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Genetic contributions to herpetofauna conservation in the British Isles

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The use of molecular genetic markers is considered in the context of their application to conservation of amphibians and reptiles in the British Isles. Aspects reviewed include population viability, connectivity and origins together with developments in molecular identification. Genetic diversity measures are not in themselves sufficient to identify risks of inbreeding or genetic erosion in the absence of direct measures of individual fitness. Neutral markers are however useful for defining populations, the extent of migration between them and the identification of permeable habitat corridors. Phylogeography has resolved previously uncertain origins of several populations and species found in the British Isles, including *Pelophylax lessonae*, *Bufo calamita* and *Triturus cristatus*. Molecular studies have clarified the species status of toads *Bufo bufo*, *B. spinosus*, and grass snakes *Natrix helvetica* in the British Isles and provided methods for species and clade identifications where this is difficult using morphology alone. DNA-based techniques have revealed the distributions of viral and fungal pathogens and environmental DNA (eDNA) has proved its worth as a technique for surveying pond-breeding amphibians.

Key words: DNA, genetics, amphibians, reptiles, conservation, British Isles

INTRODUCTION

Wildlife conservation has a long history in the British Isles and, until recently, was carried out in the virtual absence of genetic considerations. That situation has changed markedly in the past 25 years due to the development of genetic tools readily applicable to wild populations of plants and animals. A combination of DNA-based analyses and increasingly powerful computation, reviewed by Frankham et al. (2010) and Rowe et al. (2017) has brought genetics into the mainstream of conservation planning and application. Herpetofauna around the world have been among the groups attracting conservation genetic investigations, with those on amphibians reviewed recently by McCartney-Melsted & Shaffer (2015). This global analysis included case studies of several species, only one of which (the great crested newt *Triturus cristatus*) occurs in the British Isles. In this review, I consider the main contributions that genetic analysis has made to amphibian and reptile conservation specifically in the British Isles.

GENETIC ANALYSES AND THEIR IMPLICATIONS

The arrival of molecular markers

Population genetics came of age almost a century ago, at a time when variation was expected to be solely the result of natural selection. Early field studies were interpreted with this in mind and all morphological differences among individuals, however trivial, were considered to reflect variations in selection pressures over time and space. It came as a shock when allozymes, the first major family of molecular genetic markers, revealed vast amounts of variation that were impossible to explain by selection alone. Neutral theory was the result, proposing that many genetic differences were due to changes that had little or no effect on individual fitness. Subsequent development of DNA markers, primarily mitochondrial DNA (mtDNA) and nuclear microsatellites, showed that variation among these molecules too was usually neutral with respect to selection. Perhaps counter-intuitively, neutral markers are of great value in population studies but, as we shall see, the distinction between selection and neutrality is of more than academic interest to genetic applications in conservation. DNA analyses require only tiny amounts of tissue which can usually be obtained without sacrificing animals because the required sequences can be amplified by the polymerase chain reaction (PCR). The history of molecular ecology including both theoretical and practical developments is described in Rowe et al. (2017). Four main areas relevant to conservation biology have been informed by genetic analyses, notably: (1) population demography, including diversity, viability and inbreeding risk; (2) population size, structure and gene flow; (3) phylogeography, the history of populations in time and space; and (4) identification issues including those relating to species, individuals

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and hybrids. All of these approaches have been used in studies of amphibians and reptiles in the British Isles, as described below.

Population demography

Assessing genetic diversity

Conservationists have long recognised the increased extinction risk implicit in small or declining population sizes. In addition to stochastic factors such as predation, disease, adverse weather and imbalanced sex ratios, reductions in genetic diversity in small populations could predispose inbreeding depression and thus further declines not relieved by habitat or species management methods. This concern has been, arguably, the commonest reason to use genetic tools in conservation. To this end molecular genetic markers, especially microsatellites, have been developed to investigate many amphibian and reptile species. Tables 1 and 2 summarise examples of genetic diversity estimates based on microsatellites for many of the species found in the British Isles. Comparisons with populations in mainland Europe are provided to demonstrate the extent to which diversity has been affected by isolation in the island archipelago. In the cases of *Lissotriton vulgaris* (smooth newt), *L. helveticus* (palate newt), *Zootoca vivipara* (viviparous lizard), *Anguis fragilis* (slow-worm) and *Natrix helvetica* (grass snake) there was no comparable information, at least for British Isles populations, at the time of writing. Sixteen *Vipera berus* (adder) populations in England and Wales were also analysed for genetic diversity at microsatellite loci and the results, not yet published (hence their absence from Table 2), were broadly similar to those from north-east France.

Expected heterozygosity (He) and allelic richness (Ar) are the two main statistics for estimating population genetic diversity. In practice either one will usually suffice. Across all the taxa listed in Tables 1 and 2, He and Ar (or allele number, An, where Ar was not calculated from An) correlated strongly, with $r_s = 0.925$, $P<0.0001$. Suites of microsatellite loci are unique to each species, so cross-species comparisons should be treated with caution. Nevertheless *R. temporaria* populations probably are, on average, individually more diverse than those of *B. bufo* perhaps for ecological reasons considered later (see gene flow section). Intraspecific comparisons are on safer ground, though even here different investigations have sometimes used different marker sets. Range-edge populations of native species, including those in the British Isles, generally had lower levels of diversity than those in central or southern Europe. This was apparent for *R. temporaria* (common frog), *B. bufo* (common toad), *B. calamita* (natterjack toad), *C. austriaca* (smooth snake) and *L. agilis* (sand lizard). Non-native introduced species in Britain also often showed reductions in diversity compared with their populations of origin. This was true for *P. ridibundus* (marsh frog) and especially for *P. muralis* (wall lizard), but the case was different for recently re-introduced *P. lessonae* (pool frog) as the source population in Sweden was itself genetically depauperate compared with central European populations. (see references in Table 2).

Factors influencing genetic diversity

A theoretical expectation is that genetic diversity at neutral loci should correlate with population size. Testing this hypothesis is usually impossible because most genetic studies on herpetofauna have been with populations of unknown size. *Bufo calamita* in Britain is an exception because multiple populations have been studied for several decades and spawn string counts used as a proxy for population size in many of them. Averaged over time, these counts have proved reliable as estimators of relative, though not absolute population sizes. Microsatellite He correlated significantly with *B. calamita* population sizes across 33 localities in Britain, but equally strongly, and independently, with the distance of the population from those at the northerly range edges on the east and west coasts, as summarised in Table 3 (Rowe et al., 1999). Range effects are even stronger at the full biogeographical range scale. Microsatellite diversity in *B. calamita* declines continuously from south-west Europe, the probable glacial refugium, eastwards to Poland and Estonia (Rowe et al., 2006) despite the persistence of large toad populations in eastern Europe. This, too, is a theoretical expectation. Postglacial colonisation is based on successive founder events, each involving only a few individuals and thus just a fraction of the genetic diversity present in the population of origin. The important consequence of two separate factors strongly affecting genetic diversity at neutral loci is that diversity estimates cannot be taken simply to reflect population size. Many European species in various taxonomic groups exhibit this pattern of genetic depauperisation following postglacial recolonisation (Ibrahim et al., 1996).

Genetic diversity and fitness

Particularly important for conservation are the implications of genetic measurements for assessing population viability. Amphibians are good subjects for studies on fitness because larval growth and development rates are important survival factors and are easily quantified. In many species, rapid growth predicates high survival to metamorphosis, especially where animals breed in small or temporary ponds. Laboratory investigations comparing larval growth and survival of *R. temporaria* and *B. bufo* from populations breeding in garden ponds with larger ones in rural habitats showed, for both species, elevated numbers of developmental abnormalities together with reduced survival and heterozygosity at multiple allozyme loci in the urban sites (Hitchings & Beebee, 1997; 1998). Growth rates of *B. calamita* larvae from six populations of widely differing microsatellite diversities also correlated with He (Rowe et al., 1999). However, a study using microsatellites found no differences in genetic diversity between urban and rural populations of *R. temporaria* and higher larval growth rates in urban relative to rural populations (Zeisset & Beebee, 2010). This discrepancy is rather typical of variable reports on the relationship between neutral loci and fitness attributes. Perhaps it is not too surprising that correlations are often weak or inconsistent. An alternative is to look at loci likely to

Table 1. Genetic diversity estimates for amphibian populations. He, expected heterozygosity; An, mean number of alleles per locus; Ar, allelic richness (= An adjusted for sample size. Not always estimated).

Species and sample location	Number of microsatellite loci analysed	He	An	Ar	References
Triturus cristatus:					Babik et al. (2009)
Gaddesby, UK	5	0.73		5.0	
Krefeld, Germany	5	0.76		4.5	
Rana temporaria					Brede & Beebee (2006a)
Ainsdale, UK	8	0.60		4.56	
Bonn, Germany	8	0.62		5.21	
Bufo bufo					Brede & Beebee (2006a)
Ainsdale, UK	8	0.57		3.27	
Bonn, Germany	8	0.66		4.05	
Bufo (Epidalea) calamita					Rowe et al. (2006)
Ainsdale, UK	8	0.31	3.0		
Coto Donana, Spain	8	0.80	7.2		
Parnu, Estonia	8	0.28	1.5		
Pelophylax lessonae					Zeisset & Beebee (2001)
Thetford, UK	6	0.00	1.0		
Uppsala, Sweden	6	0.00	1.0		
Paris, France	6	0.49	3.3		
Pelophylax ridibundus					Zeisset & Beebee (2003)
Romney, UK	5	0.48	2.2		
Balaton, Hungary	5	0.52	3.2		

Table 2. Genetic diversity estimates for reptile populations. He, expected heterozygosity; An, mean number of alleles per locus; Ar, allelic richness (= An adjusted for sample size. Not always estimated).

Species and sample location	Number of microsatellite loci analysed	He	An	Ar	References
Vipera berus					Ursenbacher et al. (2015)
NE France, Belgium & Netherlands	9	0.39	2.46		
Massif Central, France	9	0.61	3.62		
Coronella austriaca					
Arne, UK	8	0.53	2.60	2.50	Pernetta (2009)
Pieniny, Poland	14	0.68		4.06	Sztencel-Jablonka et al. (2015)
Lacerta agilis					
Dorset, UK	15	0.75		5.32	Russell (2012)
Asketunnan, Sweden	15	0.54	4.3		Schwartz & Olsson (2008)
'Continuum', Hungary	15	0.83	11.2		Schwartz & Olsson (2008)
Podarcis muralis					Michaelides et al. (2016)
23 populations, UK	16	0.62	3.9		
21 populations, Italy	16	0.77	6.5		
13 populations, France	16	0.69	5.0		

be under selection, and which might therefore better reflect population genetic health. Such loci are harder to find and to analyse than microsatellites, but one set that has attracted widespread attention is the Major Histocompatibility (MHC) Complex. These genes are vital components of the immune system and vertebrates usually have a highly diverse array of MHC alleles. MHC class II loci have been analysed at the population level for *T. cristatus*, *R. temporaria* and *B. calamita*. For those hoping these genes might provide a window into the genetics of population viability, the results have been somewhat disappointing. In both *T. cristatus* and *B. calamita*, MHC diversity varied at the biogeographical range level in a way that largely mirrored microsatellite diversity, declining as a function of distance from their glacial refugia. It seems that random genetic drift has been more important than selection for MHC diversity in these species, a result that implies disease resistance may not have played a major role in determining their current status (Babik et al., 2009; Zeisset & Beebee, 2014). Similarly, among urban and rural *R. temporaria* populations there were no indications that MHC alleles or diversity levels related substantially to larval fitness attributes (Zeisset & Beebee, 2010).

Is there, then, any reliable way of detecting genetic problems in herpetofauna populations? Events at Saltfleetby, in Lincolnshire, suggest that the task is not impossible. The small *B. calamita* population on this nature reserve is at the species' northerly range edge on England's east coast. In the early 1970s it was on the verge of extinction and subsequently responded only slowly to extensive conservation management. Diversity at microsatellite loci was very low in the 1990s, with average He = 0.189, though two other populations in Cumbria had similarly low diversities but were apparently thriving. In laboratory trials, Saltfleetby larvae had by far the slowest growth rate of any *B. calamita* population tested (Rowe et al., 1999). Given the unreliability of lab fitness trials described earlier, experiments were also carried out in the field at Saltfleetby comparing growth

Table 3. Genetic diversity and demography in *B. calamita*. Data were from 33 British natterjack toad populations and subpopulations. Population sizes were each the averages of spawn string counts over 5-10 years between 1986 and1995 (derived from Rowe et al., 1999).

Comparison	No. comparisons	Correlation coefficient (r)	Probability of no correlation
He x Mean population size	28	0.46	<0.02
He x distance to range edge	33	0.51	<0.01
Mean population size x distance to range edge	28	0.25	0.15

Table 4. Census (Nc) and effective breeding (Nb) population size estimates for two widespread anurans. Both populations of both species bred in the same ponds in Sussex (derived from Brede & Beebee, 2006b).

Species	Site name	Mean Nc estimate	Mean Nb estimate	Nb:Nc
Bufo bufo	Pells	1,200	49	0.04
	Whitelands	1,022	34	0.03
Rana temporaria	Pells	236	86	0.36
	Whitelands	36	12	0.33

Table 5. Comparative survey results for *T. cristatus* using conventional and eDNA methods.Results are from four surveys of each of 35 ponds, and are derived from Biggs et al. (2015).

Method	Mean no. of successful detections (range)	Mean percentages of successful detections
eDNA	34.75 (34-35)	>99
Bottle trapping	27.50 (26-30)	79
Torch searching	26.25 (20-34)	75
Bottle trapping + torch searching	34.00 (33-35)	>97

rates and survival of larvae from Saltfleetby with those from a large population at Ainsdale on the Merseyside coast. These trials confirmed the lab results; Saltfleetby larvae were much less fit for survival in an ephemeral pond environment than those from the large population, even on their home ground (Rowe & Beebee, 2003). However, the Fis statistic (an indicator of local inbreeding) of Saltfleetby toads did not differ significantly from zero. This implies that inbreeding was not the primary cause of the genetic problem. Fis varies from zero (no inbreeding) to one (highly inbred) so an alternative reason for the lack of fitness must be responsible. It seems that progressive fixation of deleterious alleles, consequent upon genetic drift in the rapid decline of this small population, led to a damaging genetic load that put the population's viability at risk.

Starting in 2003, Saltfleetby *B. calamita* were subject to an attempted genetic restoration by introducing larvae (accidentally, as escapes during a replicated pond experiment) from Ainsdale and, later, deliberately from East Anglia. Spawn string counts, metamorph success and genetic diversity subsequently increased at Saltfleetby, indicating that the restoration may have made a positive contribution to population viability (Beebee, 2014). Average numbers of spawn strings per year increased significantly (Median Test P = 0.046), by about 45%, between 2009 and 2017, compared with the preceding nine years for which data were available. 2009 was the first year in which substantial numbers of post-restoration adults would have been sexually mature, However, direct measurements of larval growth rates in recent years are lacking, but necessary, to confirm a fitness effect of the restoration.

Investigations into the genetics and viability of non-native *P. muralis* showed that diversity was lower in England than in the French and Italian source populations, and that embryonic mortality was highest in the introduced British populations (Michaelides et al., 2016). Inbreeding may put the long-term future of these lizards at risk. As with Saltfleetby *B. calamita*, there was no simple correlation between microsatellite diversity and any fitness measure at the population or individual level. A *V. berus* population in Sweden exhibited both low genetic diversity and evidence of inbreeding depression, manifest as high incidences of stillborn and malformed offspring. A successful genetic rescue was accomplished by the addition of several males from an outbred population, leading to both increased offspring survival and genetic diversity (Madsen et al., 1999). The unpublished British *V. berus* study mentioned earlier was also intended to assess risks from inbreeding in British populations but looked only at genetic diversity. Based on the experiences outlined above, molecular studies alone are unlikely to identify genetic risks to British adders in the absence of quantitative information about fitness characters.

Assessing population size

Another prediction from genetic theory is the '50/500 rule' (Franklin, 1980). This infers that populations averaging 50 or fewer individuals over several generations will become subject to inbreeding effects, while in those with fewer than 500 genetic diversity will gradually erode over time. These numbers seem unrealistically large for most amphibian and reptile populations in the British Isles, especially for the rare species in restricted habitat areas. Furthermore, the theory relates to 'effective' population size, Ne, and not census size, Nc. Ecologists typically measure Nc using methods such as capture-mark-recapture, but Ne approximates to the number of individuals that successfully reproduce and is of greater relevance to population viability. Ne is typically much smaller than Nc (Frankham, 1995).

Neutral genetic markers provide methods for estimating Ne, which is difficult to do by standard ecological approaches. Because neutral genetic diversity is affected by random genetic drift, allele frequencies change more quickly in small than in large populations. It is therefore possible to compare allele frequencies across generations and thence obtain estimates of Ne.

In amphibians, this has been attempted by genotyping adults and larvae in the same year. Because many amphibians survive to breed more than once, these estimates are referred to as Nb, the effective breeding population size, which will be smaller than Ne but often not by much.

Estimates of Nb and Nc in several British *B. bufo* populations resulted in very small Nb:Nc ratios, varying from around 0.01 to 0.04; by contrast, ratios for multiple *R. temporaria* populations averaged around 0.3 (Scribner et al., 1997; Brede & Beebee, 2006b; Table 4). More recently, methods for determining Nb based on a single generation sample have been developed and applied to multiple British *B. calamita* populations yielding an average Nb:Nc ratio of around 0.15 (Beebee, 2009). These estimates are typical of those found in many other species and imply a need for census population sizes in the hundreds to avoid inbreeding risks and an order of magnitude higher to minimise genetic deterioration. These unrealistic aspirations are moderated to some extent by the discovery of genetic compensation. In both *T. cristatus* and *B. calamita* the Nb:Nc ratio increases markedly when population sizes are low (Jehle et al., 2005; Beebee, 2009) but nevertheless very few amphibian and reptile populations will ever be large enough to comply with these theoretical requirements to maintain long-term genetic health. There seems no straightforward way of relating theory and practice in this situation, though a lot hinges on the definition of a population and whether high gene flow can be interpreted as reflecting large metapopulations. This certainly could apply to relatively widespread species, but hardly to rare and geographically isolated ones.

Neutral genetic markers also provide a method for determining recent trends in population size. This is based on the theoretical expectation that He will change more slowly than Ar when populations increase or decrease. The method should reveal bottlenecks or expansions that occurred within the past 50 or so years, depending on species generation time and current population size. For *B. calamita* this approach was tested by comparing genetic inferences with known demographic histories, based on spawn string counts, for 15 British populations. The bottlenecks indicated by genetic analysis from samples taken in the 1990s corresponded almost completely with those known to have happened during the 20th century (Beebee & Rowe, 2001). Vibrant populations showed no bottleneck effects whereas some others, including recent translocations yielded significant bottleneck signatures. No recent population increases were implied. The *R. ridibundus* population at Romney in Sussex, established from just 12 founding individuals in 1935, also showed genetic confirmation of a bottleneck (Zeisset & Beebee, 2003).

Population structure and gene flow

Detecting gene flow and migration between populations

It is often useful to know the extent to which populations are interconnected by migration. Even occasional movements of individuals can help maintain genetic diversity and reduce extinction risks. This is hard to determine by standard ecological methods, especially when migrants are infrequent, as is often the case. Molecular markers offer solutions to this problem. The Fst statistic (Rowe et al., 2017) is a measure of how distinct two populations are with respect to their genetic diversities, and varies from zero (populations identical) to one (totally distinct, with no recent migration). Intermediate Fst values indicate the degree of differentiation, and thus the number of migrants moving between populations averaged over recent time; the lower the Fst, the higher the number of migrants per generation. Fst values indicate 'effective' migration, meaning the numbers of individuals that reproduced successfully in their new location, but cannot easily distinguish the directions of movement. Wherever migration is possible, a theoretical expectation is that pairwise Fst estimates will increase as a function of distance between multiple population pairs. This is a useful basis for identifying barriers to movement, as shown when a pairwise Fst is higher than expected under this 'isolation by distance' model.

Allozyme-based Fst estimates among *R. temporaria* and *B. bufo* populations in Sussex were more than twice as high among urban compared with rural breeding ponds (Hitchings & Beebee, 1997; 1998) despite the fact that distances between urban ponds were much smaller, averaging just over 2 km, than those between rural sites averaging > 40 km. However, this distinction was not seen in a similar study with *R. temporaria* in the same area using microsatellites, but did occur at an MHC locus (Zeisset & Beebee, 2010). Possibly the allozyme and MHC results reflected the fact that these markers can be subject to local selection. There was no correlation between Fst and intersite distances for microsatellites or MHC markers, indicating the existence of substantial barriers to movement between the Sussex ponds in both rural and urban habitats. Further comparisons using microsatellites with rural Sussex populations separated by an average of just 8 km produced Fst estimates for *B. bufo* averaging five-fold higher than those for *R. temporaria* using the same ponds. In this instance, with relatively close ponds, *R. temporaria* showed significant isolation by distance but *B. bufo* did not (Brede & Beebee, 2004). These results imply that *R. temporaria* is genetically a more mobile species than *B. bufo*, probably because the frog typically utilises more ponds in a landscape than does the toad. Taken together the relatively high diversity, mobility and Nb/Nc ratios of *R. temporaria* populations relative to those of *B. bufo* are in accord with differences in densities of breeding sites and operational sex ratios between these species.

Recognition that many amphibians persist as metapopulations was the basis of migration estimates, based on microsatellite analyses, in three areas of Britain with semi-continuous coastal habitat occupied by *B. calamita* (Rowe et al., 2000). Mean Fst estimates between pond clusters, essentially sub-populations in each region increased as a function of distances within suitable habitat between them. It was possible from this study to infer barrier effects of a river and of an intervening urban development, and also which subpopulations were the main sources or recipients of intersite migrants. At the

level of entire biogeographical range both microsatellites and MHC markers exhibited isolation by distance among *B. calamita* populations across Europe, but the gradient was significantly steeper for MHC than microsatellites indicating that selection has operated on the functional gene even though random drift was the most important structuring force (Zeisset & Beebee, 2014).

Defining populations

Assessing migration rates between populations requires, in the first instance, defining the populations in question. Even for pond-breeding amphibians this is often problematic because many species exist as metapopulations, with individuals moving among groups of ponds. For reptiles, there is even greater uncertainty. Are animals on one hillside really quite separate from those on the next one along the ridge? Sophisticated methods, mostly based on assignment tests, have been developed to address this issue using genetic data. In a discrete, isolated population there is a theoretical expectation that the genotypes of all the individuals present should occur at frequencies predicted by the Hardy-Weinberg equilibrium (HWE). Computer programs can analyse all the genotypes from a sample of individuals and assess whether they fall into one HWE group, or into more, in which case several different populations are implied. These groups can then be examined to see whether they are constituted by animals sampled in (say) a corresponding set of ponds or hillsides.

Applying this approach to *B. calamita* microsatellite data from all the localities in Britain where the toad occurred resulted in 38 previously defined populations contracting to about 15 metapopulations, the final number varying slightly according the exact method used (Rowe & Beebee, 2007). This result provided more clarity about 'actual' population size, which could often be considered as the totals of several sub-populations, and demonstrated where it will be important to maintain connectivity by habitat management. Assignment methods can also identify individual migrants if an animal ascribed to one HWE group was actually found at a location where the population was defined by a different HWE group. This does not reveal whether the migrant bred successfully in its new home, but merely establishes its presence. An extended type of assignment analysis can identify larvae in a pond that have mixed parentage via individuals from two separate ponds (Jehle et al., 2005) but no evidence of that was found among the British *B. calamita* populations. Fst analysis therefore remains a useful method for identifying effective migration between populations defined by assignment approaches.

Investigations of *R. temporaria* in Scotland added another dimension to understanding population structure. Microsatellite analyses of frogs from sites in central Scotland up to tens of km apart and at altitudes from <80 m to 900 m above sea level mostly failed to resolve into discrete populations, and low Fst estimates indicated universally high levels of intersite migration (Muir et al., 2013). However, larval period and growth rates did vary, with larvae from populations at the highest altitudes growing faster and metamorphosing more quickly than those from lower down the mountains (Muir et al., 2014). Differentiation of these heritable quantitative traits, quantified as Qst, was fivefold stronger than microsatellite Fst differentiation. Evidently this adaptive variation was maintained in the face of high rates of intersite migration demonstrated by the microsatellite data. Neutral markers do not tell the whole of the story.

Coronella austriaca is the rarest reptile in Britain and of high conservation concern. This snake was the subject of a genetic investigation to compare gene flow among multiple sites across Dorset, the county in which most surviving British populations occur. Smooth snakes were genotyped at microsatellite loci but assignment methods failed to define specific populations associated with the sample sites, despite them being widely dispersed (at least several km apart). Fst estimates between sampling sites were low, with no indication of isolation by distance (Pernetta, 2009). It may be that the patterns of genetic diversity seen in this long-lived reptile reflect population structures that were present decades ago, before recent barriers to movement, especially the expansion of urban areas, fragmented the snake populations to their present state. However, a study within a single area of smooth snake habitat proved more informative. In Wareham Forest, an area of mixed heath and woodland, *C. austriaca* was sampled at 10 locations separated from one another by distances between about 0.5 and 5.5 km. There was a significant isolation by distance effect (Pernetta et al., 2011) and attempts to improve the Fst – distance correlation using hypothesised habitat corridors such as forest rides failed to improve on the correlation with direct-line distances, inferring that all the habitat was available for migration by *C. austriaca*. This microsatellite study made other interesting revelations, demonstrating that males tend to move further than females and that multiple paternity of clutches is commonplace.

Populations of Britain's other rare reptile, *L. agilis*, were the subject of an extensive genetic study based on diversity at multiple microsatellite loci (Russell, 2012). Assignment methods clearly identified distinct groups in the main British distribution zones of this species in Dorset, the Weald and Merseyside. Some clusters of populations within Dorset were also resolved. However, at the finest scale investigated, within Wareham Forest, there was no population differentiation indicating substantial mobility of lizards within the Forest. An important element of the *L. aglis* study within Dorset was progression from isolation-by-distance analysis to a landscape scale investigation identifying habitat features likely to influence the movement of individuals (Fig. 1). This approach revealed probable 'least cost paths' and 'resistance surfaces' for lizards around Bournemouth. Rivers were substantial barriers to sand lizard movement and major roads were probably similar, but because some roads were relatively new their impact on genetic differentiation was not yet strong. Some agricultural land looked permeable to lizards and might become increasingly suitable as climate warming continues. This kind of investigation is valuable for the identification

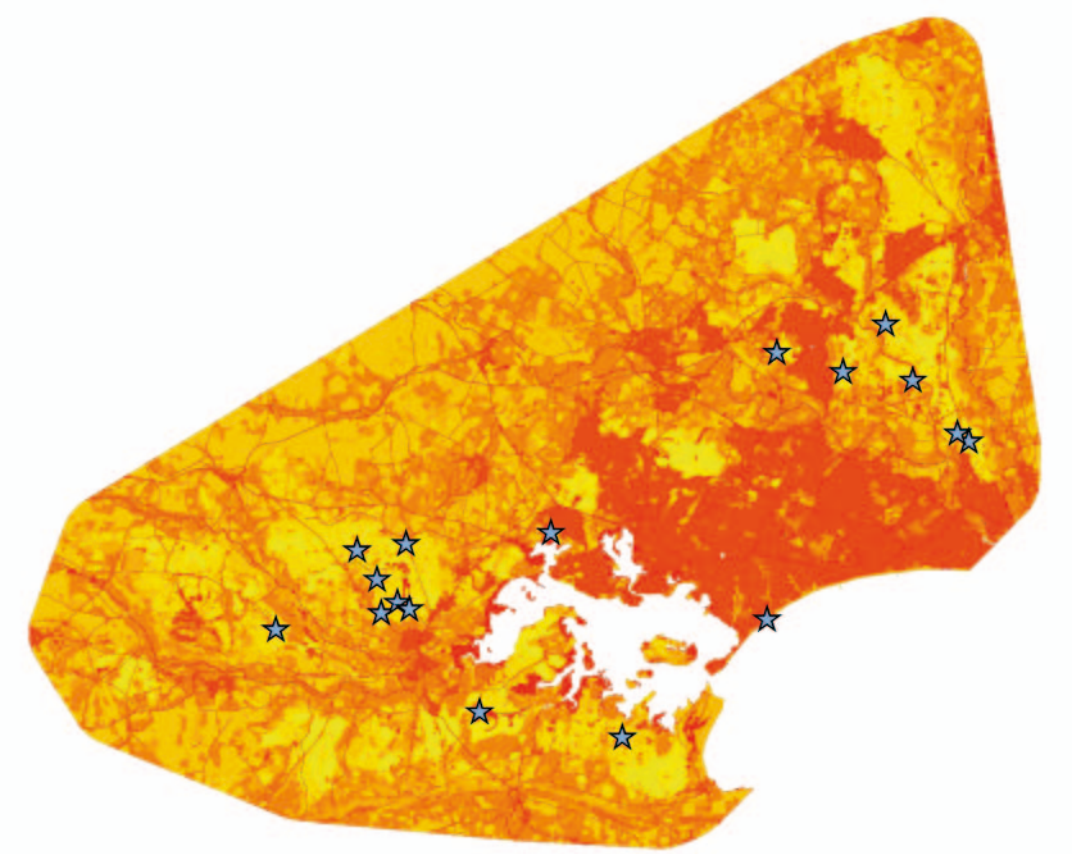

Figure 1. Landscape permeability surfaces for sand lizards in Dorset. The figure shows a resistance surface for *L. agilis* around the Bournemouth-Poole connurbation. Palest (yellow) areas are the most permeable, grading through to deep orange, the least permeable to sand lizard movement. Blue stars represent the lizard sampling sites. After Russell (2012).

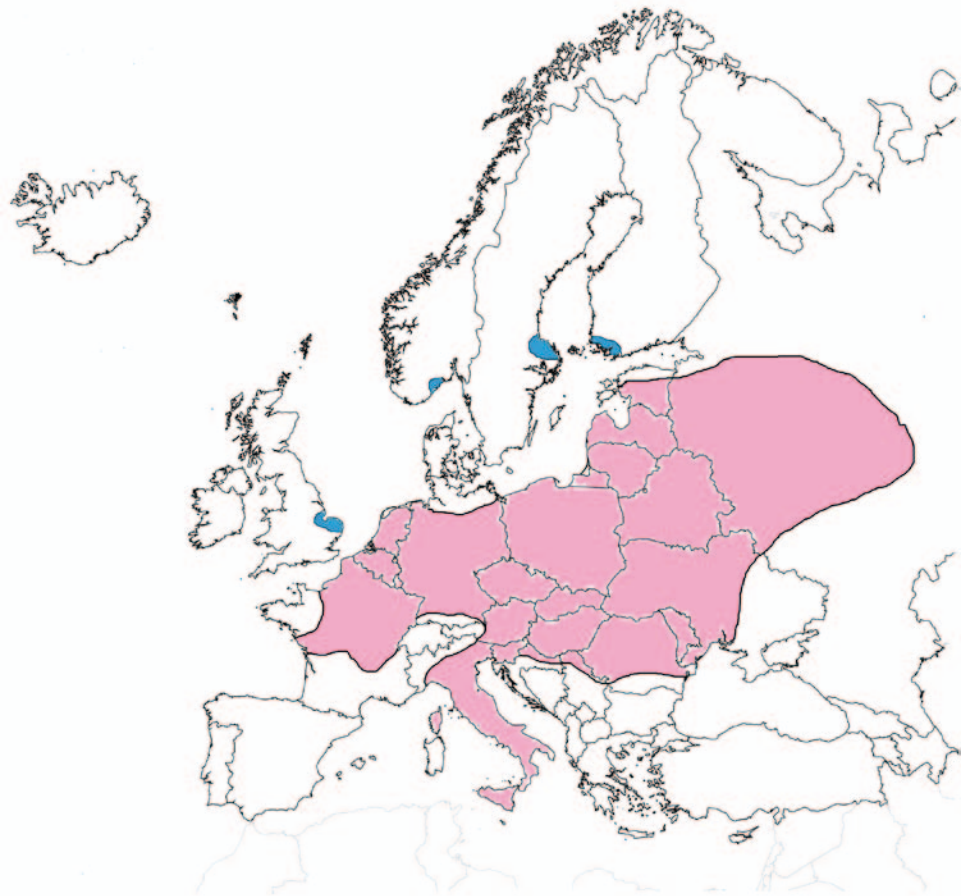

Figure 2. The pool frog in Europe. Blue = approximate distribution limits for northern clade frogs. Pink = approximate distribution of central European pool frogs.

and improvement of corridors to maintain connectivity between increasingly fragmented lizard populations and could usefully be extended to other herpetofauna species in Britain.

Phylogeography

From a conservation perspective, it can be important to know the origins of specific populations, and in particular whether they are likely to be longstanding components of a natural range or recent introductions at the hand of man. This information can be critical at the policy level for determining whether a population should be conserved or, at worst, eliminated. Comparing the genetic profiles of reference populations can provide convincing evidence to distinguish these scenarios and, in the most favourable situations, indicate both the pathways of historical colonisation and likely timing of events.

Mitochondrial DNA is a popular marker for phylogeographical analysis but for herpetofauna in the British Isles it has usually shown too little variation to work well. Fortunately, microsatellites have frequently come to the rescue. A phylogeographic study of *B. calamita* indicated that populations on the north-west coast of England were genetically distinct from those in southern and eastern England, and that the postglacial colonisation of the British Isles by this toad from mainland Europe probably followed separate western and eastern routes. It also showed that *B. calamita* populations in Ireland were very similar genetically to those on the British west coast but must have separated from them during a common original colonisation event several thousand years ago (Rowe et al., 2006). This genetic evidence ran counter to previous speculations that natterjacks were introduced to Ireland by humans, and thus confirmed the importance of conserving these isolated toad populations. A finer-scale study attempted to determine whether recently discovered large populations of *B. calamita* in a geographically separate dune system in Ireland were longstanding, or a result of recent translocation from the well-known colonies. On balance the results indicated that the newly found populations were probably not of recent origin, but this inference was weakly supported and highlighted difficulties of using genetic methods at the limits of distribution where diversity is low (May & Beebee, 2010). A similar picture to that of *B. calamita* emerged for the history of *R. temporaria* in Ireland. Populations in much of the country had west European mtDNA haplotypes like those found in mainland Britain but some in the south-west had unique sequences suggesting that, like natterjack toads, they may have survived in and colonised Ireland from a distinct and separate glacial refugium (Teacher et al., 2009).

Phylogeographical analysis with microsatellite and 'RAPD' genetic markers provided contributory evidence that *P. lessonae* was native to eastern England until the last population went extinct there in the 1990s (Beebee et al., 2005). The East Anglian frogs formed part of a distinct 'northern clade' of *P. lessonae*, only otherwise found in Scandinavia, and distinctly different from introduced populations of this frog in England originating from elsewhere in Europe (Fig 2). The genetic evidence together with that of archaeological remains and male call signatures led to formal recognition of northern clade pool frogs as a native British species and thence to a so-far successful attempt to reintroduce them from Sweden.

Great crested newts *T. cristatus* are widespread in Britain but there was speculation that an isolated group of populations in the far north of Scotland may have originated via human translocation. However, microsatellite-based analyses of genetic diversity patterns indicated that the northern Scottish populations are most probably of natural origin (O'Brien et al., 2015). There were no signs of recent genetic bottlenecks and the observed diversity patterns would have required multiple separate translocations to generate the situation seen today. Once gain the conclusion was that the populations merit continued conservation measures.

An exception to confirmation of native status followed microsatellite analysis of a *L. agilis* population at Aberffraw dunes in Anglesey (Russell, 2012). This study pointed very clearly to a recent introduction, mostly with animals originating from Merseyside but possibly 'contaminated' with some input from Dorset lizards, perhaps following captive breeding. Nevertheless, because this is a rare species that has declined severely in the UK the sand lizards certainly warrant protection both there and at nearby Newborough Warren where another illicit translocation must have taken place.

Podarcis muralis is by far the most successful non-native reptile in Britain. Using a combination of mtDNA and microsatellite markers, Michaelides et al. (2015) sought to identify the origins of 23 English populations of this lizard by comparison with potential sources in France and Italy. For at least nine of the British populations the results suggested separate introduction events from mainland Europe, while eleven probably originated as secondary translocations from the primary colonies. Some of the British wall lizard sites probably have animals hailing from more than one European locality while most British populations apparently originated from Tuscany. English wall lizard colonies are thriving and adapting to the country's relatively cool temperatures, and in some areas, could pose a threat to native *L. agilis*. However, eradication of *P. muralis* from Britain is hardly feasible and, probably not sufficiently warranted to be desirable. Wall lizards are surely in Britain to stay.

Identification issues

Species

Among the most striking recent developments have been the recognition of two new species identities in the British Isles. The taxonomic separation of toads *B. bufo* and *B. spinosus* (previously designated as the subspecies *B. bufo spinosus*) was achieved primarily on the basis of mtDNA and a nuclear DNA marker. *Bufo spinosus* occurs in Iberia and much of western France, while *B. bufo* is widespread in other parts of France, and further east, as well as in mainland Britain. However, it turns out that bufonid toads on the Channel Island of Jersey are

B. spinosus (Arntzen et al., 2014). This discovery added a new species to the British list (Fig 3). Then came a revelation about grass snakes (*Natrix* species), long known to exhibit substantial phenotypic variation across their wide European range. Based on results from both mtDNA and microsatellite studies, a strong morphological and genetic divide between grass snake populations runs north-south, more or less along the Rhine valley, in western Germany. Snakes east of this divide are now classified as *Natrix natrix* while those in France and Britain have been elevated from subspecies to full species status, becoming *N. helvetica* instead of *N. natrix helvetica* (Kindler et al., 2017). Contrary to some press reports this is a reclassification and not a 'new' species in Britain, but in a few areas, there are populations of non-native grass snakes originating in mainland Europe, and which are not *N. helvetica* (e.g. Nash, 2011).

For the most part, the few species of amphibians and reptiles found in the British Isle are easy to distinguish morphologically, though there are a few exceptions. In these cases diagnostic molecular markers can come to the rescue. Larvae of *B. bufo* and *B. calamita* are morphologically indistinguishable when small but can be separated by either protein or species-specific microsatellite analysis. Visual identification of some water frogs (*Pelophylax* species) is difficult and often unreliable. Larvae cannot be separated morphologically and the hybrid edible frog *P. esculentus* can be hard to distinguish from *P. lessonae*. Even more problematic is differentiating between central European and northern clade *P. lessonae*. Microsatellite markers are available for separating *P. lessonae* from *P. esculentus*, and from the marsh frog *P. ridibundus*, providing reference material is available from frogs of known provenance for assignment tests (Holsbeek et al., 2009). There are also RAPD primers that can distinguish northern clade *P. lesssonae* from non-native central European pool frogs (Snell et al., 2005).

Figure 3. *Bufo spinosus*, a new species for the British Isles. Photo: John Wilkinson

The newts *L. vulgaris* and *L. helveticus* occasionally hybridise in the wild. Male hybrids are readily recognised by their intermediate morphology but, probably because females of these species look very similar, no female hybrids have yet come to light. A mixture of RAPD and mtDNA sequences can not only identify hybrids but, because mtDNA has an exclusively female inheritance, can also reveal the direction of the cross (Beebee et al., 1999). Evidently hybrids can result from pairings of either type, i.e. with either *L. vulgaris* or *L. helveticus* mothers. Larvae of these two newts are morphologically indistinguishable but can also be identified non-lethally using the molecular tools appropriate for hybrids.

Disease diagnosis

Infectious diseases have caused mass mortalities of amphibians around the world in recent decades. In Britain, ranavirus outbreaks regularly decimate populations of *R. temporaria* and, to a lesser extent, *B. bufo* (Teacher et al., 2010). Although the pathology of ranavirus in dead or dying frogs is usually obvious, it may sometimes be important to make a definitive identification. This can be achieved by PCR amplification of part of the major viral capsid protein gene in DNA extracted from infected tissue. The fungus *Batrachochytrium dendrobatidis* (Bd) has caused widespread declines and extinctions of amphibians, mostly in tropical regions, and in an unpublished citizen science project (Cunningham & Minting, 2008) was found to be widespread in several British species. Fortunately, this infection seems to be benign in the UK and the fungus was only detectable by PCR amplification of a short section of chytrid-specific DNA in swabs obtained from skin surfaces. In *B. calamita* some mortality due to Bd was seen in captivity but there were no detectable consequences even for heavily infected wild populations (Minting, 2012). Essentially similar molecular analyses have been used to identify the newly emerged *B. salamandrivorans*, the causative agent of a usually fatal disease of some newts and salamanders in parts of north-west Europe (Martel et al., 2014). The effects of this fungus, with the potential to kill *T. cristatus*, look set to be anything but benign if it spreads extensively in Britain.

A fungal snake pathogen, *Ophidiomyces ophiodiicola*, has recently been found in British snakes. First detected in North America, this organism is of a different variety in Europe and can be detected in carcasses and skin sloughs by a specific PCR amplification of part of a ribosomal RNA gene. Skin lesions caused by *O. Ophiodiicola* can be fatal but so far, at least in Britain, seem not to be. In the UK Grass snakes were the species most commonly infected, although the fungus was detected on one adder (Franklinos et al., 2017).

Environmental DNA

The discovery that ponds contain DNA released into the water by animals living in them has the potential to revolutionise survey methods for amphibians. This environmental DNA, 'eDNA', can be amplified from water samples using the PCR and its subsequent analysis can identify the presence of particular species without ever seeing them. In Britain, the method has been extensively characterised for *T. cristatus* and is as or more reliable than conventional techniques such as night torching and bottle trapping for determining whether great crested

newts are present (Table 5, Biggs et al., 2015). False negatives were rare and false positives non-existent in that study. Newts were very rarely found at sites where no eDNA was detected and newts were located at every site containing their eDNA. These controls were essential to validate widespread use of the eDNA method. Because *T. cristatus* is widespread but strictly protected in Britain there is a need for a wide survey efforts to find as many newt populations as possible. Environmental DNA sampling might prove effective for achieving this goal and thus improve the long-term conservation prospects of this charismatic species. The next step in eDNA-based surveys has already been taken. Using universal primers for amplifying part of amphibian mtDNA, it proved possible to identify every species of amphibian present in a pond from a single water sample. This was achieved by 'next generation' simultaneous sequencing of all the DNA molecules produced in the PCR, generating detection probabilities close to one for all species in all the ponds sampled (Valentini et al., 2016). Environmental DNA surveys have advantages of minimal disturbance and a need for fewer site visits than standard surveys, but there are disadvantages too. DNA analyses are expensive and require dedicated lab facilities to minimise cross-contamination risks among samples. Even so, eDNA seems set to become increasingly important as a survey method in future though like any other survey method, DNA concentrations can be affected by environmental factors including in this case UV exposure, temperature and pond substrate.

DISCUSSION

Genetic investigations of amphibians and reptiles in the British Isles have generated some insights relevant to their ongoing conservation. Specifically:

(1) Simply measuring genetic diversity with neutral markers is inadequate as a reliable indicator of population genetic health and viability. This has been known for many years, but not always appreciated by conservationists. A wide-ranging meta-analysis showed that there was no significant correlation between neutral marker and adaptive variation in life history traits (Reed & Frankham, 2001). Low diversity at neutral loci can be a consequence of factors other than reduced fitness, and in particular can occur in large populations near range edges. Investigation of suspected genetic problems such as inbreeding or loss of adaptive variation requires measurements of fitness attributes directly, as shown for natterjack toads and wall lizards in Britain, and for adders in Sweden.

(2) Neutral markers are valuable for defining genetically discrete populations, and for assessing the extent of migration between them. This approach becomes all the more interesting if, as with the studies of Scottish common frogs, it can be related to selective effects on adaptive variation. More applications of landscape genetics to species other than sand lizards will surely be a valuable next step, identifying habitat permeability and putative corridors facilitating movement between populations. Climate change may make the identification of such corridors ever more important.

(3) Phylogeography has proved successful in confirming native status, in various sites, of natterjack toads, pool frogs and great crested newts. These results have informed decisions about conservation priorities and, in one case (sand lizards in Anglesey) brought to light a recent, unlicensed introduction.

(4) Molecular analysis has convincingly split two species that were previously one (each with subspecies) into two. *Bufo spinosus* as well as *B. bufo*, and *Natrix helvetica* rather than *N.n. helvetica* are, as we now know, native to the British Isles. Molecular tools are also available to aid identification in the rather few instances where this can be problematic with British species though it seems likely that, in practice, they will rarely be needed.

(5) Molecular identification is standard practise for the viral and fungal causative agents of disease in British herpetofauna. The technology involved in this work is impressive but has not, and perhaps cannot lead to ways of controlling disease outbreaks. Its value lies in demonstrating the origins, extent and spread of these unwelcome pathogens.

(6) Environmental DNA is an important new tool in the armoury of amphibian surveyors. Its reliability has been well validated as an alternative to standard survey protocols but it remains to be seen as to what degree the costs and demanding lab facilities required will limit its future use.

Inevitably there will be further developments of genetic methods in ecology and their applications to conservation are bound to increase concordantly. High throughput DNA sequencing and the ability to compare rapidly large sections of genomes will surely increase our understanding of genetic diversity and the role of selection on wild populations. DNA editing protocols may make it possible to engineer disease resistance in the face of increasing threats from the spread of novel pathogens. The future is notoriously difficult to predict but certain to be interesting.

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