown due to overlapping symbols in some cases. Shaded areas roughly indicate the outline of mountain areas of > 1,000 m (light grey) and 1,500 m (dark grey) above sea level. Colours correspond to those used in the haplotype network (Fig. 2). Bars in the upper part of the figure show the longitudinal range of each of the main haplogroups; the range of T4 extends far beyond the map and covers much of western and central Europe. Localities with sample codes correspond to the newly sampled sites listed in Table 1 (in addition to the four localities in Scotland, and one locality in northern Italy). Approximate location of type localities (TL) of nominal (subspecific) taxa described from the region are indicated by black squares; note that of these, only *parvipalmata* is currently accepted as a valid subspecies of *R. temporaria*.

Table 1. Names and geographical coordinates of localities, and collection dates and numbers of samples newly sequenced for this study. For localities and coordinates of additional sequences, see Vences et al. (2013).

Locality code	N samples	Country	Locality	Date	Latitude	Longitude
TE_AIA	21	Spain (Gipuzkoa)	Aiako harriak	2010	43.2502	-1.8283
TE_ANE	18	Spain (Araba)	Añes (Sierra Salvada)	2010	43.0337	-3.1059
TE_AVE	10	Spain (Cantabria)	Avellaneda	2011	43.0754	-4.5218
TE_ARC	19	Spain (Araba)	Arcaray (Gorbea)	2010	42.9662	-2.8240
TE_CZA	22	Spain (Cantabria)	Villasuso de Cieza	2011	43.2212	-4.1424
TE_ETX	19	Spain (Araba)	Pinar Etxebarría (Gorbea)	2010	42.9662	-2.8240
TE_HIJ	30	Spain (Burgos)	Monte Hijedo	2010	42.9329	-3.9570
TE_IZA	6	Spain (Gipuzkoa)	Izarraitz	2010	43.2079	-2.3349
TE_JUN	17	Spain (Cantabria)	Embalse del Juncal (Trebuesto, Guriezo)	2011	43.3058	-3.3175
TE_KIN	27	Spain (Nafarroa)	Kinto (Collado de Urkiaga)	2010	43.0377	-1.4690
TE_LEG	2	Spain (Gipuzkoa)	Legazpi	2010	43.0253	-2.3523
TE_MAR	23	Spain (Araba)	Marakalda (Zuia)	2010	42.9809	-2.8436
TE_MON	4	Spain (Catalonia)	Santa Fé (Parc Natural del Montseny)	2012	41.7750	2.4627
TE_MOT	10	Spain (Gipuzkoa)	Motondo (Orio)	2010	43.1620	-2.6433
TE_MUR	24	Spain (Araba)	Canteras Murua (Gorbea)	2010	42.9851	-2.7442
TE_OIA	24	Spain (Araba)	Oiardo (Gorbea)	2010	42.9866	-2.9273
TE_PAR	24	Spain (Araba)	Parketxea (Gorbea)	2010	42.9662	-2.8240
TE_REI	11	Spain (Nafarroa)	Remendía	2010	42.8744	-1.1867
TE_RON	16	Spain (Nafarroa)	Roncesvalles	2010	43.0224	-1.3171
TE_SAN	6	Spain (Bizkaia)	Barrio de Santecilla (Karrantza)	2011	43.2615	-3.3547
TE_SLD	10	Spain (Bizkaia)	Humedal de Saldropo	2010	43.0552	-2.7290
TE_SOL	5	Spain (Araba)	Solinde (Parque Natural de Valderejo)	2010	42.8545	-3.2066
TE_ULT	15	Spain (Nafarroa)	Lizaso (Ultzama)	2009	42.9432	-0.6887
TE_PDR	3	Italy	Pian del Re (Monviso Massif)	2013	44.7010	7.0957
TE_DUS	3	UK (Scotland)	Beinn Dubchraig (High Altitude)	2010	56.3951	-4.7506
TE_LAW	3	UK (Scotland)	Ben Lawers (High Altitude)	2010	56.5423	-4.2291
TE_LOM	3	UK (Scotland)	Ben Lomond (High Altitude)	2010	56.1857	-4.6478
TE_MNT	3	UK (Scotland)	Meall nan Tarmachan	2010	56.5188	-4.2958

Materials and methods

Sampling and molecular methods herein largely follow Vences et al. (2013), as DNA sequences were obtained to complement the dataset of this previous study, mostly from new locations on the Iberian Peninsula (Fig. 1, Table 1). Newly collected tissue samples included toe clips, fin clips of larval specimens or embryos at early developmental stages, all preserved in pure ethanol. When using larvae or embryos, we collected these from different clutches or ponds to avoid sampling siblings. We extracted total genomic DNA using a standard salt protocol (Bruford et al. 1992). We sequenced from all samples a fragment of the mitochondrial cytochrome b gene (cob), using primers Rana-Cytb-F2 (TTAGTAATAGCCACAGCTTTTGTAGGC) and Rana-Cytb-R2 (AGGGAACGAAGTTTGGAGGTGTGG) from Vences et al. (2013), using the following PCR cycling protocol: 94 (90 sec), [94 (30 sec), 53 (45 sec), 72 (90 sec) × 35], and 72 (600 sec).

To resolve the phylogenetic relationships of the newly identified lineage from northern Spain, we sequenced various additional mitochondrial genes (plus stretches of intervening tRNAs) of one sample from Oiardo to complement a previous data set (Vences et al. 2013): 12S rRNA (12S), using primers 12SAL/16SR3 (Palumbi et al. 1991), two fragments of 16S rRNA (16S), using primers 16SL3/16SAH (Vences et al. 2003), and 16SAr-L/16SBr-H (Palumbi et al. 1991), cytochrome oxidase subunit 1 (cox1), using LCO1490/HCO2198 (Folmer et al. 1994), NADH dehydrogenase subunit 1 (nd1), using L3914/H4419 (Macey et al. 1998b), and NADH dehydrogenase subunit 2 (nd2) and adjacent cox1, using L4437/H6564 (Macey et al. 1997, Macey et al. 1998a). For full primer sequences and cycling protocols, see the supplementary information of Vences et al. (2013).

PCR products were purified by Exonuclease I (NEB) and Shrimp Alkaline Phosphatase (Promega) digestion. Sequencing was carried out using BigDye v3.1 chemistry and sequences were resolved on an ABI 3130xl capillary sequencer (Applied Biosystems). We quality-checked chromatograms visually and corrected sequences where necessary, using the CodonCode Aligner 3.7.1 (Codon Code Corporation). Newly determined sequences were deposited in GenBank (accession numbers KT074473-KT074853).

We aligned sequences using the Clustal algorithm in MEGA 5 (Tamura et al. 2011). Only very few indels had to be added to the rRNA fragments, and those sites were excluded from further analyses; all other genes had no insertions or deletions.

To understand the clustering of *R. temporaria* haplotypes in main groups (lineages), we constructed a haplotype network based on all cob sequences available from Vences et al. (2013) and this study. We used a median-joining haplotype network reconstruction method (Bandelt et al. 1999), using Fluxus Phylogenetic Network Analysis 4.612 software (http://www.fluxus-engineering.com).

To reconstruct the phylogenetic relationships among the main *R. temporaria* haplotype lineages, we then performed a partitioned multi-gene phylogenetic analysis in MrBayes 3.2 (Ronquist et al. 2012), after determining the appropriate substitution models for each partition by applying the Akaike information criterion (AICc) in PartitionFinder 1.0.1 software (Lanfear et al. 2012), with two runs of four MCMC chains each, running for 20 million generations, and sampling every 1,000th generation. The trees corresponding to the first 5 million generations were excluded as burn-in after assessing appropriate mixing and the stationarity of chains using the software AWTY (Nylander et al. 2008). The optimal suggested model consisted of 10 partitions (P1–P10) with the following substitution models: F81: P4 (cox1-2nd codon position); K80+I: P6 (cob 1st); HKY: P7 (cob-2nd, nd2-2nd); HKY+I: P3 (cox-1st); HKY+I+G: P9 (nd1-1st, tRNAs); GTR:P8 (cob-3rd); GTR+G : P1 (12S); GTR+I: P10 (nd1-2nd); GTR+I+G: P2 (16S, nd2-1st); and P5 (cox1-3rd, nd1-3rd, nd2-3rd). *Pelophylax nigromaculatus, Rana dalmatina, R. arvalis*, and *R. pyrenaica* were used as hierarchical outgroups for the phylogenetic analysis.

Results

A total of 378 new cob sequences of *Rana temporaria* were determined for this study. Of these, 363 corresponded to

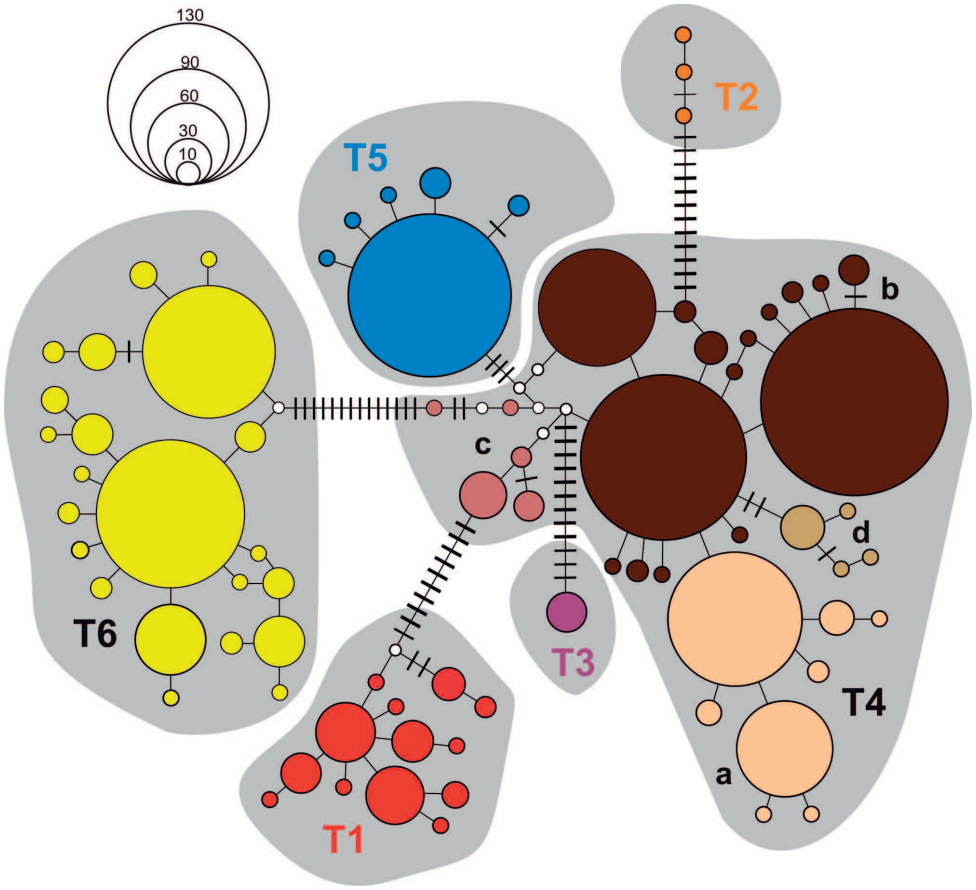

Figure 2. Haplotype network based on the analysis of DNA sequences (331 bp) of the cob gene in 1,018 specimens of *Rana temporaria*. Colours correspond to major haplogroups T1–T6 as defined in the text; T6 is the new northern Iberian haplogroup identified herein. Haplogroup T4 is represented in different colours to facilitate a better geographical visualization of the considerable variation within this group shown in Fig. 1. Within T4, the differently coloured subgroups correspond to samples from a) western Germany, France (including Mont Canigou in the Pyrenees), Switzerland, Britain, and Ireland; b) the Spanish and French Pyrenees and the eastern Basque country in Spain; c) Italy; and d) Croatia and Greece. Note that T4a corresponds to the green-coloured haplotype subgroup in the network by Vences et al. (2013); it is here sand-coloured to better fit in with the brown colours characterizing all subgroups of T4.

samples from Spain, 12 to samples from Scotland, and 3 to samples from northern Italy. Sequences were trimmed to obtain a 331 bp alignment without missing data. Analysis of the full dataset of 1,018 cob sequences (378 from the present study plus 640 from the study by Vences et al. 2013) confirmed the presence of five main haplogroups (T1–T5), separated from each other by a minimum of 6 mutational steps, and revealed a sixth deeply divergent haplogroup (T6) that differs by a minimum of 16 mutational steps from all other haplogroups (Fig. 2). This new haplogroup, T6, was recorded from 21 sites in northern Spain, mainly in the Basque country. It co-occurred with T4 haplotypes at 13 sites and with a T2 haplotype at one site. Haplotype T1 is restricted to the westernmost populations in the Spanish regions of Galicia and Asturias; T2 is so far known only from two sites in the region of Cantabria (Avallaneda and Fuente Dé); T3 is restricted to the Benasque Valley; T4 is widespread in western-central Europe, with a specific set of haplotypes (subgroup b; dark brown in Figs 1–3) centred in the Pyrenean area; and T5 is restricted to Eastern and Northern Europe.

Other relevant populations from northern Spain fell into the mitochondrial groups as follows: Samples from the Montseny Massif in Catalonia were not particularly divergent and belonged to the same haplogroup as the majority of samples from the Pyrenees (dark brown coloured subgroup T4-b). Samples from Mont Canigou, the type locality of the nomen *canigonensis*, were also part of T4; they differed from other Pyrenean samples and, as already reported by Vences et al. (2013), belonged to the sand-coloured subgroup T4-a (Figs 1–2) that is widespread in France, Germany, Switzerland, Ireland, and (according to new data herein) also in Scotland. Specimens from Respomuso (in Aragón, Spain), the type locality of the nomen *aragonensis*, fell into the brown-coloured part of T4 (Fig. 2). In the Benasque Valle (as reported previously; Vences et al. 2013), haplotypes of haplogroup T3 co-occurred in several populations with T4 haplotypes.

Phylogenetic analysis of DNA sequences (4,413 bp) from six genes plus intervening tRNAs confirmed a sister-group relationship of T4 and T5, and of T1 and T2, respectively (Fig. 3). The representative of the newly discovered haplogroup T6 turned out as a sister to a clade of all remaining haplogroups (T1–T5), with substantial support for this T1–T5 clade (PP = 0.98).

We did not observe double peaks, frameshifts or stop-codons in any of the protein-coding sequences (cob, cox1, nd1, nd2), and the sequences of the main haplogroups consistently showed important genetic divergences in all genes studied.

Uncorrected pairwise cob distances between the main haplogroups ranged from 2.4 (comparison of T4 vs T5) to 7.8% (T6 vs T3 and T4). A comparison of the 16S fragment, for which a minimum of 3% divergence has been defined as a threshold for candidate species (Fouquet et al. 2007, Vieites et al. 2009), yielded relatively low divergence val-

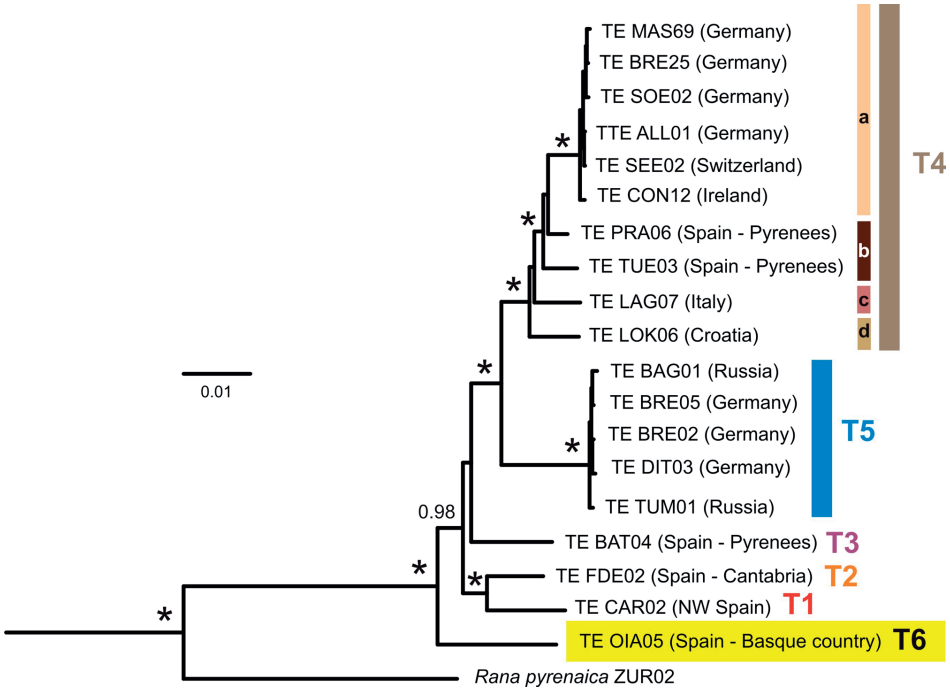

Figure 3. 50% majority rule consensus tree obtained by Bayesian inference analysis of 4,413 bp of mitochondrial DNA sequences (genes: 12S, 16S, cob, cox1, nd1, nd2, and stretches of intervening tRNAs). Samples included represent the major haplogroups identified within *Rana temporaria*. Colours and names of haplogroups (T1–T6) are as in the haplotype network and map (Figs 1–2); the newly discovered haplogroup T6 is marked in yellow. An asterisk indicates posterior probabilities of 1.0; all other nodes received PP values < 0.95. *Pelophylax nigromaculatus, Rana dalmatina, R. arvalis*, and *R. pyrenaica* were used as hierarchical outgroups, however, for graphical reasons, only *R. pyrenaica* is shown here because it was resolved as sister species of *R. temporaria*).

ues of between 0.2 (T3 vs T1 and T2) and 1.2% (T4 vs T2, T5, and T6), corresponding to only 1–7 substitutions in the fragment analysed. *Rana temporaria* differed from its sister species, *R. pyrenaica,* by 2.1–3.0% (16S) and 8.7–11.3% (cob), respectively.

Discussion

Our haplotype network (Fig. 2) and phylogenetic tree (Fig. 3) largely agree with those published by Vences et al. (2013). One inconsistency is the placement of lineage T2 relatively distant to T1; in the previous network (reconstructed using a statistical parsimony method), these two lineages were grouped rather close to each other, in agreement with their placement in the phylogenetic tree (Fig. 3). Given that the main goal of the network is to illustrate the main groups of haplotypes and that the tree is based on much longer DNA sequences from multiple mitochondrial genes, the phylogenetic relationships are more reliably depicted by the tree, which is also indicated by the high support values obtained for most of the nodes.

The discovery of yet another deep mitochondrial lineage of *R. temporaria* in Iberia (haplogroup T6), and the sister-species relationship between *R. temporaria* and the Pyrenean microendemic *R. pyrenaica,* confirms that this region most likely is the centre of diversification of this species, probably triggered by fragmentation of its range into multiple refugia or sanctuaries (Recuero & García-Paris 2011) in the north of the Iberian Peninsula. Of the various haplogroups, T1, T2, T3, and T6 all appear to be restricted to the Iberian Peninsula: T1, T2, and T6 in a west–east direction mainly along 43° latitude, to parts of the Cantabrian mountain range in northern Spain, and T3 to a small area in the Pyrenees corresponding to the Benasque valley. In addition, Iberia also hosts populations of haplogroup T4, which occupies almost all of the Pyrenees and enters deep into the Basque country and into Catalonia (in the Montseny Massif). Of all haplogroups known for the species, only T5, restricted to northern and eastern Europe, is absent from the Iberian Peninsula.

The discovery of a haplogroup (T6) apparently centred in the Spanish Basque country (and extending into the region of Cantabria) is unexpected, as such a pattern is only rarely found in other taxa (Gómez & Lund 2006). One coinciding example is another amphibian, *Lissotriton helveticus*, which has one Pyrenean haplogroup, one in the Basque country and Cantabria, one in Asturias, and a westernmost one in Galicia that extends into Portugal (Recuero & García-Paris 2011). One further example of such differentiation processes within the Basque region comes from land snails (*Elona quimperiana*) in which a distinct Basque haplogroup occurs next to a second haplogroup that occupies in a disjunctive manner western Spain and the Brittany region in France (Vialatte et al. 2008). Coincidently, a previously postulated Basque clade of the vole *Clethrionomys glareolus* has been found to probably represent pseudogenes (Filipi et al. 2015); however, we do not consider such an explanation likely for T6 found herein, because sequences of this haplogroup were distinct in each of the eight mitochondrial gene segments amplified and sequenced with different primers (although in 12S and 16S, T6 differed only slightly from other haplogroups due to overall low variation). Neither *L. helveticus* nor *E. quimperiana* show the broad admixture among the mitochondrial haplogroups seen in *R. temporaria*. Taken together, however, these examples agree with the view that in the southern European peninsulas the influence of different processes in a patchy landscape and across a varied topography has led to a complex situation. Characterizing the southern European peninsulas as single refugia or speaking of multiple unconnected refugia within each peninsula, will therefore often lead to oversimplification (Nieto Feliner 2011).

The Montseny Massif in Catalonia, an isolated forested area surrounded by Mediterranean vegetation, is known to be an important centre of endemism (Barrientos 1995) and hosts, for instance, the endemic newt *Calotriton arnoldi*, the sister taxon of *Calotriton asper*, which is widespread in the Pyrenees (Carranza & Amat 2005). However, we did not record substantial mitochondrial divergence for the *R. temporaria* population from this massif. In fact, despite being the southernmost locality of the species in Catalonia, the Montseny population of *R. temporaria* is geographically not really isolated. In the Montseny Massif the species mostly reproduces along the Santa Fe lake, but a few other reproductive sites have also been recorded (F. Amat pers. obs.), and the population is only 12 km from the next population (Guillerias Massif), which in turn is not far from Pyrenean populations. Hence, it is likely that the Montseny Massif did not act as an isolated refuge or sanctuary for *R. temporaria*, but was colonized rather recently from the Pyrenees.

Veith et al. (2012) constructed a haplotype network based on DNA sequences of the mitochondrial 16S rRNA gene for 22 Iberian populations of *R. temporaria* and found an eastern and a western group of haplotypes that are differentiated by only two mutational steps, and named them "*parvipalmata* group" and "Pyrenean group", respectively. Our data based on the more variable cob gene suggest that their network, due to low variation of the marker used, led to an oversimplification of the actual phylogeographic pattern in the region. One population from the Basque country (Puerto de Altube) was included in the study by Veith et al. (2012) and assigned to the "*parvipalmata* group". This assignment probably reflects the overall low variation in the 16S sequences (also confirmed herein), which did not allow for discerning it as the distinct T6 haplogroup. Hence, the conclusion of a homogeneous mitochondrial "*parvipalmata* group" occupying the Spanish regions of Galicia, Asturias, Cantabria, and part of the Basque country (Veith et al. 2012) requires refinement, because this area is occupied by three distinct haplogroups (T1, T2, and T6), and mitochondrial gene flow is therefore even more limited than suggested by Veith et al. (2012). Yet the conclusion of these authors regarding wide geographic introgression of nuclear (allozyme) alleles from the widespread

"Pyrenean group" westwards is valid and agrees with the haplotype distribution of the nuclear rag1 gene reported by Vences et al. (2013).

Our samplings from Great Britain and Ireland are still rather patchy. Yet, all cob sequences we analysed from this region, i.e., from five populations from Ireland (Vences et al. 2013) and four populations from Scotland (herein), were part of the T4 haplogroup, and more precisely, of the sand-coloured set of haplotypes in Fig. 2, which otherwise are widespread in northern France, western Germany, and Switzerland. This appears to be in contrast with the results of Teacher et al. (2009) who inferred postglacial expansion routes into Great Britain and Ireland based on a set of cob sequences. According to their results, one haplotype (their haplotype no. 9) was common to frogs from both Galicia in northern Spain and Scotland, and several other haplotypes were shared among frogs from different sites in Great Britain, Ireland, France, and Germany. Although these authors included a larger number of populations from France, Great Britain, and Ireland than we did, it is likely that some of their data are in error and therefore we did not merge these datasets. We hypothesize that PCR contamination, mislabeling or sample confusion might have occurred at some point of their study and accounted for the following results in Teacher et al. (2009): (i) Samples Galicia a1 and Galicia b1 of Teacher et al. (2009) were placed in a haplogroup corresponding largely to our haplogroup T4, even though the two Galician samples were supplied by ourselves (M. Vences in 2008), and all of our own samples from Galicia clearly belong to the strongly divergent haplogroup T1 in our haplotype network (although we cannot trace back exactly which of our samples correspond to the "Galicia" samples in Teacher et al. 2009). (ii) The haplogroup formed by haplotypes no. 8 and 10 in Teacher et al. (2009) contains all Eastern European and Scandinavian samples of that study and clearly corresponds to our haplogroup T5. However, it also contains samples from Spain, France, and Italy. This result likely is erroneous, as our extensive sampling from these countries, as well as the sampling from Italy by Stefani et al. (2012), did not identify any T5 haplotypes from these regions.

The morphological variation observed among populations of *Rana temporaria* has long confounded taxonomists, leading to hypotheses of distinct subspecies and species within this taxon (e.g., Dubois 1982, 1983, Sperling et al. 1996). Molecular data however do not provide any evidence for the Pyrenean taxa *aragonensis* and *canigonensis* representing valid subspecies, while the subspecies status of *parvipalmata* from Galicia in northwestern Spain (e.g., Arano et al. 1993, Galán Regalado & Fernández-Arias 1993, Galán et al. 2009) is confirmed genetically with its unique haplogroup T1. The status of the taxon *honnorati* from the Basses Alpes in France still remains to be tested with DNA-based methods following its last review in 1996 (Sperling et al. 1996). The type locality of *R. temporaria* has not been clarified and would require a historical analysis of literature, similar to the one by Dubois & Ohler (1996) for the treefrog *Hyla arborea*, but is likely to be within the range of either haplogroup T4 or T5. Whether subspecies names should be applied to different clusters of populations within *R. temporaria* remains to be discussed after fixation of the type locality, but our data agree with current taxonomy (e.g., Speybroeck et al. 2010, Sillero et al. 2014) in suggesting a single species-level lineage of high genetic, morphological, and ecological variation. In a previous study (Vences et al. 2013), we provided evidence for the syntopic occurrence of individuals representing haplogroups T4 and T5 in Germany, and T3 and T4 in the Benasque valley in the Pyrenees, without obvious differentiation in nuclear genes or other characters. Especially the latter example is indicative for the evolutionary non-independence of these lineages, as the most plausible scenario is refugial differentiation of the T3 group in the Benasque valley and subsequent complete admixture after secondary contact with the widespread T4 group. This also suggests that the species survived the last glacial periods in Pyrenean valleys (such as Benasque) rather than in Mediterranean refuges, in agreement with its tolerance of cold climatic conditions.

Our study provides additional evidence for syntopic occurrences of other haplogroups: the newly discovered T6 haplogroup exhibits wide co-occurrence with T4, with apparently increasing frequency of T4 eastwards near the range boundaries of T6. The westernmost population of T6 (AVE) is an example of the co-occurrence of T2 and T6. Together with previous evidence of mitochondrial and nuclear gradual admixture along a Cantabrian west–east gradient (Veith et al. 2012), these patterns will agree most with a taxonomic hypothesis that considers all *R. temporaria* populations part of a single, highly variable species.

From the results herein and previous publications, a series of open questions related to the evolution and systematics of the European common frog can be identified that will require future study. On the one hand, given the now relatively detailed knowledge of the range-wide genetic variation of the species, a taxonomic revision is overdue and should involve sampling the type locality of the presumed subspecies *R. t. honnorati* (Sperling et al. 1996) and a historical analysis and fixation of the type locality of *R. temporaria* following the procedure suggested by Dubois & Ohler (1996). On the other hand, a more detailed identification of the contact zones between main haplogroups is needed (in particular, the extent of the range of haplogroup T2 and the contact zone between T4 and T5 are still poorly characterized). These studies should include dense sampling of nuclear DNA, i.e., SNPs, to understand concordances and discordances between lineages defined by mitochondrial and nuclear DNA. Eventually, *R. temporaria* could become a suitable model for genomic studies assessing the degree of nuclear admixture among population groups that – as indicated by mtDNA haplogroups – are characterized by histories of ancient refugial differentiation. Most interestingly, such genomic work could be directed at identifying adaptive differences between intraspecific lineages, and the adaptive value of genetic material transferred among these groups during admixture.

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