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CONSISTENTLY DIFFERENT LEVELS OF GENETIC VARIATION ACROSS THE EUROPEAN RANGES OF TWO ANURANS, *BUFO BUFO* AND *RANA TEMPORARIA*

EDWARD G. BREDE[1] AND TREVOR J. C. BEEBEE

School of Life Sciences, University of Sussex, Falmer, Brighton, UK

[1]*Current address: Max Planck Institute for Limnology, Tropical Ecology Working Group, Plön, Germany*

We compared the genetic diversities across eight microsatellite loci of two widespread anurans, *Bufo bufo* and *Rana temporaria*, at multiple sites across their western and central European ranges. *Bufo bufo* consistently exhibited less genetic diversity than *R. temporaria*. Our evidence infers that this difference is unlikely to be a feature of the specific marker loci used, nor is it a probable consequence of the different phylogeographic histories of *B. bufo* and *R. temporaria*. No recent bottlenecks were observed in *B. bufo* or *R. temporaria* populations. Both species showed similar levels of differentiation across their European range as estimated by *F*-statistics, but whereas *R. temporaria* exhibited isolation by distance effects, *B. bufo* did not. We suggest that distinct autecological features of the two species are the most likely explanation of the diversity differences, especially more limited historical gene flow among *Bufo* compared with *Rana* populations.

Key words: amphibians, genetic diversity, interspecific variation, microsatellites

INTRODUCTION

Genetic diversity is important to the long-term viability of populations (Amos & Balmford, 2001; Hedrick, 2001). Although many factors can affect genetic diversity (Amos & Harwood, 1998), effective population size and gene flow among populations or sub-populations are among the most important. Differences in either of these features can have substantial effects on comparative genetic diversities between species. We recently found that two broadly similar anurans (*Bufo bufo* and *Rana temporaria*) had very different genetic diversities when multiple populations in Britain were analysed across eight microsatellite loci (Brede & Beebee, 2004). Although *B. bufo* had on average much larger census population sizes than *R. temporaria*, genetic diversity was consistently greatest in *R. temporaria*. This was potentially accounted for by different population structures. *Bufo bufo* had relatively few and isolated populations with little gene flow among them, whereas *R. temporaria* had multiple small populations with extensive interconnecting gene flow.

However, other factors than current population structure can affect levels of genetic diversity. Britain must have been colonized by both of these species in the immediately post-glacial period when there were land connections to mainland Europe (Vincent, 1990). If founder numbers were much smaller for *B. bufo* than for *R. temporaria*, this factor alone could have led to persistent lower genetic diversity in British *B. bufo* populations compared with those of *R. temporaria*. The potential role of founder effects in determining genetic diversity has been understood for a long time, but specific reports mainly relate to recently established populations (e.g. Merilä *et al.*, 1996; Cabe, 1998; Zeisset & Beebee, 2003). Another possible explanation is that there have been extensive recent declines, and subsequent bottlenecks, in *B. bufo* but not *R. temporaria* populations. In this study we tested the hypotheses that differences in genetic diversity between the two study species may be a result of: (1) their different post-glacial colonization histories; or (2) widespread recent population bottlenecks in the less diverse species. Our approach was to make a comparative study of the two species at multiple sites spread across their biogeographical ranges in mainland Europe. If founder effects contributed significantly to the differences in genetic diversity between them in Britain, we expected that such differences would be absent from much or all of mainland Europe, though perhaps also present in Scandinavia. By contrast, if differences were universal across the range there must be more general reasons based on differences in the ecology and population structure of the two species. Bottleneck tests should resolve whether these differences related to widespread recent declines in *B. bufo* but not *R. temporaria*. Our study focused specifically on these two species, and was not intended to test more general hypotheses about postglacial founder effects, which would require a larger number of test organisms.

We chose to use microsatellite markers as indicators of genetic diversity because they are the most polymorphic currently available. However, this carries a potential disadvantage because it was necessary to compare different sets of loci in the two species. This could generate differences based on properties of the markers rather than of the species bearing them. We address this problem by making a quantitative comparison of critical features of the microsatellite loci in the two species that might differentially affect their mutation rates, notably the repeat motifs, mean allele

Correspondence: T. J. C. Beebee, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK. *E-mail*: T.J.C.Beebee@sussex.ac.uk

lengths, frequency of repeat interruption and relative abundances of rare and common alleles.

MATERIALS AND METHODS

SAMPLING ANURAN POPULATIONS

Mainland European and Irish samples of *B. bufo* and *R. temporaria* were collected at breeding sites by colleagues in various countries (Fig. 1). Samples of the two species were not always from the same ponds, as all that was required was a representative genetic sample of each species from each region. Seven *B. bufo* and six *R. temporaria* populations were sampled altogether. The aim was to obtain a random sample of up to 40 individuals from each location, although this number was not always achieved (Table 1). The samples were either toe or muscle tissues from adults, or entire larvae harvested at stage 26 (Gosner, 1960), and all were stored in 70% ethanol prior to DNA extraction. Larvae were collected by random netting at multiple localities within breeding ponds, to ensure that as far as possible a representative sample of the genetic variation in each population was obtained. This approach has been widely used in earlier studies with amphibians (e.g. Rowe *et al.*, 1998). For comparative purposes, samples from up to seven British populations of both species used in a previous study (Brede & Beebee, 2004) were also included in some analyses (see Appendix 1).

MICROSATELLITE GENOTYPING

DNA was extracted from tissues using a Chelex 100 protocol (Walsh *et al.*, 1991). Microsatellite loci were amplified in the presence of [a^{33}P]-dATP and locus-specific primers previously developed for these species (Brede *et al.*, 2001; Rowe & Beebee, 2001). Eight polymorphic microsatellite loci (*Bbufμ*14, 15, 39, 46, 47, 54, 62, 63) were available for *B. bufo* and a further eight (*Rtempμ*1, 2, 3, 4, 7, 8, 9,10) for *R. temporaria.* Both sets of microsatellites were dinucleotide [CA] repeats although two in *B. bufo* (*Bbuf*μ14 and *Bbuf*μ39) and three in *R. temporaria* (*Rtemp*μ1, *Rtemp*μ2 and *Rtemp*μ7) had short interruptions within the repeat sequences. PCR products were electrophoresed alongside an M13 marker on standard sequencing gels (6% w/v polyacrylamide) and alleles were scored after visualisation by autoradiography (Rowe *et al.*, 1997).

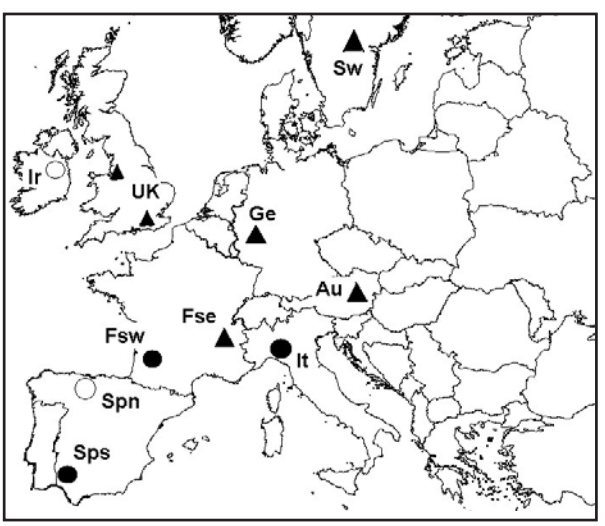

FIG. 1. Sampling site locations. Ir, Ireland; UK, United Kingdom (two sites); Sw, Sweden; Ge, Germany; Au, Austria; It, Italy; Fsw, south-west France; Fse, south-east France; Sps, southern Spain; Spn, northern Spain. Further details are given in Table 1. open circles, *R. temporaria* alone; filled circles, *B. bufo* alone; triangles, *R. temporaria* and *B. bufo*

GENETIC ANALYSIS

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were performed using BIOSYS-1 (Swofford & Selander, 1981) and GENEPOP 3.1 (Raymond & Rousset, 1995) respectively. Genetic diversity estimates including expected (H_e) and observed (H_o) heterozygosities and allelic richness were carried out using BIOSYS-1 and FSTAT (Goudet, 1995). Allelic richness estimates used samples with the same minimum sizes (n=11) for both species. This was achieved by randomly selecting 11 of the 20 *R. temporaria* samples from Austria, a country from which only 11 *B. bufo* samples were available (Table 1). Population bottleneck events were investigated using BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996). A two-phase mutation model in which the proportion of stepwise mutation (SMM) was set at 70% was employed in this analysis. As with UK populations, we found no significant differences between 70% and 90% SMM assumptions in the results of the bottleneck tests (Brede & Beebee, 2004).

Pairwise estimates of F_{st} together with their statistical significance were obtained using FSTAT, and patterns of isolation by distance using linear geographic distances were compared for the two species using the ISOLDE program (with 10,000 randomisations) in GENEPOP 3.1. We also carried out assignment tests using the program GENECLASS (Cornuet *et al.*, 1999) and the "probability of belonging" facility to estimate numbers of possible immigrants in each population. Runs used the frequency method with 10,000 simulated

TABLE 1. European sampling sites and sample details.

Sampling site	*B. bufo*	*R. temporaria*
Austria (Vienna)	11 adults	20 adults
Italy (Torino)	40 larvae	-
Germany (Koblenz)	40 larvae	40 larvae
South-east France (Chambery)	40 larvae	40 larvae
South-west France (Bordeaux)	40 larvae	-
Northern Spain (Cantabria)	-	40 larvae
South-west Spain (Sevilla)	30 larvae	-
South Sweden (Skane)	24 adults	25 adults
Ireland (Limerick)	-	40 larvae
United Kingdom (Ainsdale)	40 larvae	40 larvae
United Kingdom (Pells)	40 larvae	40 larvae

individuals, a threshold of 0.01 and the "leave one out" procedure, and assumed a constant frequency of 0.01 in cases of null alleles.

Randomisation tests were performed using RT 2.1 (Manly, 1997). In this procedure, the observed mean difference between two samples is compared with the distribution obtained by randomly allocating data values to the two samples. Significant differences (at P=0.05) in a two-tailed test are inferred if the observed mean is in either 2.5% tail of the distribution following a large number (at least 1000) of data randomizations. Standard statistical analyses (Wilcoxon Signed Rank tests and correlations) were carried out using the STATISTIX 7 analytical software package (Tallahassee, USA). Estimates of H_e were arcsin transformed and all data sets were tested for normality before analysis with parametric methods (Pearson moment correlations).

RESULTS

MICROSATELLITE LOCI IN *B. BUFO* AND *R. TEMPORARIA*

The microsatellite loci used for this study were first compared among 400 individuals of each species (250 from seven British populations and 150 from six European populations) chosen at random from the totals of >450 samples available for each species. Because different loci were used in the two species, statistical assumptions based on independent sampling from the same distribution do not strictly apply. Nevertheless, it was useful to make comparisons based on a simplifying assumption that the loci are indeed equivalent as diversity indicators provided that the results are interpreted with caution. We used randomization tests for this purpose. Mean numbers of repeats in the microsatellite loci (unweighted averages across all alleles) were 9.9 for *R. temporaria* and 8.4 for *B. bufo*. These differences between the species were not significant by randomization tests in which >28% of 10,000 permutations yielded larger differences between the species than those actually observed. The proportion of loci with interrupted repeats was higher in *R. temporaria* (0.38) than in *B. bufo* (0.25). Permutation tests indicated no significant differences between species in the mean proportions of alleles present at <1% frequency (0.25 for *B. bufo*, 0.30 for *R. temporaria*) or at >10% frequency (0.19 for *B. bufo*, 0.15 for *R. temporaria*). The distributions of allele frequencies in the two sets of loci were therefore broadly similar.

GENETIC DIVERSITY IN EUROPEAN POPULATIONS OF *B. BUFO* AND *R. TEMPORARIA*

The sample data were first tested for compliance with Hardy-Weinberg equilibrium. The *B. bufo* locus *Bb*μ63 was omitted from three populations (south-east France, south-west France, and Spain) due to difficulties with scoring alleles. One *B. bufo* population (Spain) showed significant discordance from Hardy-Weinberg equilibrium at four of the remaining seven loci after Bonferroni correction. Three of the eight Spanish *R. temporaria* loci were also out of Hardy-Weinberg equilibrium after Bonferroni correction, in this case possibly because the samples were collected from three separate ponds. In all the remaining assessments, only single loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni correction in three *B. bufo* populations (south-east France, south-west France, Italy) and four *R. temporaria* populations (south-east France, Germany, Ireland, Sweden). In all cases the deviations were an excess of homozygotes. Potential causes of these deviations include sampling bias, in which siblings were over-represented, and the presence of null alleles. We have no rigorous way of distinguishing between these alternatives, but in the Spanish populations of both species, where multiple loci showed deviations, null alleles are arguably the less likely explanation. Random netting around the ponds was designed to minimize over-representation of a few kin groups, but may not have eliminated it altogether. The tests for linkage disequilibrium showed six pairs out of 252 combinations of loci to be significant after Bonferroni correction in the *B. bufo* data set, whilst in the *R. temporaria* data set eight pairs of 224 combinations were significant after Bonferroni correction. Linkage disequilibrium was randomly distributed among populations and pairs of loci, and we therefore concluded it was due to chance effects (such as sampling sibs) rather than being of biological significance.

Within the mainland European populations, the estimated average H_e for *B. bufo* was 0.612 (range 0.431 - 0.748), with an average allelic richness of 3.81 alleles/locus (range 2.21 – 4.83). European *R. temporaria* had an estimated average H_e of 0.687 (range 0.615 – 0.745) with an average allelic richness of 5.47 alleles/locus (range 4.61 – 6.12). Fig. 2 shows in more detail that European *R. temporaria* populations tended to have higher genetic diversities than European *B. bufo* populations. With the same caveats listed above concerning the interpretation of statistics not sampling the same distribution (i.e. with different loci in the two species), randomization tests indicated that 95% of 10,000 permutations yielded smaller differences in mean H_e between European populations of the two species than the mean difference (>0.08) actually observed. Randomisation tests further showed that essentially 100% of 10,000 permutations yielded smaller differences in mean allelic richness than the mean difference (1.66) actually observed. British *B. bufo* populations (n=7) had slightly lower mean expected heterozygosities and allelic richness than European populations (n=7), but in neither case were the differences significant when tested with the group comparison permutation test (1,000 iterations) in FSTAT (for heterozygosity, P=0.530; for allelic richness, P=0.329). Exactly the same situation also held for *R. temporaria* (heterozygosity P=0.878; allelic richness P=0.278) when comparing seven British and six European populations. Genetic diversities of all the European and

British *B. bufo* and *R. temporaria* populations analysed here and in previous studies (Brede & Beebee, 2004) are summarized in Appendix 1.

Within the European populations, there was no significant correlation between mean H_e and allelic richness for *B. bufo* (r=0.497, P=0.172) unless the Spanish sample, with very low allelic richness, was omitted and after which r =0.712, P=0.048. By contrast there was a significant correlation between H_e and allelic richness for the full set of European *R. temporaria* samples (r=0.789, P=0.020).

In bottleneck tests, four of the seven European *B. bufo* populations showed heterozygote excess at >50% of the loci using a 70% stepwise mutation model, but of these only one population (south-east France) was significant (P=0.039). A similar pattern was seen in the *R. temporaria* populations where four of six populations showed heterozygote excess at >50% of loci and once again only one population (Ireland) was significant (P = 0.023). However, after Bonferroni corrections for multiple comparisons no population of either species showed evidence of a significant recent bottleneck effect. Similar results were obtained with higher levels (up to 90%) of SMM in the BOTTLENECK analyses.

Population Structure

The genetic data were analysed to determine whether differences in population structure related to differences in diversity between the two species. Pairwise estimates of F_{st} among populations of both species, from the six localities across Europe where both were sampled in reasonably close proximity, are shown in Table 2. Mean F_{st} estimates across all populations were 0.200 for *R. temporaria* and 0.167 for *B. bufo*. All the F_{st} estimates were significantly greater than zero for both species. There was no significant difference in F_{st} estimates between the species when pairwise estimates were compared (Wilcoxon Signed Rank test, n=15, P=0.118), and there was no correlation of the pairwise estimates between *R. temporaria* and *B. bufo* among the sample localities (Mantel test with 10,000 permutations, P=0.529). However, whereas *R. temporaria* demonstrated significant isolation by distance effects (Mantel test, P=0.04), *B. bufo* did not (Mantel test, P=0.11).

Assignment tests were complicated by the fact that larvae, which were the only samples available from most populations, cannot migrate between ponds. Only hybrid F1 individuals were potentially detectable in these cases. Another problem was that no local potential sources of immigrants were sampled. Overall, the proportion of individuals that could not be ascribed to the population in which they were sampled with P>0.05 was surprisingly high and not significantly different for both species (mean 0.20 for *B. bufo* and 0.24 for *R. temporaria*). Interestingly, the mean proportions for the two populations where adult tissues were available (Austria and Sweden) showed higher proportions of potential migrants (0.45 for *B. bufo*, 0.44 for *R. temporaria*) than for those with larvae (0.16 for *B. bufo*, 0.20 for *R. temporaria*). However, most individuals of both species in most populations were not ascribed unequivocally to a single population either at the default threshold (0.01) or at a threshold of 0.1.

DISCUSSION

Genetic Diversity Differences Between Species

The results of this study show that *R. temporaria* populations across Europe generally maintained greater genetic diversity than *B. bufo* populations (Fig. 2, Appendix 1). This does not support the hypothesis that founder effects generated the differences in diversity between these species in Britain, but points to a more fundamental cause throughout their geographical ranges. No previous studies of genetic diversity across the range have been reported for *B. bufo*, but other work on European *R. temporaria* populations using eight microsatellite loci (including three of those we employed) yielded data broadly similar to ours (Palo *et al.*, 2004). Their results from 29 populations gave an average of 24.8 alleles (range = 9–34) per locus with average H_e estimates between 0.35–0.72. In the present study with 13 populations we found an average of 21 alleles (range = 7 - 30) per locus and average H_e estimates between 0.601–0.745. For *B. bufo* from 14 populations we found an average of 16.7 alleles (range = 11–21) per locus and average H_e estimates between 0.431–0.748. Only one other European anuran has been investigated with respect to genetic diversity at microsatellite loci across its geographical range.

TABLE 2. Pairwise F_{st} estimates. Numbers show mean F_{st} estimates across all loci. Above diagonal, *B. bufo*; below diagonal, *R. temporaria.*

	UK (Ainsdale)	UK (Pells)	Austria	SE France	Germany	Sweden
UK (Ainsdale)		0.113	0.135	0.194	0.037	0.169
UK (Pells)	0.144		0.186	0.271	0.069	0.160
Austria	0.201	0.199		0.240	0.113	0.232
SE France	0.156	0.161	0.171		0.184	0.297
Germany	0.219	0.148	0.179	0.221		0.117
Sweden	0.293	0.260	0.199	0.211	0.241	

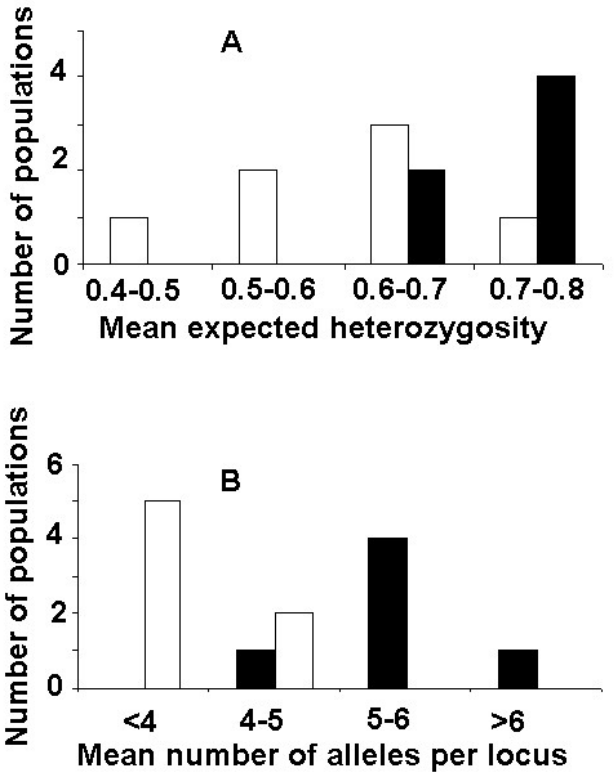

FIG. 2. Genetic diversity of European *B. bufo* and *R. temporaria* populations. (a) Frequency distributions of mean expected heterozygosities; (b) frequency distributions of mean allelic richness. Solid bars, *R. temporaria*; open bars, *B. bufo*.

Beebee & Rowe (2000) showed that across eight loci, 11 *B. calamita* populations had a mean of 13.9 (range = 11–21) alleles per locus and mean expected heterozygosities between 0.431–0.748, broadly similar to the two species studied in the present paper.

Because different loci were compared between the two species, an alternative explanation of our results is that the *B. bufo* microsatellites were inherently less variable than those of *R. temporaria*. We think this unlikely for several reasons. Firstly, the loci were broadly similar with respect to repeat motif, repeat numbers, total numbers of alleles and distributions of allele frequencies. Secondly, a higher proportion of *R. temporaria* loci contained interruptions among the repeats than was the case with the *B. bufo* loci. This factor should predispose the *R. temporaria* loci to lower mutation rates than those of *B. bufo* (Jin *et al.*, 1996), leading to the opposite result of our findings. Thirdly, a similar relationship between the two species is apparent on the basis of earlier allozyme studies. In Britain, Hitchings & Beebee (1998) found a mean observed allozyme heterozygosity of 0.035 and mean allele number per locus of 1.36 across 27 loci in four rural *B. bufo* populations. By comparison, in the same sampling area, Hitchings & Beebee (1997) found a mean allozyme heterozygosity of 0.073 and mean allele number per locus of 1.83 across 19 loci in five rural *R. temporaria* populations. Sixteen loci were common to both species. These differences in heterozygosity and allele numbers were both highly significant, with $P<0.0001$ (Wilcoxon signed rank tests) in both comparisons. Twelve of the allozyme loci were polymorphic in both species when sampled across 12 British populations. Again mean heterozygosity in *R. temporaria* (0.177) was significantly higher than that of *B. bufo* (0.048) by the Wilcoxon rank sum test ($P=0.034$) with these common loci. Difference in mean numbers of alleles per locus (4.17 in *R. temporaria*, 2.17 in *B. bufo*) was close to significance in these common loci ($P=0.052$).

A further possible explanation of interspecific differences in genetic diversity is that one of the two species has experienced widespread recent declines and/or population bottlenecks. However, tests using the BOTTLENECK program gave no indication of such differences. This was similar to the situation in Britain (Brede & Beebee, 2004). It remains possible, of course, that sensitivity to detect such effects was too low or that bottlenecks occurred too far back in time (>4 N_e generations) to be detected by the heterozygosity excess method used in this analysis (Luikart & Cornuet, 1998). The bottleneck test also assumes closed populations, and this may not always be true of *R. temporaria* because local gene flow among ponds might be substantial (Brede & Beebee, 2004).

Population Structure

Comparison of pairwise F_{st} estimates among the sites where both species were sampled in reasonably close proximity indicated similar levels of differentiation at this geographical scale, where populations were separated by hundreds of kilometers. This contrasts sharply with local F_{st} estimates among ponds in southern England where inter-site migration is possible over short time scales. In these circumstances the F_{st} estimates for *B. bufo* were similar to the larger scale estimates reported here, whereas those for *R. temporaria* were some five-fold lower (Brede & Beebee, 2004). This supports the hypothesis that population structure, with much higher historical rates of gene flow among local demes in *R. temporaria* compared with *B. bufo*, contributes to overall differences in levels of genetic diversity between these species. The similar levels of differentiation observed at the larger geographical scale may reflect the effects of occasional, relatively substantive barriers to movement of both species, such as mountain ranges or major river systems. The lack of isolation by distance in *B. bufo*, but its occurrence in *R. temporaria*, also supports a more fragmented population structure with genetic differentiation dominated more by random drift effects (rather than gene flow) in *B. bufo* than in *R. temporaria*. The assignment tests, however, did not suggest that in current populations there are more first generation migrants in *R. temporaria* than in *B. bufo*. Unfortunately the relatively low power of the tests with our data to ascribe a high proportion of individuals to a unique population strongly limits any interpretation of migrant designations. It may be that more loci are required to carry out this type of analysis with high confidence.

The lack of correlation between the pairwise F_{st} estimates for each species across the European sampling sites may reflect different phylogeographic histories of *R. temporaria* and *B. bufo*. Our sampling was insufficient to generate credible postglacial colonization histories for either species. For *R. temporaria* Palo *et al.* (2004), using mitochondrial DNA sequences and microsatellites, provided clear evidence of two distinct lineages, one in eastern and the other in western Europe, and suggested northerly colonization routes during the postglacial warming. No comparable study has yet been made on *B. bufo.* Partial support for a south-west France/Iberian refugium for this species, and for a Germanic clade derived from Balkan/Italian refugia, has been found with both morphometric data and allozyme analyses (Hemmer & Böhme, 1976; Lüscher *et al.*, 2001).

In conclusion, our studies indicate a very widespread and consistent difference in the genetic diversities of two anurans with broadly similar natural histories and geographical distributions. Autecological differences between *B. bufo* and *R. temporaria* seem more likely to explain this difference than chance events in their population histories. In particular, the preference of *B. bufo* for permanent ponds often results in a lower density of breeding sites in the landscape than is the case for *R. temporaria*. This may result in lower gene flow between ponds in *B. bufo* than in *R. temporaria*. Also, we have found that ratios of effective:census population size are much lower in *B. bufo* than in *R. temporaria*, perhaps because the former but not the latter species has a sex ratio at breeding sites highly skewed in favour of males (Brede & Beebee, 2006). Taken together, these features (low gene flow and low effective population sizes) might be expected to generate lower genetic diversity in *B. bufo* compared with *R. temporaria*.

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APPENDIX 1: Genetic diversity across eight microsatellite loci within *B. bufo* and *R. temporaria* populations.

Population	Average *He* (S.E)	Average *Ho* (S.E)	Average Allelic Richness
BUFO BUFO			
Austria	0.600 (0.040)	0.516 (0.055)	3.62
SE France	0.624 (0.093)	0.595 (0.101)	4.60
SW France	0.431 (0.119)	0.420 (0.120)	3.53
Germany	0.662 (0.041)	0.635 (0.056)	4.05
Italy	0.748 (0.031)	0.625 (0.100)	4.83
Spain	0.599 (0.113)	0.614 (0.124)	2.21
Sweden	0.618 (0.072)	0.509 (0.097)	3.00
Ainsdale (UK)	0.568 (0.052)	0.509 (0.054)	3.27
Crematorium (UK)	0.568 (0.036)	0.503 (0.055)	3.09
Pells (UK)	0.609 (0.041)	0.535 (0.045)	3.62
Saltfleetby (UK)	0.623 (0.041)	0.580 (0.038)	3.50
St Annes (UK)	0.411 (0.100)	0.401 (0.112)	2.24
Whitelands (UK)	0.622 (0.038)	0.586 (0.034)	3.33
Withdean (UK)	0.647 (0.044)	0.611 (0.065)	3.00
RANA TEMPORARIA			
Austria	0.719 (0.055)	0.612 (0.080)	5.44
SE France	0.745 (0.035)	0.699 (0.041)	5.76
Ireland	0.642 (0.055)	0.618 (0.061)	4.61
Germany	0.615 (0.053)	0.576 (0.062)	5.21
Spain	0.702 (0.072)	0.641 (0.080)	6.12
Sweden	0.702 (0.054)	0.605 (0.062)	5.69
Ainsdale (UK)	0.601 (0.090)	0.586 (0.089)	4.56
Crematorium (UK)	0.682 (0.053)	0.645 (0.054)	5.12
Pells (UK)	0.659 (0.093)	0.595 (0.090)	5.46
St Annes (UK)	0.732 (0.060)	0.641 (0.056)	5.99
Halesworth (UK)	0.694 (0.045)	0.594 (0.071)	5.04
Whitelands (UK)	0.668 (0.048)	0.528 (0.064)	4.97
Withdean (UK)	0.687 (0.046)	0.740 (0.064)	5.32