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Phylogeography of the pool frog (*Rana lessonae* Camerano) in Europe: evidence for native status in Great Britain and for an unusual postglacial colonization route

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The pool frog, *Rana lessonae*, has historically been considered an introduction into Britain, with Italy the most likely source. Recently, the possibility of native status for a Norfolk pool frog population was raised. We used random amplified DNA (RAPD) analyses to clarify the status issue. Nine arbitrarily designed primers detected 160 polymorphisms in 174 pool frogs from 11 European locations. Polymorphism levels varied widely, being lowest in the northern populations and highest in the south. As with many isolated populations, the peninsular and insular populations of Sweden, Norway and Britain showed relatively little polymorphism. Principal component and cluster analysis showed clear geographical groupings. The Norfolk, Norwegian and Swedish individuals formed a closely related group – a northern clade, substantiating native status for the Norfolk population, which, interestingly, had idiosyncratic features suggestive of a distinctive evolutionary history. Phylograms constructed from RAPD data were consistent with an unexpected postglacial colonization route, in which the northern clade derived from migration out of Italy, via Hungary and Poland, though cryptic glacial refugia in Eastern Europe are an alternative explanation. Our RAPD analyses concur with a parallel microsatellite investigation. Both genetic studies and bioacoustic and archaeozoological findings support native status for Norfolk pool frogs and have prompted a programme for re-establishing the northern clade in England. *Biological Journal of the Linnean Society*, 2005, **85**, 41–51.

ADDITIONAL KEYWORDS: cryptic refugia – northern clade – RAPD – reintroduction.

INTRODUCTION

Until the mid-1990s, mainland Britain was considered to have only three native anurans: the common frog, *Rana temporaria* L., 1758, the common toad, *Bufo bufo* L., 1758 and the natterjack toad, *Bufo calamita* Laurenti, 1768. Other anurans in Britain – e.g. the 'water frogs', *Rana ridibunda* (Pallas, 1771), *R. esculenta* (L., 1758) *and R. lessonae* (Camerano, 1882) – were presumed to derive from introductions (Lever, 1980). Early in the 20th century the pool frog, *Rana lessonae* was restricted to Norfolk, although it had been widespread in East Anglia. It was presumed to be an introduction from Italy (Boulenger, 1884a, b; Gleed-Owen, 2000). Snell (1994) noted that these Norfolk pool frogs differed significantly from known introduced pool frogs in a number of respects, including their behaviour and brown coloration, and argued that these facts, together with the historical record, suggested a native origin for the Norfolk population. Snell (in Gent, 1996) also pointed out that these frogs looked, sounded and behaved much more like those in Norway and Sweden than the *R. lessonae* populations in neighbouring and other parts of mainland Europe.

English Nature, the statutory nature conservation organization in Britain, became interested in the possible native status of the Norfolk population, and commissioned a variety of research initiatives to explore this possibility. These included a further literature review, analysis of East Anglian subfossil finds, bioacoustic studies, and genetic investigations comprising microsatellite, mitochondrial DNA, and random amplified polymorphic DNA (RAPD) analyses. The archaeozoology demonstrated pool frog bones in Saxon

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sites dated to around 600–950 AD (Gleed-Owen, 2000), substantially predating known or probable introductions. The bioacoustic (Wycherley, Doran & Beebee, 2002) and microsatellite studies based on six loci (Zeisset & Beebee, 2001) supported the postulate, founded originally on field observations, that the Norfolk *R. lessonae* is more closely related to the Scandinavian than to the mainland European populations.

RAPD analysis (Williams *et al*., 1990) has well documented limitations including, in particular, the dominance of RAPD markers and the need to assume a maximum of two alleles per locus – problems which can be mitigated by maximizing sample size and numbers of loci examined (Lynch & Milligan, 1994). Another problem can be reproducibility (Williams *et al*., 1993; Fritsch & Rieseberg, 1996) though we were able to obtain good band reproducibility by careful use of an optimized protocol, including standardization of DNA concentrations.

However, the RAPD approach has proved very useful in quantifying genetic variation in amphibia at population and species level (Masters & Forrester, 1995; Kimberling *et al*., 1996; Zeisset & Beebee, 1998) and is particularly advantageous in the genetic study of rare and endangered organisms, where there are concerns about small sample sizes and maximizing the genetic information obtained (Kimberling *et al*., 1996). Two further positive attributes of RAPD are that the portion of the genome sampled is relatively unbiased, and that essentially unlimited numbers of loci can be generated (Fritsch & Rieseberg, 1996). Nevertheless, we certainly concur with the suggestion made by Fritsch & Rieseberg (1996), that RAPD is best used in conjunction with other types of molecular marker, and that is the case here, where a microsatellite approach (Zeisset & Beebee, 2001) has been applied to the same populations.

The RAPD study reported here represents the most extensive sampling of European pool frog population genomes to date (160 polymorphic loci examined in 11 frog populations), and strongly substantiates the proposition that the Norfolk *R. lessonae* population is native and belongs to a distinct northern clade of this species, occurring in Sweden, Norway and Britain. Furthermore, our analysis indicates the possible presence of distinctive genetic features in the Norfolk population and is consistent with an unexpected colonization route from Europe. Unfortunately, by the mid-1990s the known Norfolk population was reduced to a single adult male, which died in 1999. This work and the other studies cited above have prompted English Nature to attempt the re-establishment and conservation of the *R. lessonae* northern clade in England, using pool frogs from Scandinavia.

MATERIAL AND METHODS

Population samples

The populations examined were from Norway, Norfolk (UK), Sweden, Poland, Hungary, the Netherlands, France (three populations: Cherbourg, Dijon and Paris), Switzerland and Italy (see Fig. 1). The sample number was 20 for all populations except in three cases where availability was limited: Norway (7), Cherbourg (6) and Norfolk (1). Sample sizes here were limited by population size and vulnerability. However, examination of a large number of loci, which is possible with RAPD, can compensate for small sample sizes when estimating diversity and genetic distance (Nei, 1978; Gorman & Renzi, 1979). The tissue samples were mainly larval tail fin tips. Larvae were randomly caught from ponds known to have secure pool frog populations and were subsequently checked for species identity using RAPD with a 10-mer primer (5′–AGGT GACCGT–3′), which was found in initial trials to clearly separate the 'water frog' species (data not shown). In cases where larvae were not available (Norfolk and Sweden) small pieces of adult toe tips were used; the toes were then treated with 'New Skin' (Bayer, UK) and the frogs released when this had formed a firm seal. The excised tail fin/toe tips were stored in 100% alcohol.

Reagents

Agarose and 100 base-pair DNA marker ladders were obtained from Gibco-BRL (UK). Chelex 100 resin was from Bio-Rad (CA, USA). DNA polymerase derived from the organism *Thermus islandicus* ('Red Hot Polymerase') was obtained from Advanced Biotechnologies (Epsom, UK). 'Taq Supreme' (one of the so-called 'Super Taqs') and the PCR master mix 'Megamix Blue' were from Helena Bioscience (Sunderland, UK). Primers were synthesized by Operon Technologies (Gosforth, UK), Cruachem (Glasgow) and Microzone (Lewes, E. Sussex, UK).

DNA extraction

Small amounts of tissue samples (*c*. 4 mg) were statically incubated in 160 μL of sterile distilled water (SDW) and 40 μL Chelex-100 resin overnight, in a water bath at 55 °C (Zeisset & Beebee, 1998). The samples were then briefly vortexed, boiled for 8 min in a water-bath, re-vortexed and centrifuged at 5000 *g* for 3 min at room temperature. The resulting supernatant, stored at –20°, was used as the DNA source for subsequent PCR amplifications. DNA concentrations were determined by measuring absorbance at 260 nm, and were adjusted to approximately 50 μg/mL by dilution or freeze-drying (100 μg/mL and 25 μg/mL gave

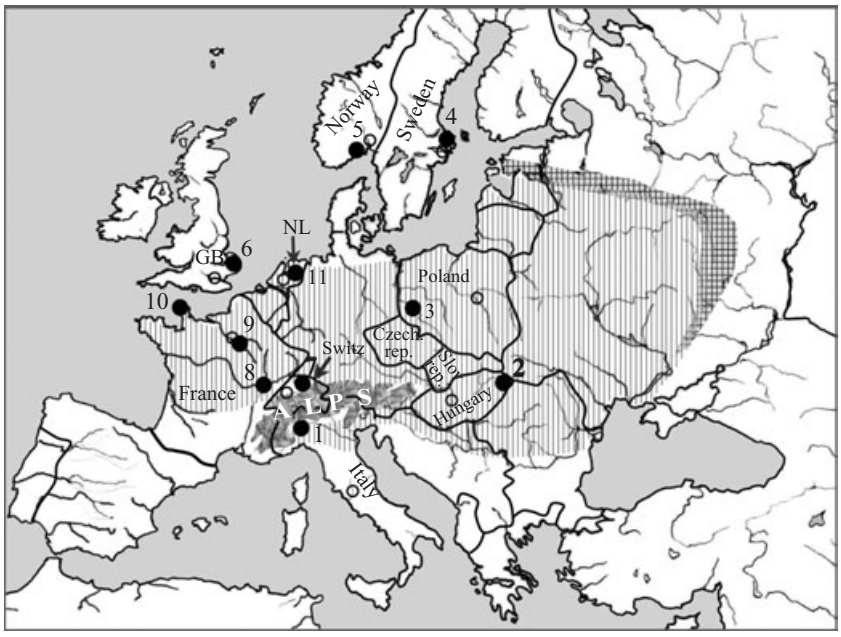

Figure 1. Map of Europe showing sampling stations and species range. Populations sampled shown as black dots: 1, Po Valley, Nr. Torino, Italy. 2, Bockerek, Hungary. 3, Wroclaw, Poland. 4, Uppland, Sweden. 5, Arendal, Norway. 6, Norfolk, Britain. 7, Zurich, Switzerland. 8, Dijon, France. 9, Nr. Chamont, SE of Paris, France. 10, Cherbourg, France. 11, Nunspeet, the Netherlands. The vertical hatching represents areas in Europe where *Rana lessonae* coexists with *R. esculenta*. The cross-hatching represents areas where pure pool frog colonies can be found. Dots 4, 5 and 6 represent isolated pure pool frog colonies. Open circles are respective capital cities (for orientation purposes only). For extra details of species and ranges see Arnold & Ovenden (2002), Gasc (1997), Günther (1990), Nöllert & Nöllert (1992).

the same PCR amplification results as 50 μg/mL). All procedures were carried out in dedicated pre- and post-PCR areas. All solutions and apparatus were also confined to these areas and rigorously sterilized where appropriate. Freshly autoclaved pipette tips and PCR-dedicated, thin-walled microfuge tubes (Advanced Biotechnologies) were used throughout.

RAPD ANALYSIS

RAPD analysis was essentially as described by Williams *et al*. (1993), with some modifications. Each PCR assay contained 2 μL extracted supernatant (with DNA) plus 18 μL of a stock solution containing all other components. This stock, based on Megamix Blue which contained a tracker dye and density-raising solute, provided the final concentrations specified in parentheses: $MgCl_2$ (2.15 mM), dNTPs (170 μM each), Tris (pH 9.0, 42 mM), DNA polymerase (1.1 units, of which 0.3 units of licensed *Taq* were a component of the Megamix and 0.8 units were contributed by additions of Red Hot Polymerase and/or Taq Supreme; see Table 1), and primer (0.2 μM).

One hundred and seven arbitrary 10-mer primers were screened to see which would be most useful in showing repeatable, inter-population polymorphism. Four different polymerase preparations were screened for their utility, and two (Red Hot Polymerase and Taq Supreme) were chosen as having different advantages: some primers were used with one polymerase whilst others were used with a combination of the two enzymes (see Table 1 and Results for details).

The thermocycling protocol started with a denaturation cycle of 94 °C for 4 min followed by 40 cycles of three segments: 94 °C × 1 min, 36 °C × 1 min and 72 °C × 2 min, followed by a final extension cycle of 72 °C for 6 min. The resulting amplified samples were pipetted into wells in 1.5% agarose gels in 0.75 × TBE (67.5 mM Tris-borate, 1.5 mM EDTA) running buffer, then electrophoresed at 4 V/cm until the tracker dye had migrated 5–6 cm, then the gel was stained in a bath of SDW containing 1 mg/L ethidium bromide. Each gel typically had three wells with DNA molecular weight standards and included a reaction with all of the reagents but no DNA, as standard procedure. Gel images were captured by a video camera linked to a computer and digitized (GDS-7600 Gel Documentation System, UVP Ltd, Cambridge, UK).

Table 1. List of primers, sequences and polymorphic markers observed. For each primer the name is followed by the sequence (5'–3'), the DNA polymerase used in amplification (RHP, Red Hot Polymerase; TS, Taq Supreme) and polymorphic bands sizes in base pairs, rounded to nearest 5 bp

Primer	Sequence	Polymerase	Band
B13	TTCCCCCGCT	RHP/TS (3 : 2)	290, 330, 375, 400, 420, 490, 530, 550, 600, 635, 670, 695, 715, 755, 770, 800, 820, 840, 880, 915, 950, 980, 1090
B16	TTTGCCCGGA	RHP	315, 345, 605, 645, 670, 720, 755, 800, 845, 895, 920
B17	AGGGAACGAG	RHP	280, 340, 370, 400, 445, 485, 510, 535, 570, 610, 640, 710, 750, 790, 830, 870, 900, 945, 1000
B2	TGATCCCTGG	RHP/TS (3 : 2)	315, 335, 355, 395, 440, 470, 495, 520, 535, 565, 585, 630, 660, 720, 775, 810, 835, 870, 900, 980, 1050
B3	CATCCCCCTG	RHP/TS (3 : 2)	320, 340, 380, 420, 460, 485, 495, 520, 560, 595, 630, 680, 730, 770, 830, 865, 910, 960, 1005, 1070
L10	CACGGCGAGT	RHP/TS (3 : 2)	340, 365, 395, 420, 450, 495, 620, 665, 680, 705, 725, 760, 800, 820, 895
C17	CCTTTTTTAG	TS	235, 285, 305, 350, 380, 410, 445, 480, 515, 535, 575, 640, 680
C20	ACTTCGCCAC	RHP	310, 370, 395, 430, 475, 515, 560, 605, 630, 655, 735, 785, 815, 865, 940, 1000
L6	CAGCGAACTA	RHP/TS (3 : 2)	310, 330, 370, 385, 415, 445, 485, 525, 585, 610, 675, 710, 740, 775, 820, 870, 940, 1030, 1090, 1165, 1240, 1350

Data analysis

From the results for each set of gels for each primer, upper and lower size limits of clear band resolution were determined visually, and within this range, the same for each gel in the set, all bands were identified and molecular weights determined using proprietary software (LabImage, Labsoft-GmbH, Germany). Specially written software (R. Edwards, pers. comm.) was then used to classify bands according to molecular weight and then to transform this into a matrix in which the presence or absence of each fragment was signified by 1 or 0, respectively. The matrix was extended to include data for all primers and used to assess the genetic distances between individuals following Nei & Li (1979), from which a bootstrapped phylogram was inferred by the neighbour-joining (NJ) method (Saitou & Nei, 1987; Studier & Keppler, 1988), and geographical clustering of the individuals by principal component analysis (PCA) (Reyment & Joreskog, 1993); all analyses were performed in the MatLab (2003) computational environment using the singular value decomposition (SVD) technique (Johnson, 1963).

The matrix also allowed computation of frequencies for each band within each population, which were then used in the PHYLIP suite of programs (Felsenstein, 1993) to produce bootstrapped phylogenetic tree constructions using different statistical approaches, notably, maximum likelihood (ML) (Cavalli-Sforza & Edwards, 1967; Felsenstein, 1981; Gomberg, 1996); UPGMA (Sokal & Michener, 1958) based on Nei's genetic distance (Nei, 1972, 1978); Cavalli-Sforza chord distances/Neighbour-joining (CSC) (Cavalli-Sforza & Edwards, 1967; Saitou & Nei, 1987); and Nei/neighbour-joining (NNJ) (Nei, 1972, 1978; Saitou & Nei, 1987; Studier & Keppler, 1988).

RESULTS

Preliminary findings

In initial experiments, nine of the 107 primers that were screened were deemed the most useful on the basis of polymorphic band resolution and repeatability, were selected for use in later analyses. The sequences of these nine primers used for this analysis and the molecular weights of the polymorphic bands they amplified (in the range of band sizes used in this study) in the complete set of frog DNA samples are given in Table 1.

As previously reported by Williams *et al*. (1993), we found that different preparations and sources of thermostable DNA polymerases could give different patterns of band amplification with the same DNA samples, but utilization of a particular polymerase preparation under standardized conditions gave highly reproducible band patterns. With the conditions and reagents given in this paper, all replicates and repeat extractions tested (even when DNA concentrations were doubled or halved), repeatedly gave the same banding patterns within the chosen fragment length ranges.

Of the primers selected, C17 (TTCCCCCCAG (all sequences shown 5′–3′)) exhibited its most useful polymorphism at the lower end of its spectrum of band sizes (230–680 bp). Since Taq-Supreme was most efficient at amplifying the shorter band lengths, it was used as the DNA polymerase of choice with

Table 2. Estimates of RAPD polymorphism in the pool frog, *Rana lessonae*. PBPP, polymorphic bands per population; TP, total polymorphism

	No. PBPP (A)	% TP	Sample no. (B)	A/B
Dijon, France	132	83	20	6.1
Italy	116	73	20	5.8
Switzerland	101	63	20	5.0
Hungary	100	63	20	5.0
Paris, France	94	59	20	4.7
the Netherlands	83	52	20	4.2
Poland	68	43	20	3.9
Cherbourg, France	61 (70)	38 (44)	6 (20)	10 (3.5)
Sweden	49	31	20	2.5
Norway	26 (41)	16 (26)	7 (20)	3.7 (2.1)
Norfolk	N/A	N/A	1	N/A
All populations	160	100	174	0.92

primer C17. For three primers (TTTGCCCGGA [B16], AGGGAACGAG [B17], ACTTCGCCAC [C20]), Red Hot Polymerase was found to be the most efficient at amplifying the mid- to higher range of band sizes. For five primers (TTCCCCCGCT [B13], TGATCCCTGG [B2], CATCCCCCTG [B3], CAGC GAACTA [L6], CACGGCGAGT [L10]), where interest ranged from the short all the way through to the longer (300–1350 bp) fragment sizes, we found that a mixture of three parts Red Hot Polymerase to two parts Taq Supreme enhanced band amplification and resolution.

Population genetic diversity

Table 2 gives estimates of polymorphism for each population as a proportion of total polymorphism, and indicates that polymorphism levels were lowest in the Norwegian and Swedish populations and highest in the Italian and Dijon (France) populations. A graphical extrapolation to a sample number of 20 (using Excel) was applied as a correction for low sample number for the Norwegian and Cherbourg (France) populations (the extrapolated numbers being parenthesized in all columns).

Genetic distances and inferred relationships

The binary data produced from the RAPD analyses using all nine primers constituted an extensive data set (160 polymorphic loci, 174 individuals), which was used to estimate genetic distances (Nei & Li, 1979) from which a consensus (1000 boot-strap cycles) phylogram was inferred via NJ techniques using TREECON (Van de Peer, 1994). The resulting population clustering very closely reflected the geographical origins of the individuals. The Norwegian and Swedish individuals formed a distinct cluster including the Norfolk individual, which showed closest affinities to the Norwegian group.

Band frequencies for all 160 polymorphic loci were determined for each geographical population and then used to generate consensus phylograms via the four theoretical approaches cited in Material and Methods: ML, UPGMA (Nei, 1972, 1978), CSC and NNJ. These analyses were implemented through PHYLIP (Felsenstein, 1993) using 1000 bootstrap cycles. All four trees were very similar (the ML and UPGMA trees are illustrated in Fig. 2A, B), with, in each case, populations from Norway and Sweden, and the Norfolk individual, showing a marked, close affinity. The Hungarian and Polish populations were the nearest to these. One interesting ambiguity was the position of the Dutch population. In the UPGMA and NNJ results the Dutch branch of the phylogenetic tree was associated with the mainland populations of France, yet with ML and CSC analyses it forked off before the branch leading (in order) to Hungary, Poland, Sweden Norway and Norfolk. However, the low bootstrap numbers for the basal nodes should be noted.

Principal component analysis

PCA of the binary RAPD data set showed a smooth non-linear relationship between cumulative percent variance and the number of principal components, approximately 150 of the latter being needed to obtain 100% of the variance. However, 20% of the cumulative percent variance was contributed by the first four principal components, and pair-wise plots of all these components showed clustering of the individuals according to their geographical origin, though there was variable overlap between the populations.

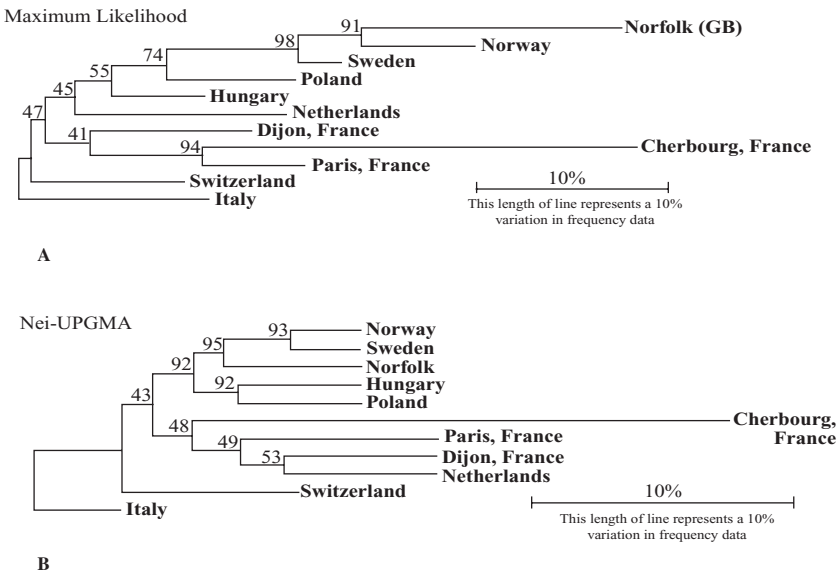

Figure 2. Consensus phylograms for European pool frog populations based on band frequencies. The trees were constructed using frequencies of all 160 polymorphic bands, determined for each population via (A) Maximum Likelihood and (B) UPGMA, both implemented through PHYLIP (Felsenstein, 1993). Bootstrap numbers are percentage of 1000 iterations.

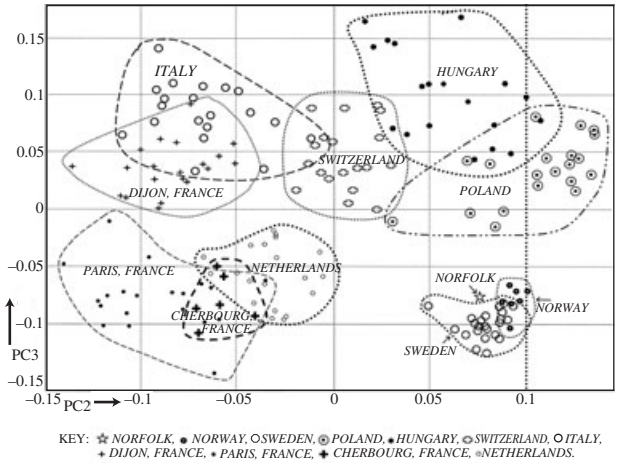

Figure 3. Principal component analysis of European pool frog populations. The dotted and dashed lines show the clustering and overlap of the populations.

In all these cases the Norwegian and Swedish populations and the Norfolk individual co-clustered or overlapped more closely with each other than with any other populations. This phenomenon was particularly pronounced in all plots involving the second principal component, and can be seen very clearly in the plot of PC2 vs. PC3 shown in Figure 3. Another feature of these plots is that the Scandinavian populations tended to be closest to those of Poland and Hungary – which tended to abut the Swiss population. Thus the PCA closely supports the consensus phylogram analyses given above.

Norfolk pool frog individuality

A striking feature of all the gel analyses with the various RAPD primers was the relative homogeneity of the band patterns for the Norwegian and Swedish populations and the Norfolk individual. However, consistent with some evolutionary differentiation of the Norfolk population from the rest of the northern clade, the DNA from the Norfolk individual yielded distinctly and reproducibly different band patterns from the Scandinavian individuals with two of the primers – L10 and C20. This is shown for L10 in Figure 4.

DISCUSSION

Patterns of genetic diversity in European pool frogs

A limitation of the RAPD data set analysed here is the small size of some of the population samples (Table 2), the worst being the single individual in Norfolk (now believed to be extinct). In the case of Norway this limitation was imposed by the small and vulnerable nature of the population and in the case of Cherbourg by sample availability, but this limitation was somewhat mitigated by the very large number of polymorphic loci (160) that the RAPD approach has made available for analysis. Allowing for the limitation of sample sizes, Table 2 indicates that polymorphism lev-

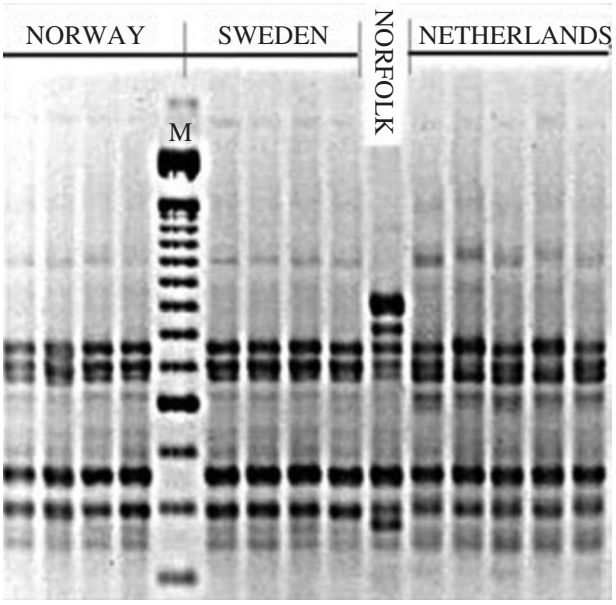

Figure 4. RAPD-PCR analysis of DNA samples from European pool frogs. Each lane represents a different individual from the geographical populations indicated. DNA samples were amplified and analysed as described in the Material & Methods, using primer L10. The marker lane (100 base pair ladder, lowest band 300 bp) is represented by the letter 'M'.

els were highest in the southern and western populations (Italy and France) and lowest in the northern, especially Scandinavian, populations. Isozyme analysis in Sweden also indicated low genetic variability in pool frog populations there (Sjögren, 1991). This is suggestive of a geographical trend in genetic diversity, with the Scandinavian populations being relatively genetically impoverished.

The geographical coherence of the data was attested by cluster analysis (data not shown). The 174 individual frogs fell into discrete 'national' clusters reflecting geographical origin, with only one exception – a Swedish individual lying in a Polish cluster. Even the three French populations, from quite well separated sites (Cherbourg, Paris, Dijon), formed a discrete 'French' cluster. Other geographically adjacent populations, such as Hungary and Poland, or Norway and Sweden, were also closely connected. Certain groupings, such as Norfolk/Norway/Sweden, were particularly convincing in terms of bootstrap values.

The cluster analysis was supported by other analyses: ML (Fig. 2A), UPGMA (Fig. 2B), CSC and NNJ population phylograms and by PCA of the same data set (Fig. 3), PCA being a form of analysis which does not imply phylogeny. As reported above, cumulative variance was not strongly associated with one or even a few principal components in the PCA, suggesting that many different loci have contributed to the genetic differentiation between these European populations. However, pair-wise plots using all of the first four principal components, which accounted for one fifth of the cumulative variance, gave coherent geographical clustering of all populations in each case, reflecting the other analyses. PCA of the data set is thus highly consistent with the clustering-phylogram analyses, supporting the robustness of the data.

The consensus phylograms yielded by the analyses clearly indicate that the Norfolk individual and the Norwegian and Swedish populations form a distinct group, with genetic distance increasing through the more southerly populations and reaching its maximum in Italy. The phylograms raise the possibility that a separation occurred between ancestral populations colonizing eastern and northern Europe and those ancestral to the French populations. All phylograms emphasize that the Norfolk individual's closest genetic affinities are with the Norwegian and Swedish populations, and not at all with the French or Italian populations, originally considered to be the source, via introduction, of the Norfolk population (Boulenger, 1884a, b).

In a study to which this present study was a parallel investigation, analysis of the same populations, including many of the same individuals, with microsatellite markers was undertaken by Zeisset & Beebee (2001). They identified six polymorphic microsatellite loci and found that the total number of alleles per locus varied from 6 to 21. For all the animals from Norfolk, Sweden and Norway, all six loci were monomorphic and fixed for the same allele at five of the loci. The allele at one locus, RICA18, was specific for Norway, Sweden or Norfolk. Mean expected heterozygosity (H_e) was negatively correlated with linear geographical distance from Italy. A phylogram constructed on the basis of the allele frequencies, using Cavalli-Sforza chord distances and NJ, closely resembled the trees presented here.

Thus, results with two different types of molecular marker – a small number of microsatellite loci and a much larger number of RAPD generated loci – provide strong, mutually supportive and independent evidence that the now extinct Norfolk pool frog population was far more closely related to distant populations in Norway and Sweden than to those across the English Channel in France or the Netherlands. These English, Norwegian and Swedish pool frog populations have been described as a distinct northern clade by Zeisset & Beebee (2001). Further evidence for the existence of this northern clade has come from bioacoustic analysis of the calls of frogs from several European populations, including Norfolk, Norway and Sweden (Wycherley *et al*., 2002).

Implications for postglacial colonization routes

The existence of the northern clade raises the obvious question of the origin of the English/Norfolk population – and indeed, of the very isolated Scandinavian populations. Sjögren (1991) analysed allozymes at 31 loci in Swedish populations of *R. lessonae* and found 29 were monomorphic, contrasting with much greater variation in a Polish sample, agreeing with our RAPD data. Sjögren argued that his findings did not support the hypothesis of recent introduction of the Swedish population by man. Similarly, it seems highly unlikely that the Norfolk population arose from recent human translocation of Scandinavian pool frogs: there are no records of humans moving frogs between Scandinavia and Britain, and pool frogs are now known to have occurred in Britain at least as long ago as Saxon times (Gleed-Owen, 2000).

Acceptance that the Norfolk *R. lessonae* population was native, and a member of the northern clade, suggests that colonization of eastern Britain took place via land/fresh-water connections. Amphibians cannot cross large expanses of seawater, such as the current North Sea. A land bridge, which connected Britain to mainland Europe and also stretched to the Scandinavian region (Denmark and southern Norway), was extant in postglacial times up to *c*. 8000 BP (Masters & Flemming, 1983; Lambeck, 1995).

The ultimate origin of vertebrate fauna re-colonizing northern Europe after the most recent ice age is usually considered to be one of the peninsular glacial refugia (Taberlet *et al*., 1998; Hewitt, 1999; Petit *et al*., 2003). Our RAPD and the microsatellite data are consistent with an Italian origin, the Italian population having high genetic diversity, and the northern fringe populations – the northern clade, the furthest outposts of the colonization movement – being genetically most impoverished. With climatic amelioration and alpine glacier recession starting *c*. 16 000 years BP, major northward colonization routes to the north would have opened up east and west of the Alps, our data indicating the easterly route for the northern clade, via Hungary and Poland, though the shortest route to Britain would have been westward, through France.

Glaciation and eustatic and isostatic movements had lowered sea levels in the Mediterranean (Lambeck, 2000). At the time of climatic amelioration there was a relatively narrow corridor for migration to the west of Italy, whereas a wide area of the Adriatic remained dry land, allowing for the possibility of earlier eastward migration via Slovenia/Croatia into Hungary. In this route, high land south of the Julian Alps would need to be crossed, although the pool frog is known to be an early colonizer of newly formed ice-free habitat in the present day (K. Grossenbacher, pers. comm.). This tolerance of cold is reflected in the present day range of *R. lessonae.* Also, interestingly, temperate plant species ranges expanded postglacially faster in eastern than in western and central Europe (Huntley & Birks, 1983).

Another factor may have been glacial refugia much further north than the Mediterranean peninsulae (Stewart & Lister, 2001), which appear to be regions of endemism (Bilton *et al*., 1998; Pekkarinen, 2001; Schmitt & Seitz, 2001; Stewart & Lister, 2001). In this context the cryptic refugium occurring *c*. 26 000 years BP identified in north-eastern Hungary (Willis, Rudner & Sumegi, 2000), may be particularly significant (see Fig. 5). While no amphibian remains have yet been recovered, the site – about 150 km from our Hungarian sampling site – would have been a convenient springboard for earlier northward migration and it is noteworthy that our Hungarian population showed a high level of polymorphism (Table 2).

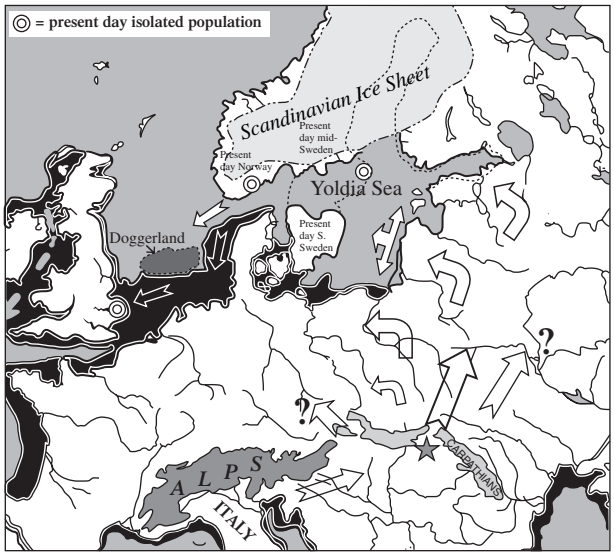

Figure 5. Northern Europe *c.* 9500 years BP. Arrows represent possible colonization routes for 'northern clade' *Rana lessonae*. Black areas indicate dry land now submerged. This includes 'Doggerland' (picked out in grey and black) which remained dry land long after the black areas had become submerged (still extant 7000 years BP). (Sources for coastline: Yalden, 1982; Masters & Flemming, 1983; Berglund, 1986; Björk, 1995; Rainio, Saarnisto & Ekman, 1995; Flemming, 1968, 1998; Lambeck, 1995, 2000). The question marks represent areas where the penetration of the eastern clade could usefully be sampled (no samples were available for this study). The star represents the position, 26 000 years BP, of a cryptic refugium which contained temperate plant species (Willis *et al.,* 2000; Stewart & Lister, 2001).

There is also recent evidence of more northerly, cryptic refugia for the field vole *Microtus agrestis* L. (Jaarola & Searle, 2002); the root vole *Microtus oeconomus* Pallas, 1776 (Brunhoff *et al*., 2003), the land snail *Arianta arbustorum* L. (Haase *et al*., 2003); the land snail *Trochoidea geyeri* Soós, 1926 (Pfenninger, Posada & Magnin, 2003) and the butterfly *Erebia medusa* Denis & Schiffermüller, 1775 (Schmitt & Seitz, 2001). Regarding herpetofauna, Carlsson (2003) has argued that the adder *Vipera berus* (L.) survived the Weichselian glaciation in at least three areas north of the more traditionally accepted refugia, and Babik *et al*. (2004) have data implying that the moor frog *Rana arvalis* (Nilsson, 1832) survived several glacial cycles in the Carpathian Basin (including Hungary), one lineage subsequently colonizing much of Eurasia to the north.

Rapid northward expansion by the easterly route might have occurred, at least in part, by a leptokurtic mechanism (Ibrahim, Nichols & Hewitt, 1996), with or without northern refugia; this would be likely to generate low polymorphism levels in the advanced colonist, as seen in the pool frog's northern clade.

The precise routes for colonization of Scandinavia and Britain remain to be considered. Climate in this region in postglacial times fluctuated (Berglund, 1986; Atkinson, Briffa & Coope, 1987; Lowe & Walker, 1997a, b; Bradley, 1999) and coastlines were very different (Yalden, 1982; Flemming, 1968, 1998; Masters & Flemming, 1983; Berglund, 1986; Björk, 1995; Lambeck, 1995, 2000; Rainio, Saarnisto & Ekman, 1995). A representation of coastlines of northern Europe *c*. 9500 BP is given in Figure 5 which depicts our view of the spread of the northern clade from Italy and/or cryptic northern (Hungarian) refugia. Animals migrating from this or an Italian refugium would eventually reach the Yoldia Sea (approximating to today's Baltic Sea, but much less saline; Tikkanen & Oksanen, 2002). With ice melt encouraging a westward flow, peri-Yoldia colonization toward the North Sea area might thus have been promoted and colonization of eastern England via Doggerland, with its freshwater habitats (Coles, 1998), would have been fairly straightforward.

This unexpected case of a NE–SW postglacial colonization may not be unique. Bilton (1994) showed that the Norfolk population of the freshwater beetle *Hydroporus glabriusculus* Aubé, which has very limited flight and dispersal ability, is more closely related to the northern Swedish population than to the Scottish or Irish populations. In Bilton's view the reduced genetic variability of the British colony favoured it being an offshoot from the Swedish lineage rather than vice versa.

Thus, on the basis of emerging evidence and that presented here, postglacial movements of biota in north-western Europe were not necessarily events based straightforwardly on considerations of temperature, latitude and geography.

Implications for conservation

The results of this study and others have already encouraged English Nature to mount a programme to re-introduce the northern clade of *R. lessonae* to eastern Britain. The source of the stock used will be Sweden because, even though the habitat and climate of the Norwegian group is more comparable to East Anglia, the Norwegian population is smaller and more vulnerable than the Swedish.

Before its death in 1999, an attempt was made in Britain to breed the last male Norfolk pool frog with northern clade females from Sweden. Unfortunately, these females showed no interest in breeding with either the Norfolk male, or indeed, males from Sweden. However, females from mainland Europe bred readily with the Norfolk male and offspring exist. It is hoped that molecular genetic methods can now be exploited to track genetic contributions of the Norfolk genome (which may have some adaptive value) to the offspring. These offspring, should the Swedish introduction to England fail, may eventually be used in later introduction attempts.

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