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Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*

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Understanding how species are constrained within their biogeographical ranges is a central problem in evolutionary ecology. Essential prerequisites for addressing this question include accurate determinations of range borders and of the genetic structures of component populations. Human translocation of organisms to sites outside their natural range is one factor that increasingly complicates this issue. In areas not far beyond presumed natural range margins it can be particularly difficult to determine whether a species is native or has been introduced. The pool frog (*Rana lessonae*) in Britain is a specific example of this dilemma. We used variation at six polymorphic microsatellite loci for investigating the phylogeography of *R. lessonae* and establishing the affinities of specimens from British populations. The existence and distribution of a distinct northern clade of this species in Norway, Sweden and England infer that it is probably a long-standing native of Britain, which should therefore be included within its natural range. This conclusion was further supported by posterior probability estimates using Bayesian clustering. The phylogeographical analysis revealed unexpected patterns of genetic differentiation across the range of *R. lessonae* that highlighted the importance of historical colonization events in range structuring.

Keywords: *Rana lessonae*; microsatellites; phylogeography

1. INTRODUCTION

Biogeographical ranges and the delimitation of species' distributions have fascinated naturalists for hundreds of years and numerous ecological and genetic theories relate to the problem (e.g. Hoffmann & Blows 1994; Lennon *et al*. 1997; Gaston 1998). However, central to any study of this issue is accurate determination of range borders. Recent human activities, notably the widespread movement of many species around the globe, increasingly complicate this apparently straightforward matter. Thus, in Britain most populations of wild cabbage (*Brassica oleracea*) were probably introduced by Roman or Saxon invaders, but some may have arrived naturally (Mitchell 1976). Several small mammals, including shrews (*Sorex coronatus*, *Crocidura russula* and *Crocidura suaveoleni*) and the mouse *Apodemus flavicollis*, have uncertain origins on many small islands and, in the case of *Apodemus*, even on mainland Britain (Lever 1979). Other examples include the frog *Discoglossus pictus* in southern France (Martens & Veith 1987) and the chameleon *Chamaeleo africanus* in southern Greece (Kosuch *et al*. 1999). Knowledge of origins in such cases is also important for deciding local conservation priorities and may even be invoked in choosing between conservation and eradication.

The pool frog *Rana lessonae* is a good illustration of the range border problem. This amphibian is widespread in much of mainland Europe, with isolated northerly populations in Scandinavia that are generally accepted as native (Gasc 1997). However, in Britain several introductions of water frogs, including *R. lessonae* and its hybridogenetic relative *Rana esculenta*, have been documented since the 1830s (Smith 1951) and it has been widely assumed that all British pool frog records are of their descendants. *Rana esculenta* is a viable, fertile hybrid formed by crosses between *R. lessonae* and another water frog *Rana ridibunda*. The hybrid is perpetuated hemiclonally in mixed populations with one or other of the parent species. This is made possible by a complex process (hybridogenesis) involving the pre-meiotic loss of one parental genome in primordial germ cells (Graf & Polls Pelaz 1989). Most of the introduced water frog populations died out within a few decades (Smith 1951), but one mixed population of *R. lessonae* and *R. esculenta* has thrived in Surrey since its initial establishment in approximately 1905 (Gillett 1988).

Irrespective of these introductions, the natural range of *R. lessonae* includes countries neighbouring Britain and a recent re-evaluation of historical records together with new fossil evidence has indicated that some British pool frogs, specifically those from an area in Norfolk with long-standing records, might be native (Buckley 1986; Snell 1994; Gleed-Owen 2000). We hypothesized that a robust phylogeography should clarify the likely origins of British pool frogs and, thus, whether Britain is within the natural range margin. Molecular genetic markers have been used successfully with other European species in tracking post-glacial migration patterns (e.g. Taberlet *et al*. 1998; Garcia-Marin *et al*. 1999; Hewitt 1999; Beebee & Rowe 2000) and we carried out such a study on *R. lessonae* using six polymorphic microsatellite loci.

2. METHODS

Depending on availability, between 5 and 40 individual *R. lessonae* were sampled at ten sites across Europe (figure 1) and their tissues stored in ethanol prior to use. The species is very rare in Scandinavia and only five and 12 specimens were available from Norway and Sweden, respectively. Only one potentially native population of *R. lessonae* survived until recently in

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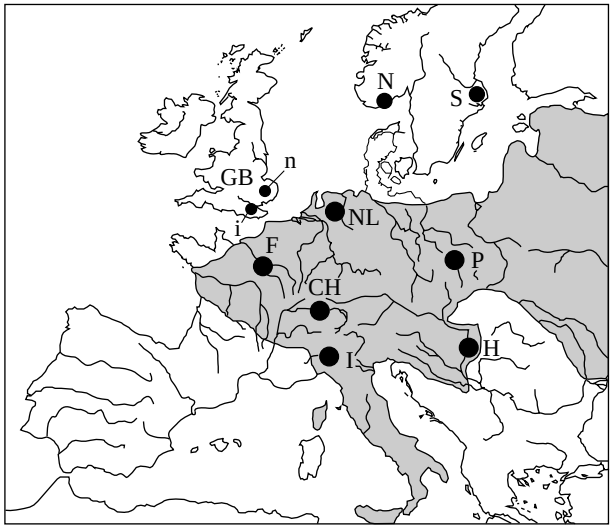

Figure 1. Distribution of *R. lessonae* in Europe. Shading indicates the approximate limits of the central range area. Sampling sites are as follows: I, Italy; CH, Switzerland; H, Hungary; P, Poland; F, France; NL, The Netherlands; S, Sweden; N, Norway; GB, Great Britain (i, introduced; Surrey and n, potential native, Norfolk).

Britain but the last individual from this Norfolk site died in 1999. However, tissues from this animal and from four museum specimens of *R. lessonae* collected in the same area of Norfolk (Stow Bedon district) over the past 150 years were available for analysis. The museum specimens included two from the Norwich City Museum collected by A. and E. Newton in 1853 and two from the British Museum of Natural History, London (store reference 44–50), collected in 1884. In addition, we took samples from living animals at the Surrey site where *R. lessonae* was introduced from France and Belgium *ca.* 100 years ago and from an East London Museum specimen (no. 1451) of *R. esculenta* taken in 1884 from a separate Norfolk site (Wereham Fen) where water frogs were introduced early in the nineteenth century. DNA was mostly extracted from adult digits or, in the case of museum specimens, from skin and tongue fragments and used in polymerase chain reaction (PCR) assays with primers designed for six dinucleotide-repeat microsatellite loci. Four of these are described elsewhere (Zeisset *et al.* 2000) while the other two were characterized and the primer information kindly communicated to us by T. Garner (Garner *et al.* 2000). Briefly, the latter two loci were RICA18 (a clone including a $(CA)_{22}$ microsatellite, generating a PCR product of 177 base pairs) and RICA19 (a clone including a $(CA)_{15}$ microsatellite, generating a PCR product of 129 base pairs). Museum DNA was extracted in a separate laboratory with dedicated equipment (Beebee *et al.* 1998) using standard precautions against cross-contamination including blank extraction controls. ^{33}P-labelled products were electrophoresed on 6% polyacrylamide gels and alleles identified by autoradiography using M13 sequence markers (Rowe *et al.* 1998).

Allele frequencies and mean allele numbers per locus, heterozygosities and polymorphism estimates were calculated using BIOSYS-1 (Swofford & Selander 1981). Phylogeographical analyses including genetic distance estimates and tree construction were carried out using the PHYLIP programs (Felsenstein 1993). The population affinities of British *R. lessonae* were tested using STRUCTURE (Pritchard *et al.* 2000), a Bayesian-based clustering model. Mantel tests of migration route correlations were performed using GENEPOP 3.1 (Raymond & Rousset 1995).

3. RESULTS

Microsatellite diversity was estimated in frogs from each sampling area as shown in table 1. The total number of alleles per locus identified across all populations varied from 6 (Res 16 and RICA19) to 21 (Res 20 and RICA18). One locus (Res 20) consistently exhibited homozygote excess and was largely responsible for the differences between the observed and expected mean heterozygosities that were evident to varying degrees in most of the sampled populations. Although there was more than one sampling site in France and Hungary, the pooled subsamples were used in the summary analysis of table 1 in order to generate the largest possible total regional sample. After exclusion of Res 20 neither the subsamples alone or pooled together nor the full samples from the other populations deviated significantly from Hardy–Weinberg equilibrium. All the animals from Norway, Sweden and Norfolk were monomorphic at all six loci and fixed for the same allele at five loci (table 2). However, the allele at locus RICA18 was Norway, Sweden or England specific. Between three and five of the six loci (mean 4.25) amplified successfully from the four *R. lessonae* museum specimens and all yielded homozygous genotypes identical to the last surviving Norfolk animal. Although it is possible that not all alleles amplified from the museum specimens, we believe it is unlikely that allelic drop-out significantly affected our results. There was insufficient material for a multiple tubes approach (Taberlet *et al.* 1996) but the consistency of genotypes between all the potentially native British individuals was striking. Samples from living specimens in Norway and Sweden were similarly monomorphic at all loci and repeat analysis of individuals from various populations, including one museum sample for which enough DNA was available, always yielded reproducible microsatellite genotypes.

Genetic diversity in the *R. lessonae* populations, as quantified by the mean expected heterozygosity (H_e), declined as a function of distance from Italy, the prospective glacial refugium (see below), as expected from colonization theory and in accord with various similar studies (e.g. Taberlet *et al.* 1998; Hewitt 1999; Beebee & Rowe 2000). H_e was normally distributed and its negative relationship with linear geographical distance from Italy (figure 2*a*) was highly significant ($r = -0.764$, d.f. $= 7$ and $p < 0.02$). Measures of genetic diversity were significantly intercorrelated (e.g. mean allele number per locus per population sample and expected heterozygosity) ($r_s = 0.6522$ and $p = 0.05$) and, therefore, exhibited a common general trend. As noted above, the Scandinavian and potentially native British populations, which were small and isolated at the northernmost range edges, were homozygous at all six loci. This was not true of the Surrey population of introduced British animals which had a mean H_e of 0.736.

Allele frequencies across all six loci were used for constructing phylogeographical trees for *R. lessonae*, one of which is shown in figure 2*b*. Omitting locus Res 20 or including it with estimated null allele frequencies

Table 1. *Microsatellite diversity of pool frog populations*

(P_{95}, percentage of loci polymorphic at the 95% criterion; H_o, observed mean heterozygosity; H_e, expected mean heterozygosity. The sample size for Norfolk in Great Britain included one living animal and four museum samples.)

sample area	sample size	mean number of alleles/locus	P_{95}	H_0	H_e
Italy (Torino)	18	5.5	83.3	0.443	0.611
Switzerland (Zurich)	40	3.0	100.0	0.337	0.399
Hungary (Little Batalon/Bockerek)	31	7.0	100.0	0.405	0.501
Poland (Wrocław)	41	4.5	66.7	0.216	0.311
France (Paris Lac du Der/Chantecoq/Le Brenne)	19	3.3	100.0	0.326	0.485
The Netherlands (Diever)	34	2.5	100.0	0.285	0.380
Sweden (Uppsala)	12	1.0	0.0	0.000	0.000
Norway (Arendal)	5	1.0	0.0	0.000	0.000
Great Britain (Norfolk)	5	1.0	0.0	0.000	0.000
Great Britain (Surrey)	11	5.2	100.0	0.483	0.736

Table 2. *North European pool frog microsatellite genotypes*

(The numbers are the allele sizes (base pairs) in the homozygotes at each locus. n = number of individuals genotyped from each country.)

sample origin	Res 3	Res 5	Res 16	Res 20	RICA18	RICA19
Great Britain ($n=5$)	129	131	108	92	168	92
Norway ($n=5$)	129	131	108	92	174	92
Sweden ($n=12$)	129	131	108	92	176	92

(Brookfield 1996) had negligible effects on the tree topology or bootstrap values. The neighbour-joining tree in figure 2 is based on the Cavalli-Sforza chord distance D_c (Cavalli-Sforza & Edwards 1967), a measure that assumes that drift is more important than mutation and allows for variable population sizes. D_c has proved particularly appropriate for microsatellite analyses of population structure (Takazaki & Nei 1996; Beebee & Rowe 2000). However, trees with very similar or identical topologies to that in figure 2 were also obtained using Nei's (1987) standard distance and maximum-likelihood (Felsenstein 1993) approaches (data not shown).

The results were consistent with Italy being the main glacial refugium for *R. lessonae* and subsequent northward colonization as a bifurcation in which animals moved west into France, Switzerland and The Netherlands and north and east into Hungary, Poland and Scandinavia. Italy is the only Mediterranean peninsula in which *R. lessonae* occurs today (figure 1), a distribution pattern that, on the basis of many studies with other organisms, is likely to indicate where the species survived the last glacial maximum (Hewitt 1999; Beebee & Rowe 2000). The potentially native British specimens clustered robustly with the Scandinavian populations, whereas animals from the Surrey introduction site in Britain clustered with those from areas close to their known origins in Western Europe. In addition, DNA from the single museum specimen of *R. esculenta* taken from a Norfolk introduction site (Wereham Fen) *ca.* 20 km from the possibly native *lessonae* population also amplified successfully at three loci (data not shown). The *lessonae* component of this animal's genome was very different from that of the nearby pure *lessonae* population, with typical west European alleles again accurately reflecting its known origin.

The affinities of the British *R. lessonae* were also investigated by a model-based clustering procedure (Pritchard *et al.* 2000). This approach allocates individual genotypes into clusters that most probably represent discrete populations or, in the case of migrants, into their most probable population of origin. It can also be used to ascribe the affinities of individuals that are not recent migrants to their likely origins if intervening genetic differentiation has been small. This was the case with the northern clade pool frogs, presumably because their genetic variation was low from the start due to strong founder effects at the leading edge of the colonization (Ibrahim *et al.* 1996). We used a model that assumed no migration and no admixture, a 100 000 burn-in period and 100 000 replications. The locus Res 20 was excluded from the analyses described below because there was a requirement for populations to be in Hardy–Weinberg equilibrium, although essentially identical results were obtained in other runs of the program when it was included. Initially we used the microsatellite data from five sampling sites, assuming five populations, in order to ascertain how discretely they clustered. These included the four sites geographically closest to Britain (France, The Netherlands, Norway and Sweden) and, thus, likely to include the closest post-glacial relatives of the potentially native British frogs and the site in Surrey where frogs were introduced *ca.* 100 years ago. As shown in table 3, all four of the non-British sampling sites showed high proportional membership (>0.9) to single inferred clusters. Those of France and The Netherlands were each unique, while a third cluster was ascribed with high probability to

Table 3. *Allocation of pool frogs using STRUCTURE*

(The numbers indicate the proportion of membership of each predefined population in each of five clusters.)

given population	inferred clusters				
	1	2	3	4	5
France	0.001	0.936	0.062	0.001	0.001
The Netherlands	0.009	0.052	0.939	0.000	0.000
Norway	0.984	0.000	0.016	0.000	0.000
Sweden	1.000	0.000	0.000	0.000	0.000
Great Britain (introduced)	0.019	0.005	0.003	0.495	0.479

frogs from Norway and Sweden. In contrast, the introduced Surrey population was distributed almost equally between two other and completely separate clusters. We then used prior population information for the four European sites, excluding the introduced one in Surrey (because the Norfolk frogs are known to pre-date the existence of this population), in order to test the affinities of the Norfolk genotype. It was unequivocally allocated to the Scandinavian clade with posterior probabilities of 0.394 for Sweden and 0.604 for Norway. However, a reservation for this approach stems from the absence of detectable genetic variation within each of the northern clade populations. This inevitably violates some of the assumptions upon which the model is based, notably those with respect to independent sampling of alleles and individuals. It is not clear to what extent this compromises the model, but its inferences were evidently compatible with those from the other data analyses described earlier.

Assuming that the current range limits of *R. lessonae* were reached naturally (see § 4), the genetic information also provides clues as to how the distribution of *R. lessonae* became established during the post-glacial warming period. Figure 3 illustrates some hypothetical post-glacial migration routes for *R. lessonae* that are broadly compatible with the genetic data and the autecology of the species. These anurans were likely to have moved mostly at low altitudes and particularly along river valleys. The genetics imply a bifurcating colonization of northern and northwestern Europe, but cannot distinguish between route A (with a relatively late separation after the Alpine–Carparthian gap) or route B (with initially separate east and west migration routes out of Italy) for the latter. The correlations between D_c and the linear, northerly plus route A and northerly plus route B geographical distances were 0.255, 0.707 and 0.575, respectively, using all 36 intersite distance measures. Since these comparisons were not independent the significance of the relationships was tested by Mantel permutations. Mantel tests using 10 000 iterations yielded probabilities of 0.0003 and 0.008 for the northerly plus route A and northerly plus route B correlations, respectively. However, the correlations between H_e and the distance from the Italian refugium were improved more by the northerly plus route B (to $r = 0.958$) than by the northerly plus route A (to $r = 0.845$) geographical distances relative to the linear distance correlation of figure 2. It is of course quite possible that the frogs took both of these routes and others.

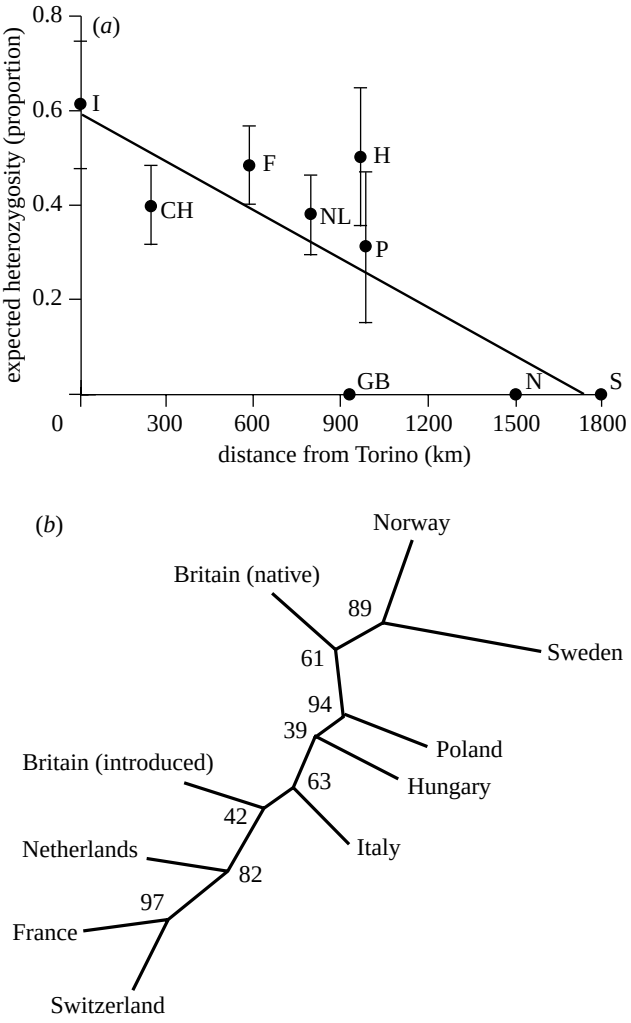

Figure 2. Phylogeography of *R. lessonae* in Europe. (*a*) The relationship between expected heterozygosity and linear distance of *R. lessonae* populations from the likely glacial refugium referenced as Torino in Italy. I, Italy; CH, Switzerland; NL, The Netherlands; H, Hungary; P, Poland; F, France; S, Sweden; N, Norway; GB, putative native population in Britain. Bars show the standard errors of the means across the six loci. (*b*) A consensus phylogeographical tree was constructed using Cavalli-Sforza chord distances and the neighbour-joining method. The numbers are percentages of bootstraps (out of 1000) in which population clusters distant from that node were conserved.

4. DISCUSSION

Although some genetic distance measures can be biased by variations in genetic diversity (Paetkau *et al.* 1997), tree topologies should be unaffected when clades are robustly supported by high bootstrap values, as was generally the case here. Taken together, the molecular phylogeographical analyses strongly inferred the existence of a distinct, genetically depauperate *R. lessonae* clade persisting in Norway, Sweden and Britain. The sample sizes from this clade were small but all three populations were each invariant at all six loci, making it unlikely that larger numbers would have significantly altered the

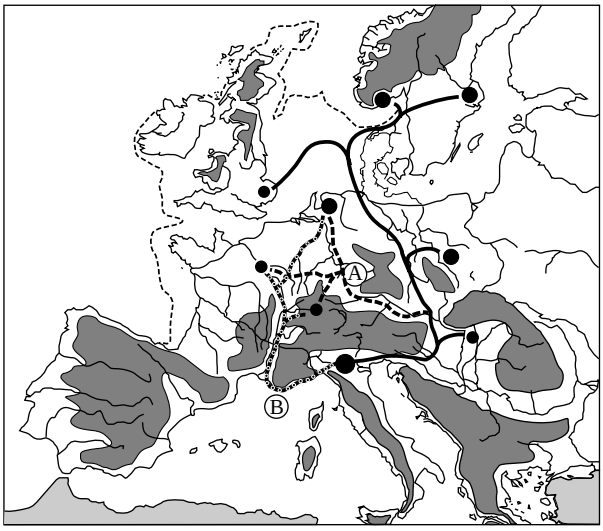

Figure 3. Hypothetical post-glacial migration routes of *R. lessonae*. Densely shaded areas represent mountainous regions and solid circles represent the genetic sampling sites. Major rivers are indicated, as is the likely coastline *ca.* 12 000 years before the present (dashed line).

analytical results. Various minor morphological and behavioural characters also support the existence of such a clade (Snell 1994), the current distribution of which could have two possible origins. It must be either a long-standing relict of post-glacial migration patterns or the result of recent introductions including the movement of frogs between Norway, Sweden and Britain. The latter explanation seems highly unlikely. No mechanisms are known by which amphibians can colonize naturally across such large intervening distances, particularly where this involves crossing large expanses of seawater. There are no records of humans moving frogs between these countries, which in any case seems improbable between localities that are all near the range edge where *R. lessonae* will always have been relatively rare. The current genetic pattern within the clade would also be hard to explain on this basis. The frogs in Norway, Sweden and Britain were genetically very similar but nevertheless nationally distinct at one of the six loci examined. On the basis of allozyme analyses of Swedish *R. lessonae* across 31 loci, Sjogren (1991) argued that the current population genetic structure could not be easily explained on the basis of a recent introduction by man. As with microsatellites, Swedish pool frogs exhibit very little polymorphism at allozyme loci (29 were monomorphic) whereas animals from Poland had much greater variability. The similarity of the patterns exhibited by both genetic markers was therefore striking. Although microsatellite mutation rates are higher than those of allozymes, they vary greatly between taxa (from 6×10^{-6} in *Drosophila* to *ca.* 5×10^{-4} in mice) (Goldstein & Schlotterer 1999) and have not been determined in amphibians. However, the mutation rates of nuclear loci in ectothermic vertebrates in general tend to be approximately ten times lower than in endotherms (Martin 1999). A generation time for *R. lessonae* of approximately four years in northern Europe (Sjogren 1991) and a microsatellite mutation rate of between 1×10^{-5} and 5×10^{-5} could account for the maintenance of similarity at five loci over 10 000 years with a probability (based on a simple stepwise mutation model) of >0.5 (Wilson & Balding 1998). More distant common ancestry or higher mutation rates reduce this probability substantially (e.g. to 0.28 at 1×10^{-4} and 0.0056 at 5×10^{-4}) and our phylogeographical model is clearly dependent on the dates and lower rates invoked above.

On the other hand, post-glacial migrations of frogs would very probably have reached their northern limit *ca.* 12 000 years ago when temperatures were higher than now but when most of the North Sea was still dry land (Vincent 1990). Evidence from the pollen record has indicated that northward colonization after the last glaciation was more rapid in the east than in the west of the continent (Taberlet *et al.* 1998; Hewitt 1999). This could explain why pool frogs did not reach Britain naturally by the apparently more direct route through France, since slower migration in this direction might have resulted in late arrival after the establishment of an uncrossable sea barrier. A plausible scenario is that a small number of vanguard *R. lessonae*, perhaps somewhere in the current North Sea region, were severely bottlenecked by the Younger Dryas cooling *ca.* 11 000–10 000 years ago. The survivors of this episode, now isolated and genetically impoverished, subsequently gave rise to the populations that have persisted in Norway, Sweden and Norfolk. North European *R. lessonae* have probably maintained mean effective population sizes of only a few tens over long periods of time (Sjogren 1991). Observations of fixation for a single allele at every locus are also in line with theoretical expectations for such small populations at equilibrium (Nei 1987). The much higher genetic diversity of pool frogs descended from introduced animals in Britain together with their clustering close to their documented sources of origin in Western Europe provides further confidence in the phylogeographical approach.

Phylogeographical analysis of *R. lessonae* has thus provided some useful and unexpected results. It supports a range that includes Britain, but with a surprising inference about the way in which the northerly range limits of this amphibian were probably attained. The role of history in generating genetic structure across biogeographical ranges can evidently be more complex than is widely realized. Molecular phylogeography based on variation at hypervariable loci has considerable potential in resolving such questions and identifying cryptic clades of potential significance for future evolution. Episodic range expansions and contractions during intermittent glaciations and interglacials have probably played important roles in the generation of temperate biodiversity (Willis & Whittaker 2000) and studies of this kind should also be of use in conservation biology.

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