Two clades of north European pool frogs *Rana lessonae* identified by cytochrome *b* sequence analysis

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A 410 base-pair region of the mitochondrial cytochrome *b* (cyt*b*) gene, including the coding region for amino acids 44–179, was amplified and sequenced from a total of 53 pool frogs (*Rana lessonae*) sampled in nine European countries across the species' biogeographical range. Just two haplotypes were found, differing by single base pair (G–A transition) at a codon second position, corresponding to a conservative (serine–asparagine) change at amino acid 75. Only haplotype A (serine 75) was found in the samples from Italy, Hungary, Switzerland, Poland, Sweden, Norway and Britain (including museum specimens). Only haplotype B (asparagine 75) was found in France, and there were equal numbers of A and B haplotypes in a small sample from the Netherlands. Comparisons with other ranid cyt*b* sequences together with an absence of stop codons indicated that the sequences were mitochondrial rather than nuclear copies. Using these north European cyt*b* sequences and three more from other work based on individuals from Italy, Ukraine and Luxembourg, we discuss haplotype distribution with respect to the phylogeography of *R. lessonae*.

Key words: diversity, mtDNA, phylogeography

INTRODUCTION

The pool frog *Rana lessonae* is a member of the European water frog complex and has a broad distribution across north-central Europe (Gasc, 1997). Across much of its range *R. lessonae* occurs syntopically with the edible frog *R. esculenta*, a fertile hybrid of *R. lessonae* and the marsh frog *R. ridibunda* (Berger, 1973). However, isolated populations of *R. lessonae* without other water frog species occur at various sites along the northernmost range edge (e.g. Sjögren, 1991). Phylogeographic analysis of *R. lessonae* using six polymorphic microsatellite markers and multiple RAPD loci indicated that these northerly populations in Norway, Sweden and Britain constitute a clade quite distinct from those in mainland Europe (Zeisset & Beebee, 2001; Snell et al., 2005). We set out to test the hypothesis that these "northern clade" pool frogs are also distinct from those in southern Europe at the level of mitochondrial (mt) DNA, the molecular genetic marker most commonly used in phylogeographic studies because it permits genealogical analysis uncomplicated by recombination (Avise, 2000). Since mtDNA sequences have much higher copy numbers per cell than do nuclear ones, we also hoped that a larger proportion of the museum specimens in Britain would provide information for analysis than was the case with our earlier microsatellite analysis.

Initial studies with *R. lessonae* D-loop mtDNA failed to generate PCR products that consistently provided good sequence data, perhaps because the D-loop region is unusually large in the genus *Rana* (Sumida et al., 2000a). We therefore analysed part of the cytochrome *b* (cyt*b*) gene, since sequence information from four individuals already lodged in GenBank indicated that substantial polymorphism existed within this gene in *R. lessonae*. There is also a substantial amount of published research on the phylogeography of European anurans based on cyt*b* analyses with which to compare our results. These include the evolutionary histories of the major brown and water frog groups (Veith et al., 2003; Lymberakis et al., 2007) and the postglacial colonization histories of *Pelobates fuscus* (Eggert et al., 2006; Crottini et al., 2007), *Rana arvalis* (Babik et al., 2004) and *R. temporaria* (Palo et al., 2004).

MATERIALS AND METHODS

Materials

Rana lessonae samples were a subset of those used in an earlier microsatellite study (Zeisset & Beebee, 2001). They were collected in nine European countries, and sample sizes for the mtDNA sequencing were: France (Paris), six individuals; Netherlands (Diever), four individuals; Switzerland (Zurich), two individuals; Italy (Torino), ten individuals; Hungary (Balaton), nine individuals; Poland (Wroclaw), six individuals; Sweden (Uppsala), six individuals; Norway (Arendal), four individuals; Britain (East Anglia), six individuals including the last surviving frog and five museum specimens.

DNA extraction and analysis

DNA was extracted from frog tissues as described elsewhere (Zeisset & Beebee, 2001). In the case of museum specimens, all extractions and subsequent PCR amplifications were carried out in a separate laboratory to minimize the risk of contamination. Primers for cyt*b* amplification by PCR were designed using sequence data from Tanaka et al. (1996). For all except the museum specimens, the primers employed were: CytbF1, 5'-CCTGGGAGTCTGC GTAATC-3' and CytbR1, 5'-ATGTAAGGTGAAATAAGAGGGG-3'. These generated a fragment of 451 base pairs (bp), the intervening 410

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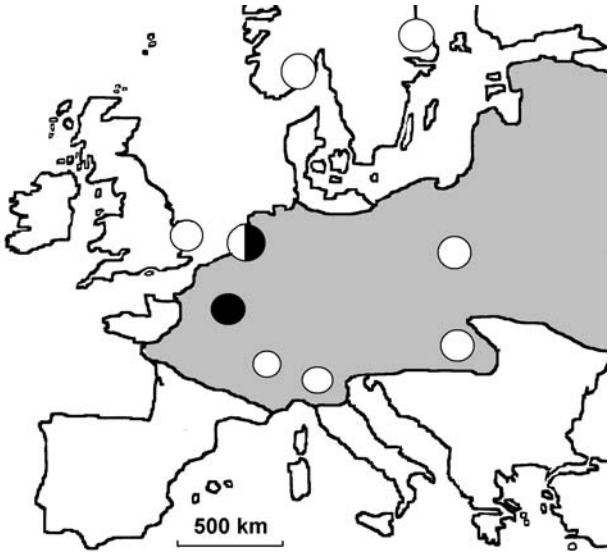


Fig. 1. Sampling sites and cyt*b* haplotype diversities of *R. lessonae*. Unshaded, haplotype A; shaded, haplotype B. Grey shading: approximate central European range of *R. lessonae*.

bp of which (excluding primer regions) coded for amino acids 44–179 inclusive of the cyt*b* protein. For museum specimens a smaller (177 bp) fragment, internal to the 451 bp sequence, was amplified using CytbF2 (5'-CGCCCAAATCGC AACAGG-3') and CytbR2 (5'-GCCTCGTCCGATGTGTAG-3'). This fragment was designed to include the single polymorphic site detected in the pool frog samples (see Results).

PCRs each contained 25–50 ng DNA (or unknown amounts from museum specimens), 50 mM Tris-HCl pH 8.5, 16 mM ammonium sulphate, 3.5 mM $MgCl_2$, 100 μM each of dATP, dGTP, dCTP and dTTP, 0.2 μM of each (forward and reverse) primer and 0.5 units of GENPAK (New Milton, U.K.) *Taq* express DNA polymerase, all in a final volume of 20 μl. PCRs were carried out using 1) a single cycle of 94 °C × 4 min; 2) 30 cycles with 94 °C × 1 min, 55 °C × 1 min and 70 °C × 1 min; and 3) a single cycle of 70 °C × 5 min. Two to four replicates were included for every DNA sample to ensure adequate total yields, and all replicates were pooled together after the PCR for subsequent product purification. Samples (<10%) of the PCR products were electrophoresed through 1.5% agarose gels and stained with ethidium bromide to assess quality and quantity. In the case of museum specimens it proved necessary to purify all the products on agarose gels, followed by electroelution of the DNA fragments, either to separate them from primer dimers or to provide a substrate for a second round of amplification because quantities were very low.

PCR products were in all cases purified using Qiagen mini-columns (Qiaquick PCR purification kit, Qiagen, U.K.) and recovery was assessed by electrophoresis of 5% of the products on agarose gels together with marker DNA (lambda phage *Hind*III digest) of known concentration. Between 500 and 1000 ng of each sample were then dispatched for automated sequencing (both strands) by Genetix Ltd, New Milton, U.K.

Data analysis

Forward and reverse sequences were processed to generate consensus sequences using SEQMAN, trimmed to remove primer sequences using EDITSEQ, and aligned using the clustal procedure in MEGALIGN (all part of the DNASTAR computer program package). Our results were also compared with three other different *R. lessonae* cyt*b* sequences available from GenBank, one each from Italy (GenBank accession number AB029942), Ukraine (AB029944) and Luxembourg (AB029943). We used TCS 1.21 (Clement et al., 2000) to generate haplotype networks, using the 95% confidence limit, and the PHYLIP 3.66 package (Felsenstein, 1993) to generate phylogenetic trees. For tree construction, data were bootstrapped 1000 times using SEQBOOT, distance matrices were generated with DNADIST using its default evolutionary model (substitution model F84, transition/transversion = 2.0, and single category of substitutions), trees were created using NEIGHBOUR and summarized using CONSENSE.

RESULTS

Haplotype diversity in *R. lessonae*

Only two haplotypes, differing by a single base pair, were identified among the 53 pool frog cyt*b* sequences from our samples. Both translated completely, without stop codons, using the mitochondrial but not the nuclear genetic code. The difference between them, an A–G transition at the second position of a codon, resulted in a conservative substitution (serine–asparagine) at amino acid residue 75. Both haplotype sequences are lodged in GenBank (accession numbers AY057099 and AY057100 for A and B respectively). Sampling sites and the distributions of the two haplotypes are shown in Figure 1. Haplotype A was widely distributed and at 100% abundance in most of the European samples (in 45 frogs altogether), including in the northern clade populations of Norway, Sweden and Britain. However, only a few (five out of 17) museum specimens yielded reliable sequence information, though these were not in all cases the same individuals that provided microsatellite data. In particular two specimens (British Natural History Museum, collective accession number 2-20-5-7) from Fowlmere Fen in Cambridgeshire, neither of which generated microsatellite results, were successful with mtDNA. The three other successful specimens, from the same museum (collective accession number 44-50), were from Stow Bedon in Norfolk. Animals from this area, close to the last surviving pool frog population which died out during the 1990s, were among those successfully analysed with microsatellites. All the museum specimens were collected during the nineteenth century, and the frog population at Fowlmere became extinct during that period (Smith, 1951). Haplotype B was universal in the northern France (Paris) samples while in the small sample of Netherlands frogs both haplotypes occurred equally (eight frogs altogether had haplotype B).

Comparison with other ranid cyt*b* sequences

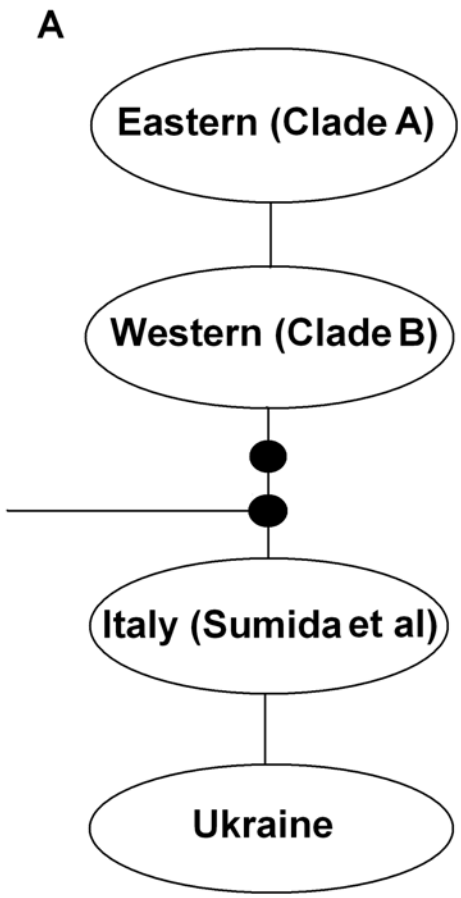
Although cyt*b* sequences have been obtained for many species of European *Rana/Pelophylax*, we focused in our

Table 1. Comparison of cyt*b* protein sequences among three *Rana* species.

	Amino acid		
Amino acid position	*R. lessonae*	*R. ridibunda*	*R. nigromaculata*
67	Valine	Valine	Isoleucine
75	Serine (A) Asparagine (B)	Asparagine	Asparagine
97	Leucine	Leucine	Phenylalanine
122	Leucine	Phenylalanine	Phenylalanine
133	Valine	Asparagine	Valine
150	Leucine	Phenylalanine	Leucine
157	Valine	Valine	Isoleucine
159	Threonine	Threonine	Proline*
178	Arginine	Arginine	Serine*

*Non-conservative change

analysis mostly on those from the well-characterized *lessonae–esculenta–ridibunda* hybridogenetic complex. In September 2007 GenBank held four *R. lessonae* partial cyt*b* sequences other than those described above. Two were obtained from *R. lessonae* specimens in Italy and Luxembourg, and one was from a *R. esculenta* in the Ukraine (Sumida et al., 2000b). It is well established that the hybrid *R. esculenta* usually carries the *R. lessonae* mitochondrial genome (Spolsky & Uzzell, 1986). A second Italian sequence from *R. lessonae*, as yet unpublished (AY043051), was also on the GenBank database. This was identical to our haplotype A, the only haplotype we found in the Torino samples. The Italian sample from Sumida et al. (2000b) differed from our haplotype B at three sites. These involved two transversions (A–T at site 23, G–T at site 65) and a T–C transition at site 322. The Luxembourg individual was very different from all the rest, with between nine and 11 variant sites. It was, how-



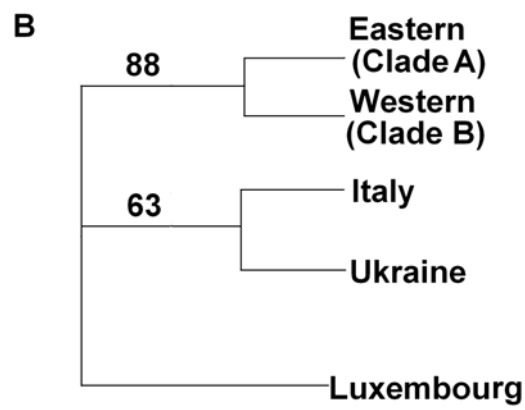


Fig. 2. Phylogenetic relationships of *R. lessonae* cyt*b* sequences. A) Haplotype network connected with 95% confidence. Haplotype A was found in Italy, Switzerland, Hungary, Poland, Sweden, Norway, the Netherlands and Britain. Haplotype B occurred only in France and the Netherlands. The Italy and Ukraine sequences are from Sumida et al. (2000b). The extraneous arm shows where Luxembourg connects, with a further 10 mutational steps, at 90% confidence. Solid black circles represent individual mutational steps generating haplotypes that were not detected. B) Phylogenetic tree (neighbour-joining method) showing percentage bootstrap support for the two clade groups.

ever, equally different from *R. ridibunda* (the only other frog species with which it is likely to be confused) and the Luxembourg sequence translated using the mtDNA genetic code without stop codons (analyses not shown). A comparison of ranid cyt*b* protein sequences is provided in Table 1. *Rana ridibunda* was included because of its widespread sympatry with *R. lessonae* in Europe, and the propensity of the two species to hybridize successfully. The gene sequence was obtained by Sumida et al. (2000b). *Rana nigromaculata* was included because a complete mtDNA genomic sequence is available for this species in GenBank, making it an invaluable reference point. Amino acid differences were mostly conservative and there was therefore no indication that either of the *R. lessonae* haplotypes A and B might be nuclear copies.

Haplotype relationships in *R. lessonae*

A network showing relationships between the four closely related haplotypes was connected with 95% confidence by the TCS program (Fig. 2A). The Luxembourg sequence could only be connected, via 10 extra mutational steps, with 90% confidence. For this reason, Luxembourg was used as an outgroup to construct the phylogenetic tree, using the neighbour-joining method (Figure 2B). Maximum parsimony generated a tree of identical topology (not shown). Evidently the two haplotypes found across northern and central Europe were more closely related to each other than to either of the more southerly sequences, from Italy and the Ukraine, obtained in other studies.

DISCUSSION

We set out to determine whether "northern clade" pool frogs were differentiated from those in central Europe on the basis of mtDNA sequence diversity, but found no evidence for this. The very low diversity of *R. lessonae* cyt*b* sequences no doubt contributed to this result. Sampling was carried out across the full range, and data included many individuals from the southernmost areas (northern Italy and Hungary, 22 individuals from at least five populations, including those obtained by others) likely to include glacial refugia. Further south in the Mediterranean peninsulas, *R. lessonae* is replaced by closely related species (such as *R. bergeri* in Italy and *R. perezi* in Spain). It is possible that a major refuge with higher diversity exists in the east, perhaps in Ukraine or beyond, that has not yet been sampled. However, if *R. lessonae* was excluded from more southerly refugia during the most recent glacial maximum around 20,000 years ago (perhaps by the presence of the closely related species), an alternative explanation is that it may have been severely bottlenecked at that time.

Low levels of cyt*b* polymorphism are common among north European amphibians. The northern crested newt *Triturus cristatus* shows a strikingly similar distribution to *R. lessonae*, and also exhibits unusually low polymorphism both in allozymes and in mtDNA RFLP analysis (Rafinski & Arntzen, 1987; Wallis & Arntzen, 1989). In a sample of 74 newts from across the biogeographical range, no substitutions were detected in the 258 bp of DNA sampled across the entire mitochondrial genome by restriction enzyme digestion. A single mitochondrial haplotype may therefore exist for this species over all northern Europe. Another comparable situation is the moor frog *R. arvalis*, which may have shared a similar (northern Balkan) refuge area with *R. lessonae*. Most of Europe is occupied by moor frogs containing only a single cyt*b* lineage, though three lineages occur in the southern refugia (Babik et al., 2004). A similar story was found with common frogs *R. temporaria*, with just two cyt*b* lineages (with seven haplotypes) across its European range, among which just two haplotypes accounted for 31 of the 37 frogs analysed (Palo et al., 2004). The spadefoot toad *Pelobates cultripes* has just three closely related cyt*b* haplotypes across northern and western Europe, but 12 in its Balkan refuge (Eggert et al., 2006). Low diversity is even the case for the control region, usually a very variable part of mtDNA, in *Bufo calamita* across northern and central Europe, although there is substantial haplotype diversity in its Iberian refuge (Rowe et al., 2006). *Rana lessonae* has moderate to high levels of diversity at microsatellite loci and this diversity is highest in the southern part of the range (Zeisset & Beebee, 2001), but microsatellites are much more sensitive indicators of polymorphism than either allozymes or mtDNA.

Our *R. lessonae* cyt*b* sequences were in most cases very similar or identical to those obtained from the same species in other studies (Sumida et al., 2000b), with the notable exception of one Luxembourg individual. We have no explanation as to why the Luxembourg sequence is so very different from all the rest obtained both by others and ourselves. One complicating issue with mtDNA analysis is that, quite often, nuclear copies of mitochondrial genes exist (Zhang & Hewitt, 1996). These copies mutate at the much lower rates characteristic of nuclear compared with mitochondrial DNA, and are therefore less diverse. However, several lines of evidence suggest that the sequences reported here are not nuclear. Firstly, there was always a single strong band of PCR product as visualized by agarose gel electrophoresis, with no hint of secondary bands. Such bands can arise where substitutions and deletions occur in nuclear copies. Secondly, there were no translation stop codons using the mtDNA genetic code but several such stops, in all reading frames, using the nuclear genetic code. Thirdly, it is unlikely that nuclear rather than mitochondrial sequences would be amplified from museum specimens. Finally, there was concordance of amino acid sequence with other ranids including only a few conservative changes relative to the sympatric *R. ridibunda*. Although the possibility that we have characterized a gene recently translocated to the nucleus cannot be completely ruled out, this seems unlikely on the basis of the above evidence.

The current haplotype distributions and relationships as shown in Figures 1 and 2 are compatible with relatively long occupation of some southerly regions (Italy and Ukraine with three haplotypes in a total sample of 12 frogs) and more recent colonization of northern Europe (two haplotypes in a total sample of 43 frogs, excluding Luxembourg). Survival somewhere in northern Europe may, however, have persisted through the last glacial

maximum. If haplotype B is ancestral to A, as Figure 2A implies from its closer relationship to the Sumida et al. (2000b) Italian sequence, a recent refuge in the west (France) may have permitted range expansion north-eastwards at the end of the glacial period. There is evidence that other ectotherms, including *Bufo calamita*, may also have survived the last glacial maximum in this region north of the Pyrenees (Rowe et al., 2006). Of course, other explanations are also possible. Haplotype B might have become regionally dominant in parts of western Europe by lineage sorting as a result of range expansion from a more easterly refugium during the postglacial warming. Microsatellite data also indicate a genetic east–west divide for *R. lessonae* and are compatible with recent colonization routes from Italy and/or Hungary (Zeisset & Beebee, 2001). For both types of loci, low diversity in the north is evident and predicted from predominantly leptokurtic dispersal and repeated founder effects, a likely pattern of events during postglacial colonization (Ibrahim et al., 1996).

The low diversity of cyt*b* in *R. lessonae* limited its usefulness with respect to inferring the origins of British frogs. There has been considerable debate about whether *R. lessonae* is native to Britain or was introduced by man (Snell, 1994). Microsatellite and RAPD analyses (Zeisset & Beebee, 2001; Snell et al., 2005), together with subfossil discoveries (Gleed-Owen, 2000) and call character analyses (Wycherley et al., 2002), strongly support native status (Beebee et al., 2005). Since known introductions of water frogs (including, presumably, *R. lessonae*) into Britain during the nineteenth century were from Paris and Belgium (Smith, 1951), the observation that haplotype B is common there but absent from British frogs is also compatible with native status. However, haplotype A clearly occurs in the Netherlands and the possibility that introductions with this genotype could have come from northwest Europe cannot be rigorously excluded.

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