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European phylogeography of the common frog (*Rana temporaria*): routes of postglacial colonization into the British Isles, and evidence for an Irish glacial refugium

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We use phylogenetic techniques to investigate the postglacial re-population of Europe by the common frog and, in particular, the colonization of Ireland. Three main hypotheses have been proposed for the re-establishment of the Irish fauna after the last ice age: arrival across a late-glacial land bridge from Britain; expansion from a glacial refuge in the south of Ireland and, for some species, re-introduction by humans from Iberia. We examined the phylogeographic structure of 52 populations of the common frog (*Rana temporaria)* throughout Europe using 476-bp mitochondrial cytochrome *b* gene sequences. Our data replicate earlier studies in showing substantial sequence divergence (3%) between Eastern and Western European common frog haplotypes. However, we uncover a new evidence that these haplotypes co-exist in Spain, Switzerland and France, and infer an expansion of the eastern clade along the Mediterranean coastal corridor. All the British samples fall within the Western European clade, but the Irish data imply a different history. Genetically distinct haplotypes occur in populations from the south-west of Ireland. This local genetic differentiation may be a consequence of a local glacial refuge, possibly combined with natural colonization or introduction from Western Europe.
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Introduction

The earth's climate appears to have warmed and cooled in cycles of approximately 100 000 years, with the last ice sheet last retreating approximately 10 000 years ago (Webb and Bartlein, 1992). These glaciations had profound effects on the distribution of temperate species, causing severe fragmentation of populations. In Europe, most temperate species were displaced southwards towards non-glaciated refugia. After glaciation, the species expanded northwards to occupy territories with improved climates and habitats, losing genetic variation along the way by founder events (Nichols and Hewitt, 1994). Isolation in refugia and the subsequent re-colonization events allowed populations to diverge genetically. These events shaped the genetics of many species so fundamentally that geographical differences can still be detected in contemporary populations, and colonization history can be re-constructed (Hewitt, 2000).

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Although still debated, the majority of evidence supports a general pattern of Southern European refugia in which temperate fauna survived and North-Eastern European refugia in which boreal or cold-temperate species survived (Malez, 1972; Taberlet *et al*., 1998; Hewitt, 2004). A study of 10 temperate taxa (including five amphibians; *Triturus* spp.) revealed similarities in their re-constructed colonization routes, with Northern Europe being colonized through three main routes, from Iberian, Italian and Balkan refugia (Taberlet *et al*., 1998). However, in addition to these large refugia, some authors argue that there were many small Pleistocene refugia in central and Northern Europe; such refugia would have been in sheltered regions with relatively stable microclimates and have been identified in Norway, Belgium, the United Kingdom, Hungary, Ireland and Slovakia (for review, see Steward and Lister, 2001).

Despite similarities in the broader patterns of colonization, differences have been inferred in the history of colonization of different species (Taberlet *et al*., 1998). For example, a thorough study on the white oak (*Quercus* spp.) using chloroplast DNA variation concluded that these trees appear to have colonized Britain through France, having followed a route from a refugium in Iberia (Dumolin-Lapegue *et al*., 1997). Studies on pool frogs (*Rana lessonae*) show an interesting alternative route into Britain through Poland from a refugium in Italy,

based on microsatellite and random amplified polymorphic DNA analyses (Zeisset and Beebee, 2001; Snell *et al*., 2005). These distinct histories might be explained by differences in the ecology of the species, differences in the refugial origins of the colonizers or by events affecting the rate of expansion from different refuges. The postglacial colonization of Ireland is particularly interesting due to the wealth of conflicting evidence. There are three main hypotheses explaining the postglacial re-establishment of the Irish fauna, which are not mutually exclusive: a species may have arrived by immigration across a land bridge from Britain, it could have survived throughout the glaciations in a refuge in the south of Ireland, and then expanded, or it could have been translocated by humans.

Some authors have argued that a late-glacial land bridge existed between Ireland and Britain, based on sea-level modelling (Wingfield, 1995; Lambeck, 1996; Lambeck and Purcell, 2001) and biostratigraphic evidence (Preece *et al*., 1986). Although there is limited evidence for species colonizing Ireland through a land bridge, existing evidence comes from the fossil remains of the mountain hare (*Lepus timidus*) and the stoat (*Mustela erminea*) found in Ireland. The samples date to the late-glacial period before humans arrived in Ireland, ruling out human introduction (Woodman *et al*., 1997; McCormick, 1999). However, these data could be re-interpreted not as an evidence of colonization through a land bridge but as the animals having survived the glaciations within Ireland, as both species are cold tolerant (Stuart and Van Wijngaarden-Bakker, 1985). The position (and existence) of any land bridge remains unresolved, with suggestions that it spanned from Scotland (Devoy, 1985) or from France through the south-west of Britain (Lambeck, 1996; Lambeck and Purcell, 2001). Under the land-bridge hypothesis, we would expect to see British and Irish samples occurring within the same monophyletic group. However, a high divergence between British and Irish populations has been found in certain species. For example, Hamill *et al*. (2006) found that populations of mountain hares (*L. timidus*) in Scotland and Ireland belonged to different mitochondrial DNA (mtDNA) clades and showed high divergence based on nuclear microsatellite data, reporting F_{ST} values above 0.4.

Another possible colonization route into Ireland is anthropogenic introduction. Some evidence suggests that humans could have introduced species from Iberia, as trade routes are thought to have existed between Ireland and Iberia over 2000 years before present (Praeger, 1939; Corbet, 1961, 1962; O'Rourke, 1970). Several species are either found only in South-West Ireland and Iberia, or have very different distributions in the geographically intermediate countries (for example, the pygmy shrew (*Sorex minutus*), Mascheretti *et al*. (2003); the strawberry tree (*Arbutus unedo*), Mitchell (1986); and the Kerry slug (*Geomaculosus maculosus*), Platts and Speight (1988)). Under this hypothesis, we would expect to see Irish and Iberian species falling within the same monophyletic group.

An alternative explanation for divergence between British and Irish populations is that of a refugium in Ireland. The Pleistocene ice sheets may not have covered the whole of Ireland and there is evidence that the south-west of the island remained an ice-free area of open steppe tundra (Forbes, 1846; Yalden, 1999). This ice-free region is hypothesized to have acted as a refugium in which species could have survived the glaciations (Forbes, 1846; Hoarau *et al*., 2007). Under this hypothesis, we might expect to find unique haplotypes in Irish populations, which are divergent from those found elsewhere in Europe, forming a distinct monophyletic group. Microsatellite and mtDNA evidence from Natterjack toads (*Bufo calamita*) indicates higher divergence times between Northern England and Ireland compared with Southern England and Ireland (Rowe *et al*., 2006). This finding led Rowe *et al*. (2006) to propose a source population between South-West England and Ireland, an area that would have been a partly dry land at the time (approximately 11 000 years before present) (Devoy, 1985).

The study organism

The common frog, *Rana temporaria*, inhabits the United Kingdom and Ireland and is widespread throughout Europe (Figure 2). This species provides a good model system for examining postglacial colonization as it is widespread throughout Europe, and has the greatest genetic variability of all western Palaearctic brown frogs (Reh and Seitz, 1990). Common frogs can survive in very cold environments and can be found at high altitudes; the highest recorded are in the French and Swiss Alps at approximately 2630 m (Gasc *et al*., 1997). Palo *et al*. (2004) surveyed European populations of common frogs and carried out analysis of mitochondrial cytochrome *b* sequences and variation in allele frequency at nuclear microsatellite loci. They found evidence for separate western and eastern lineages; the differences in DNA sequences were interpreted to mean that they shared a common ancestor approximately 700 000 years ago, coinciding with the onset of Pleistocene glaciation. However, common frog populations have not been well studied in the British Isles and could shed light on how these islands were colonized following the glacial periods. This study provides additional phylogeographic coverage of Europe, with particular reference to assessing evidence for the various potential colonization routes into Britain and Ireland. To approach these questions, we examine variation in mtDNA cytochrome *b* (Cyt*b*) gene sequences in populations of *R. temporaria* throughout Europe, with focussed sampling in Ireland. Mitochondrial DNA can be used to infer population histories dating back to the Pleistocene as the mutation rate is rapid enough to create variability, whereas the absence of recombination makes the inference of relationships between haplotypes straightforward (Avise, 2000).

Materials and methods

Samples were collected from the United Kingdom and the Republic of Ireland, and were donated from colleagues in Russia, France, Switzerland, Poland, Spain, Denmark, Italy, Finland, Germany, Sweden and Austria. Samples from Sweden, Denmark and Finland were extracted using a standard salt-extraction method (Aljanabi and Martinez, 1997), whereas those from Italy were extracted using the protocol as described by Boyle *et al*. (2004). All other samples were extracted using the Wizard SV96 Genomic Purification System (Promega, Southampton, UK). In total, 131 individuals from 52

locations ($n = 1$–6 per location) were used for mtDNA sequencing and analysis.

DNA was amplified using PCR, which was performed using primers L14850 (5′-TCTCATCCTGATGAAAC TTTGGCTC-3′; Tanaka *et al*., 1994) and H15410 (5′-GTCTTTGTAGGAGAAGTATGG-3′; Tanaka *et al*., 1996), which amplify a 605-bp Cyt*b* segment (Palo *et al*., 2004). Reactions consisted of 8 μl of Qiagen Taq PCR Master Mix (Qiagen, Crawley, UK), 1 μl of each primer at 100 pmol μl^{-1}, 8 μl of autoclaved and double-distilled water and 4 μl of extracted DNA. The PCR programme was as follows: 12 min of denaturation at 95 °C followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s and extension at 72 °C for 40 s, followed by a final extension phase at 72 °C for 7 min. PCR products were run on a 1.2% agarose gel using 5 μl of PCR product with 3 μl of loading buffer and a 100-bp ladder (Microzone Ltd, Haywards Heath, UK). When the PCR produced a clear band at the expected size, the band was cut from the gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, UK). Gel-extracted samples were used for direct sequencing. For sequencing, the reagents used were 5 μl of Better Buffer (Microzone Ltd, UK), 1 μl of BigDye Terminator 3.1 (Applied Biosystems, Warrington, UK), 1.5 μl of primer at 16 pmol μl^{-1}, 4.5 μl of autoclaved double-distilled water and 3 μl of purified PCR product making a total reaction volume of 15 μl; one reaction was performed using each of the two original primers to obtain overlapping forward and reverse sequences. The sequencing reaction PCR programme specified 3 min of denaturation at 96 °C followed by 25 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 10 s and extension at 60 °C for 40 min. The 15-μl sequencing reaction products were then cleaned to remove unbound terminators using an EDTA/ethanol-based protocol (see Supplementary information). Cleaned products were denatured at 95 °C for 2 min followed by snap cool before automated sequencing on an ABI 3100 Genetic Analyzer (Applied Biosystems).

Sequences were aligned using Sequencher v.4.8 (Gene Codes Corporation, Michigan, MI, USA), and clean sequence was obtained for 476 bp for all samples. For locations where multiple samples were of the same haplotype, a consensus sequence was produced for each haplotype present. From these condensed data (GenBank accession numbers: FJ030808–FJ030872), polymorphic sites and haplotypes were identified using TCS v.1.21 (Clement *et al*., 2000). MODELTEST (Posada and Crandall, 1998) was used to test 56 possible DNA-substitution model parameters. The best-fit model was used to generate a maximum likelihood phylogenetic tree using Phyml Online (Guindon *et al*., 2005), with 100 bootstrap replicates to assess node support. Bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist, 2001) with random start trees, sampling every 100 generations for 5×10^5 generations using the Hasegawa-Kishino-Yano (HKY) model of evolution. Neighbour-joining and maximum parsimony trees were constructed using Phylip programmes (Felsenstein, 1989) with 100 bootstrap replicates. Trees were constructed using a published *Rana arvalis* Cyt*b* sequence (GenBank accession number: AY156954, Palo and Merilä, 2003) as an outgroup. Multiple phylogenetic methods were used to allow for the collation of consistent results and the avoidance of artefactual results (as per Holder and Lewis, 2003).

Results

Eighteen haplotypes were identified from the 131 sequences analysed (see Supplementary Table 1). More than one sequence was obtained for 40 of the 52 locations, often with identical haplotypes (see Figure 1). We identified 26 polymorphic sites, four with transversions and no insertions or deletions. MODELTEST (Posada and Crandall, 1998) determined that the least-complex model of evolution with the best fit to the data under the hierarchical likelihood ratio tests was the HKY + G model, which allows for different transition and transversion rates and different rates of evolution at each nucleotide position (Hasegawa *et al*., 1985). The parameters of the model were base frequencies: $A = 0.2370$, $C = 0.2770$, $G = 0.1644$, $T = 0.3216$; ti/tv ratio = 8.2987; and a γ distribution shape parameter of 0.1991 to describe the among-site rate variation at variable sites.

Bayesian, maximum likelihood, maximum parsimony and neighbour-joining trees all showed support for the same major clades, with few minor variations (Figure 1). Two deep lineages were indicated, separated by mutations at 12 nucleotide positions. One lineage was found predominantly in the Eastern Europe (to the east of France and Germany) and the other in the west. The extended sampling also revealed a distribution undescribed earlier of the eastern clade along the French Mediterranean coast and into Spain (Figure 2). English and Welsh samples clustered in the main western clade, together with one Scottish sample. The majority of the Scottish samples clustered with Spain and one South-Eastern French sample in a western sub-clade. Samples from throughout Ireland grouped with the main western clade; however, distinct Irish haplotypes also diverged from the western clade and consisted almost exclusively of Southern Irish samples (Figure 2). One Irish sub-clade (haplotypes 13–15) showed a unique change in one amino acid from alanine to valine.

Discussion

A split within Europe

Expansion from different glacial refugia has affected the current distributions of genetic lineages. Northern Europe was colonized from three main refugia, in Iberia, the Balkans and Italy (Taberlet *et al*., 1998). Italian lineages of some taxa (for example, the grasshopper *Chorthippus parallelus*) did not colonize Northern Europe as the Alps acted as a barrier (Taberlet *et al*., 1998). A substantial split between eastern and western mtDNA sequences in the common frog was identified by Palo *et al*. (2004) and is replicated in our data. Schmeller *et al*. (2008) used mtDNA to investigate five common frog populations in Northern Germany and established that there is a contact zone between the eastern and western lineages in North-East Germany; this finding is consistent with our data (Figure 2).

However, our data demonstrate an overlap between the eastern and western lineages not found earlier. The eastern clade penetrates along the French Mediterranean coast and over the Pyrenees into Spain. The clades co-occurred in the Ceillac population (France, Figures 1 and 2); in this population, the western clade is

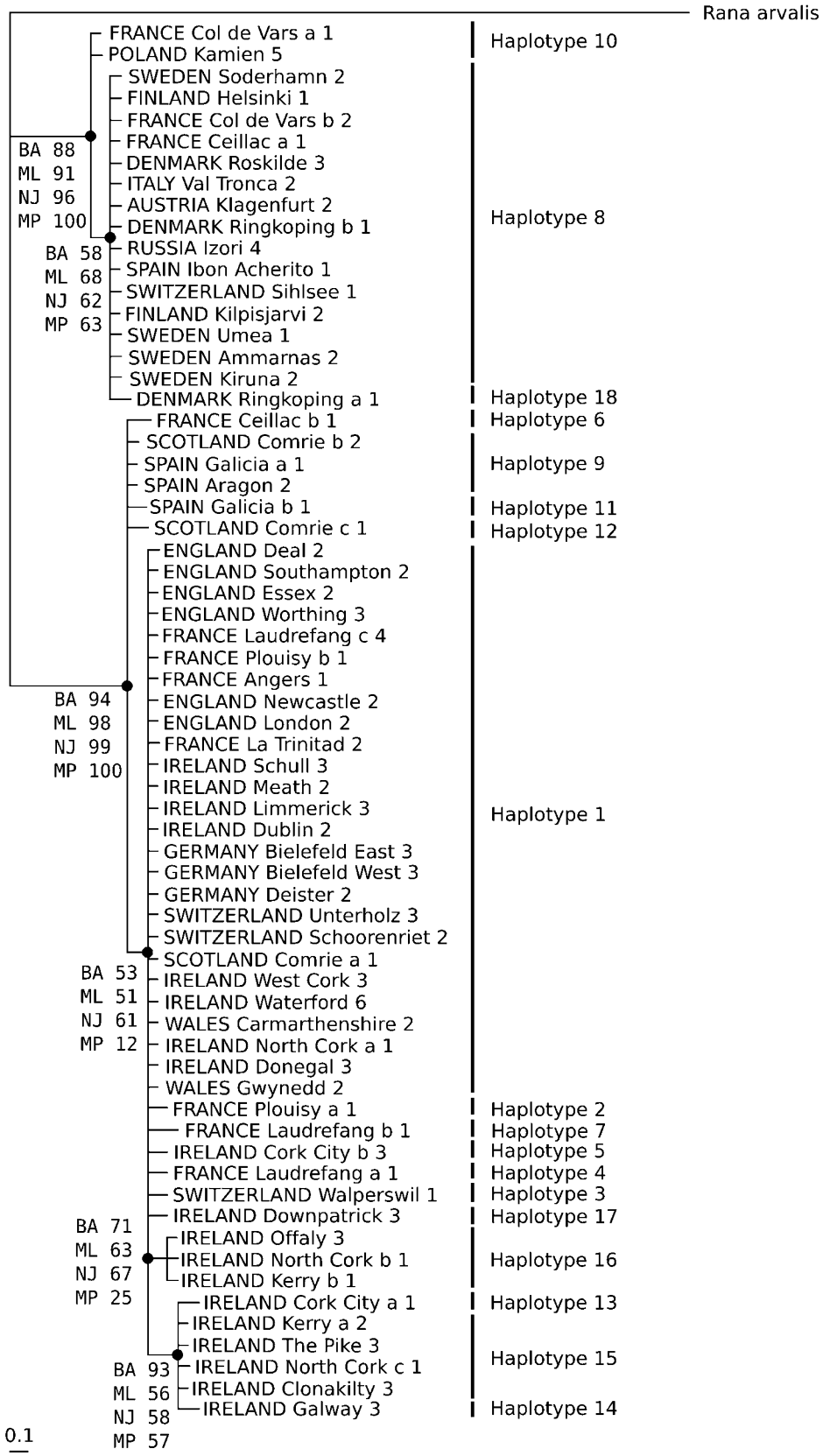

Figure 1 Phylogenetic tree. Bayesian tree based on 476-bp Cyt*b* sequences, with haplotypes marked. Support values are represented at major nodes for all statistical methods of tree building used (BA, Bayesian, ML, maximum likelihood, NJ, neighbour joining, MP, maximum parsimony). If more than one haplotype was found at a single location, the location name is followed by a letter (a, b and c). Following each location name is a number that represents the number of sequences obtained of the same haplotype at the same location.

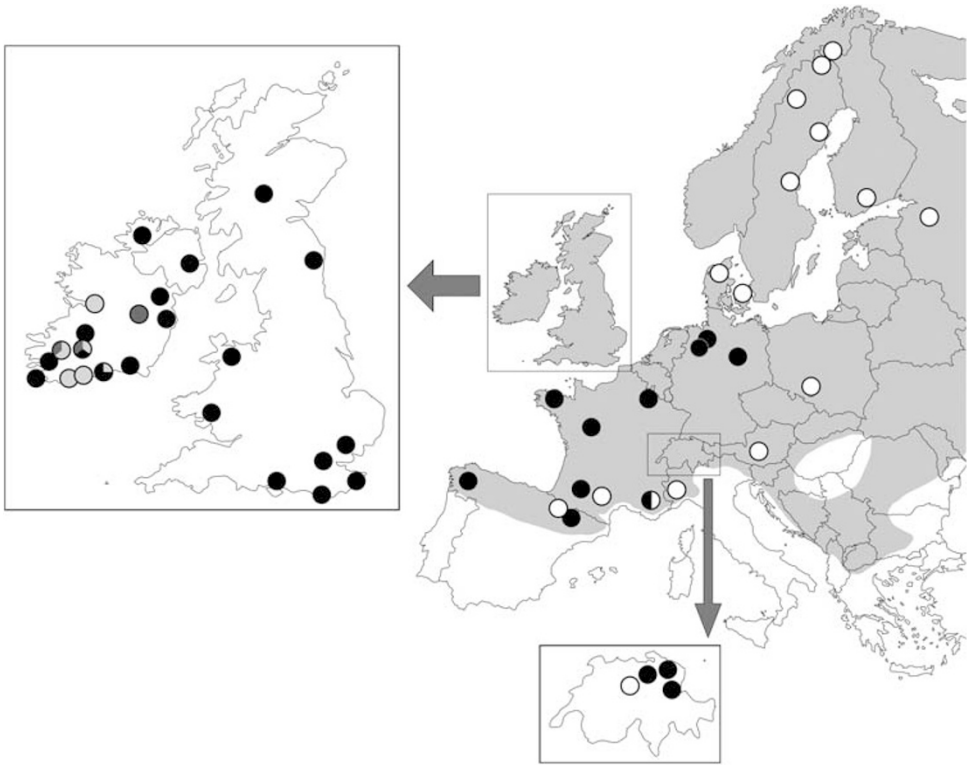

Figure 2 Haplotype distribution in Europe. Map to show the distribution of European clades. The eastern clade (haplotypes 8, 10 and 18) is shown by white circles, and the main western clade (haplotypes 1–7, 9, 11, 12 and 17) is shown by black circles. The approximate range of *Rana temporaria* is shaded in grey (Gasc *et al*., 1997). On the inset, map of the British Isles, Irish sub-clade one (haplotypes 13–15) is shown in pale grey, and Irish haplotype 16 is shown in dark grey.

represented by a unique haplotype (haplotype 6) that differs from the main western clade (haplotype 1) by three nucleotide polymorphisms, two of which are identical to those found in the main eastern haplotype (haplotype 8). It is possible that this is caused by homoplasy or by the retention of an ancestral state that has been lost in other western haplotypes.

North-Eastern Spanish, South-Eastern French, Northern Italian, one Swiss and all Austrian samples group with Fennoscandian samples in the eastern clade. Regardless of the direction of colonization, the route linking these populations follows a contour around the major mountain ranges of the French and Italian Alps and the Pyrenees. Although the Alps appear to have acted as a barrier to some species (including newts, *Triturus carnifex*; Taberlet *et al*., 1998), common frogs survive at very high altitudes in these mountains (Gasc *et al*., 1997). Our sampling locations in the Italian Alps (Val Troncea) and French Alps (Ceillac) imply that the Alps form a permeable barrier to the common frog, restricting rather than preventing gene flow. Nevertheless, the distribution of the eastern clade along the Mediterranean coast suggests that the mountains may have channelled colonizing founders. Ancestors of the western clade most likely originated in an Iberian refugium, colonizing North-Western Europe as the climate warmed. The distribution of the eastern clade indicates that the refugial populations could have been situated in Italy or the Balkans, with expansion both into North-Eastern Europe and to the west along the Mediterranean coast (Figure 2). Pidancier *et al*. (2003) also identified an east/west divergence in this species; however, they found that the western clade extended east along the Mediterranean coast to Croatia. No overlap was found between the lineages, and the authors interpreted these patterns as representing a westerly expansion from Italy and an eastern expansion from the Balkans. It would be interesting to perform intensive sampling along the contact zone in the south of France and Northern Spain to provide a more complete picture of the region where the ranges of the eastern and western haplotypes overlap.

Earlier studies on other amphibian species in Eastern Europe have implied multiple refugia in the east. For example, a study on mtDNA haplotypes of fire-bellied toads (*Bombina bombina* and *Bombina variegata*) in South-Eastern Europe showed that there were three distinct clades and suggested refugia in the Carpathians and Apennines as well as in the Balkans (Hofman *et al*., 2007). A study on mtDNA haplotypes in the moor frog (*R. arvalis*) indicated that multiple lineages co-exist in Eastern Europe and may originate from refugia in the Carpathians and Southern Russia (Babik *et al*., 2004). In our study, the eastern clade provides no indication of multiple refugia in this region; however, our sampling range did not extend as far east as these other studies; sampling further into Eastern Europe could prove fruitful for assessing the existence of additional refugia in this area.

The colonization of Britain

Zeisset and Beebee (2001) provided evidence for the colonization of pool frogs (*R. lessonae*) into Britain from

Poland through an eastern route originating in Italy; microsatellite data (six loci) grouped British samples into a distinct northern clade with Norway and Sweden. Our data provide no support for this hypothesis in the common frog, as British frogs fall within the western clade whereas Polish and Scandinavian frogs are in the eastern clade. All samples from England and Wales are representative of the main western clade, and so colonization of these areas is likely to have occurred through France or nearby surrounding regions. However, it is possible that microsatellites (as analysed for *R. lessonae*) and mtDNA may give different results for the same populations; microsatellite markers are faster evolving, and so are better suited to provide information on fine-scale and/or contemporary distributions and genetic diversity (for example, Bowcock *et al*., 1984).

Three samples were obtained from the same location in Scotland, comprised of three distinct haplotypes. Two of these Scottish haplotypes group closely with Spanish samples. One speculative explanation is that they provide evidence for an anthropogenic translocation from Iberia. If such an introduction has occurred, it is likely to be recent or isolated as these haplotypes are not found elsewhere in the British Isles. However, a more parsimonious explanation might be that of retained ancestral polymorphism, or of homoplasy. It is not possible to distinguish between these possible explanations using our data; however, additional molecular markers and sampling within Scotland could provide an answer in the future.

The colonization of Ireland

The haplotypes found in Ireland are particularly interesting, as some form monophyletic groups that are unique to Ireland whereas the others group within the main Western European clade. We propose two hypotheses to explain this pattern: (1) Irish frogs survived in a refugium in Ireland where some acquired novel mutations and others retained their ancestral state and (2) a dual colonization occurred in which some frogs survived in a refugium in Ireland (the Irish haplotypes) and some frogs colonized through a land bridge or were introduced from Western Europe (the ancestral haplotypes). Interestingly, there is a historical reference to a Fellow of Trinity College Dublin moving common frogs from England to Dublin in 1696, and others have hypothesized that this was the route of colonization (Smith, 1964). An alternative hypothesis—that of an anthropogenic introduction to Ireland from Iberia—gains no support from our data as populations in Ireland show no specific similarity to those in Spain. A study by Veith *et al*. (2003) found a unique common frog haplotype in one sample from North-Western Spain (Serra da Capelada), which they interpreted as representing a sub-species (*R. t. parvipalmata*). On inspection of the nucleotide sequence, this sample fits most closely with the main eastern haplotype (sharing 7-bp mutations) and is highly divergent when compared with all Irish haplotypes, thus again providing no evidence to support an Iberian-Irish link in the common frog.

Conclusions

Postglacially established common frogs (*R. temporaria*) are divided into Western and Eastern European lineages. The ranges of these lineages overlap along the French Mediterranean coast and as far west as the Spanish side of the Pyrenees, and both lineages co-occur in at least one population in Southern France. Britain appears to be colonized from the western lineage, with a probable origin in an Iberian refugium. Ireland has haplotypes belonging to the western lineage as well as some unique mtDNA sequences that are consistent with survival in an ice-free refugium in Ireland. Thus, a dual colonization of Ireland may have occurred, and contemporary Irish common frog populations may have ancestors both from a refuge within Ireland and from the Western European lineage. Our results indicate that the postglacial colonization of Europe by common frogs may be more complicated than has previously been assumed, as an additional potential refugium for this species has been identified. It is possible that a more detailed sampling within Europe could reveal further small, cryptic refugia.

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