Volume 28 (April 2018), 63-72

Herpetological Journal

FULL PAPER

Published by the British Herpetological Society

A reassessment of the biogeographic range of northern clade pool frogs (*Pelophylax lessonae*)

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Distinguishing between native and introduced species can be difficult, particularly at range borders where patchily distributed populations may occur away from a species' natural core range. The case of native pool frog (*Pelophylax lessonae*) populations at their northern range limit in Europe is particularly interesting. These are morphologically and genetically distinct populations that are patchily distributed and have been reported from the UK, Sweden and Norway, but up until 2013 were thought to be absent from Finland. When pool frog populations were discovered in south-western Finland they were morphologically classified as belonging to this northern clade. However, the origin of these populations has been unclear and it is possible that the Finnish populations originated through human aided introductions, established themselves recently through natural migration, or are indeed previously undiscovered relic populations. To establish the origin and relationship of these frogs to other populations across Europe we used phylogeographical analysis based on microsatellite and mitochondrial DNA markers. Our results indicate that the Finnish, Norwegian, Swedish, UK, as well as Estonian populations belong to the northern clade. The Finnish frogs are most closely related to Swedish northern pool frogs, but are genetically more diverse. This suggests that the Finnish pool frogs are most likely a relic from postglacial migration, though we could not entirely rule out the possibility of a recent natural or human aided colonisation from Sweden. This has implications for the conservation status of the pool frog in Finland, where it thus far has been considered an invasive alien species.

Key words: Pelophylax lessonae, microsatellites, phylogeography, northern clade, pool frogs

INTRODUCTION

Determining natural biogeographic ranges, particularly at range borders, is central to conservation biology and important for establishing local conservation priorities, often deciding between conservation and eradication (Simberloff, 2003). Where populations occur away from a species' continuous range and beyond their natural dispersal abilities, their origin can be particularly difficult to establish. Such populations may be the result of natural demographic processes, e.g. relics from a previously larger range, or the result of human activities that have caused the translocation and global movement of many species. For conservation purposes, it is important to distinguish between native and introduced populations, as the former is usually deemed more valuable in terms of conservation. Today, genetic tools can be used to investigate the origin of such populations, distinguish between native and introduced populations, and sometimes even pinpoint the source of introductions. Genetic markers have, for example, been used successfully to establish the origin and species identity of water frogs on Cyprus (Plötner et al., 2015), as well as the likely origin of a non-native population of crested newts in Geneva (Arntzen, 2001). They have also been used to determine that potentially native populations of water frogs in Switzerland stem from human introductions (Dubey et al., 2014), as well as establishing the likely number of translocations giving rise to non-native populations in the case of American bull frogs in Europe (Ficetola et al., 2008). Recently, molecular tools have also been used to establish that great crested newt (*Triturus cristatus*) populations in Scotland were native to the Scottish Highlands and not the result of a human translocation, as previously assumed (O'Brien et al., 2015).

Pool frogs (*Pelophylax lessonae*; previously *Rana lessonae*) are another good example of the importance of distinguishing between native and introduced populations. These members of the western Palearctic water frog complex have a European-wide distribution and for most of their range they are found in a hybridogenetic complex with the hybrid edible frogs (*Pelophylax kl. esculentus*). Edible frogs are hybrids between the pool frog and the marsh frog (*Pelophylax ridibundus*) and reproduce by hybridogenesis with either parental species, discarding one parental genome during gametogenesis (Graf & Polls-Pelaz, 1989). This system allows the hybrids to coexist with only one parental species, which in most cases is the pool frog (Berger,

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1973; Ragghianti et al., 2007). Hybrid edible frogs can also reproduce without either of the parental species if there are triploid individuals in the population. Such all-hybrid populations are common in northern Germany, Denmark and southern Sweden (Fog et al., 2001; Christiansen & Reyer, 2011). The distribution of pool frogs and the hybrid edible frog extends from France and Italy to Estonia and the western parts of Russia (Sillero et al., 2014) and partially overlaps with that of other water frogs, such as the marsh frog.

Only at the northernmost edge of the species' range do isolated populations of *P. lessonae* occur without any other water frog species or hybrids present (e.g. Sjögren, 1991a). These northern pool frogs form a distinct clade, which differs genetically, morphologically and behaviourally from central European populations (Zeisset & Beebee, 2001; Buckley & Foster, 2005; Snell et al., 2005; Snell, 1994; Sjögren, 1991b, Fog et al., 2001). The northern pool frog is currently known to naturally occur along to the Baltic coast of Uppland, central Sweden (Sjögren, 1991a; Edenhamn & Sjögren-Gulve, 2000; Nilsson, 2013; Lindgren et al., 2014) and in three ponds in southern Norway, which were first discovered in 1986 (Dolmen, 1997; Dolmen, 2012). It was present in the UK until the 1990s, when it went extinct due to habitat degradation and lack of conservation efforts. At the time it was tragically assumed to be an introduced species, and only after its disappearance genetic data established native species status for this species in the UK (Zeisset & Beebee, 2001; Snell et al., 2005; Beebee et al., 2005). A species recovery program has since helped to re-establish this species in the UK through the translocation of individuals from Sweden (Buckley & Foster, 2005). In Estonia pool frogs are often found in mixed populations, together with the hybrid edible frog, but pure pool frog populations have been recorded in the northern parts of the country (Talvi, 1992; Kuzmin, 1995). A study considering vocalisation patterns in pool frogs across Europe indicated that the Estonian frogs also belong to the northern clade of pool frogs (Wycherley et al., 2002), but so far genetic evidence has been lacking.

The northern clade populations are now recognised as being distinct conservation units of evolutionary importance and the pool frog is now a UK priority species (Joint Nature Conservation Committee, 2010), red-listed as vulnerable in Sweden (Nilsson, 2013), critically endangered in Norway (Direktoratet for naturforvaltning, 2006) and protected under EU legislation as a European Protected Species (EPS, schedule 2 of the Conservation of Habitats and Species Regulations, 2010).

In Finland, water frogs are not known to occur naturally. Apparently introduced populations of marsh frogs (*P. ridibundus*) occurred in the estuaries of the rivers Vantaa and Porvoo in the 1930s-1950s but had gone extinct by the 1960s (Terhivuo, 1993). However, since 2008 water frogs have been reported from several locations near Turku in south-western Finland. On the basis of their morphology these have been identified as hybrid edible frogs (*P. kl. esculentus*) in most locations. No parental species (e.g. pool frogs or marsh frogs) have been found in these populations, indicating the likely presence of polyploid edible frogs. However, populations of pool frogs were more recently reported from at least two locations (Hoogesteger et al., 2013, 2014). These pool frogs closely resemble the northern clade pool frogs in Sweden and have been assumed to belong the northern clade based on morphology (Hoogesteger et al., 2013, 2014). The Finnish pool frog populations are within the natural range of northern clade pool frogs, but the edible frogs are outside of their normal range, indicating possibly different origins of these two species in Finland.

Considering the rarity and precarious status of the few isolated populations of northern clade pool frogs in Europe, the question of whether the Finnish populations present a valuable addition to the northern clade is of great importance. Like many temperate species, *P. lessonae* survived the last glacial maximum in warmer refugia, such as in Italy, where climatic conditions were less extreme (Hewitt, 1999; Zeisset & Beebee, 2001; Snell et al., 2005). As a result of postglacial recolonisation processes pool frog populations at northern range edges have reduced genetic diversity but also carry distinct microsatellite alleles in some populations (Hewitt, 1996; Zeisset & Beebee, 2001). Mitochondrial DNA, the protein-coding gene for cytochrome b in particular, is frequently used to establish phylogeographic relationships and a number of partial cytochrome b haplotypes have been identified in pool frogs (e.g. Canestrelli & Nascetti, 2008; Hofman et al., 2012; Dufresnes et al., 2017). Here we present data on the phylogeography and genetic diversity of pool frog populations across Europe (using mtDNA and microsatellites), with a particular focus on the northern clade populations. We were particularly interested in the question of whether the Finnish pool frogs belong to the northern clade and if their presence in Finland can be explained by recent human translocations, natural colonisation from nearby populations, or whether they may indeed be previously undiscovered, relic populations, which reached Finland through postglacial colonisation processes. We also collected, for the first time, genetic data on Estonian pool frogs to establish their genetic relationship to the northern clade.

MATERIALS AND METHODS

Samples

Tissue samples from adult and juvenile frogs were taken from eight Finnish pool frogs (three from Kaarina and five from Raisio), as well as from nine edible frogs from nearby locations (six from Piikkiö, two from Rusko and one from Kaarina; all museum specimens collected between 2008 and 2015 and preserved in 95% ethanol). Morphological characteristics that distinguish pool frogs and edible frogs, as described in Hoogesteger et al. (2013), were used to separate the two. DNA extraction was subsequently carried out using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions.

We verified morphological species identification using molecular markers as follows: the hybrid edible frogs (*P. kl. esculentus*) contain the marsh frog (*P. ridibundus*) genome and *P. ridibundus* specific markers can be expected to amplify in hybrids. We

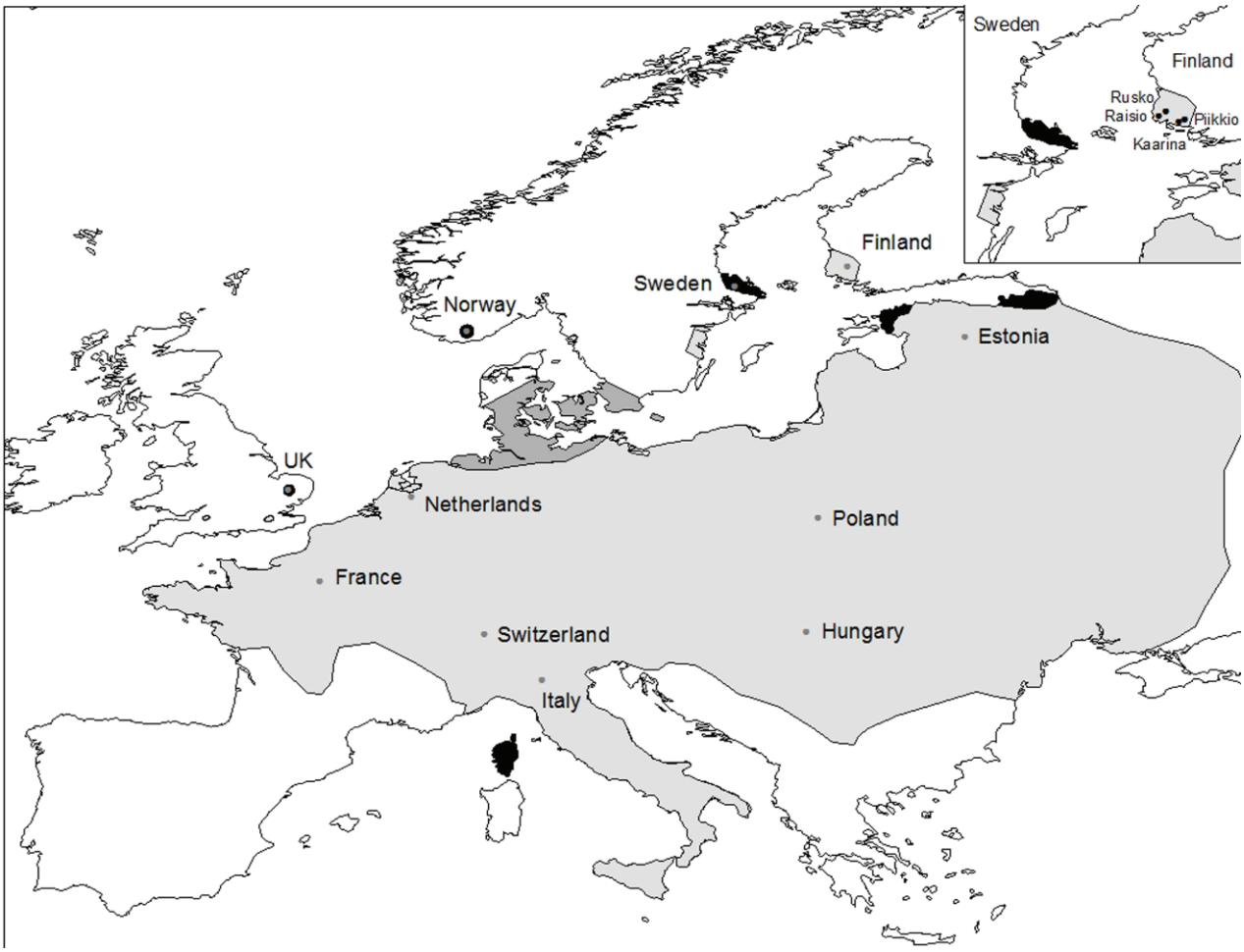

Figure 1. Sampling sites and approximate distribution of *P. lessonae* and *P. kl. esculentus* in Europe. Light grey shading indicates the approximate extent of the European range of both species, dark grey shading indicates pure *P. kl. esculentus* populations and pure *P. lessonae* populations are indicated in black (based on Sillero et al., 2014 & Arioli et al., 2010). Sampling sites are indicated as grey dots; the UK sample was based on historic (native) samples. Details of Finnish populations sampled are presented in map insert.

used two molecular markers to test for the presence of *P. ridibundus* DNA in all Finnish individuals: one *P. ridibundus* specific microsatellite marker, res22 (Zeisset et al., 2000) and a primer pair designed to amplify part of the serum albumin intron in *P. ridibundus* (458bp; Psai1F: TGTGCTAAGTAGGTTTGAGTGT (as in Hauswaldt et al., 2012); Psai1ridR: GTTTTAGTGAGTGGCCCGTG based on GenBank sequence MF667646). PCR reaction mixtures (20µl total) contained 1x standard reaction buffer (NEB), 0.2µM of each primer, 100µM of each dNTP and 0.5 units of Taq DNA polymerase (NEB). All PCRs started with 3 min denaturation at 94 ˚C, then 35 cycles of denaturation at 94 ˚C for 30 sec, annealing (with a touchdown protocol starting with two cycles at 62 ˚C reducing in 2 ˚C steps to 50 ˚C) for 30 sec and elongation at 72 ˚C for 30 sec, with a final elongation of 3 min. We used two *P. ridibundus* and two *P. lessonae* DNA samples as controls. PCR products were visualised using agarose gel electrophoresis.

Pool frog samples from across Europe stemmed from an earlier microsatellite study (Figure 1; Zeisset & Beebee, 2001). The following European countries represented in that study were used: France (N=19), Switzerland (N=40), Netherlands (N=34), Italy (N=18), Hungary (N=31), Poland (N=41), Sweden (N=12), Norway (N=5), UK native (prior to re-introduction), including museum specimens (N=5). Additionally, tissue samples from eight Estonian pool frogs (from Karula national park) were collected and analysed in 2003 (unpublished data).

Microsatellite DNA analysis

Microsatellite genotype data for populations across Europe were obtained from a previous study (Zeisset & Beebee, 2001). Individuals from Estonia and Finland were genotyped at five microsatellite loci using PCR conditions as in Zeisset et al. (2000) for res3, res5, res16 and res20 and Garner et al. (2000) for RlCA18 and RlCA19, and fluorescently labelled primers. Fragments were genotyped using an ABI Prism 377 sequencer (Applied Biosystems) and GENESCAN 3.1.2 software (Estonian samples) or using an automated capillary DNA sequencer (Applied Biosystems, model 3730) at DNA Sequencing and Services (University of Dundee) and scored using Peak Scanner 1v.0 software (Finnish samples). Due to the different allele sizing methods in the current study, we adjusted the sizing of alleles to previously obtained genotypes by including DNA samples with known genotypes from Zeisset & Beebee (2001). One locus (res3) failed to amplify consistently and was excluded from this study. As the hybrid *P. kl. esculentus* contains the marsh frog and pool frog genome, some

Table 1. Microsatellite diversity and mtDNA haplotypes of *P. lessonae* populations (and *P. kl. esculentus*). N= number of individuals, N_l= number of polymorphic loci, N_a= mean number of alleles, N_p= number of private alleles, R=allelic richness based on three individuals, H_o= observed heterozygosity; H_e=expected heterozygosity, N_{mt}= number of individuals sequenced for mtDNA haplotype, hap= name of haplotype obtained.

	N	N_l	N_a	N_p	R	H_o	H_e	N_{mt}	hap
Netherlands	34	5	2.6	1	1.9	0.33	0.36		
France	19	5	3.4	1	2.2	0.30	0.47	3	CC-01 (2x) CC-03 (1x)
Switzerland	40	5	3	0	2.0	0.29	0.39		
Italy	18	4	5.6	6	3.1	0.40	0.58	1	NC-01
Hungary	31	5	8	15	3.1	0.47	0.58	1	CC-02
Poland	41	3	5	1	2.3	0.24	0.36		
UK	5	0	1	0	1.0	0.00	0.00		
Estonia	8	1	1.2	1	1.1	0.05	0.05		
Norway	5	0	1	0	1.0	0.00	0.00	1	NC-01
Sweden	12	0	1	0	1.0	0.00	0.00	4	NC-01
Finland	8	3	1.6	0	1.4	0.05	0.20	8	NC-01
(***P.kl.esculentus***)	9	3	1.6	0	1.5	0.18	0.22		

Table 2. Northern clade pool frog microsatellite genotypes and frequencies. The numbers represent allele sizes in base pairs, with allele frequency given in brackets where more than one allele was present; N=number of individuals genotyped from each country.

Origin	res16	res5	res20	RICA18	RICA19
UK (native) (N=5)	108	131	92	168	92
Norway (N=5)	108	131	92	174	92
Sweden (N=12)	108	131	92	176	92
Estonia (N=8)	108	131	92	172 (0.875) 186 (0.125)	92
Finland (N=8)	108 (0.937) 114 (0.063)	131	92 (0.750) 102 (0.250)	172 (0.313) 176 (0.687)	92
Finland *P.kl. esculentus* (N=9)	108 (0.556) 114 (0.444)	131	92 (0.111) 102 (0.889)	172 (0.778) 176 (0.222)	92

of the microsatellite loci can be amplified from both genomes (e.g. res16) whilst others cannot (Zeisset et al., 2000, Garner et al., 2000). Therefore the data from *P. kl. esculentus* do not represent the actual genetic makeup of these individuals, but do provide some information on the *P. lessonae* part of their genome.

Microsatellite polymorphism was quantified by the mean number of alleles per locus (N_a), number of private alleles (N_p), allelic richness R, observed (H_o) and expected (H_e) heterozygosities for each of the populations studied using GenAlEx vs. 6.4 (Peakall & Smouse, 2006; 2012), Genepop on the web (Raymond & Rousset, 1995; Rousset, 2008) and FSTAT vs. 2.93 (Goudet, 2001). Calculation of genetic distance estimates (D_A, Nei et al., 1983) and neighbour-joining tree construction were carried out in POPTREE2 (Takezaki et al., 2010).

The population affinities of the Finnish *P. lessonae* and *P. kl. esculentus* to other European populations were also tested using STRUCTURE v2.3.4 (Pritchard et al., 2000) with a model that assumed admixture and correlated allele frequencies. We tested from 1 to 12 groups (K), with five replicate runs per K, a 50 000 burn-in period and 10 0000 iterations. The ΔK test was used to determine the most likely number of groups (Evanno et al., 2005) and CLUMPAK (Kopelman et al., 2015) was used to generate a consensus solution and compare the clustering results across different K values.

Mitochondrial DNA

To investigate mtDNA sequence diversity in pool frogs we sequenced 552bp (excluding primer sequences) of the cytochrome *b* gene corresponding to positions 129-680, encoding amino acids 44 to 226 (based on *P. lessonae* mtDNA sequence JN627426) from a total of 18 *P. lessonae* individuals from Finland (N=8), Sweden (N=4), Norway (N=1), France (N=3), Italy (N=1) and Hungary (N=1), using primer cytbPelophylax F1 (CTCCTGGGAGTCTGCCTAAT) and cytbPelophylaxR1 (CGAAGCCTAGAAGATCTTTG) as described in Dubey et al. (2014). This represents a larger section of the cytochrome *b* gene sequenced in a previous cross-European study (based on 410bp, corresponding to positions 129-539; Zeisset & Beebee, 2007). Sequencing was carried out at DNA Sequencing and Services, University of Dundee (https://www.dnaseq.co.uk). Native British and Estonian samples were no longer available. However, we additionally used the haplotypes we obtained to carry out a nucleotide BLAST search on the genetic sequence database GenBank

in order to identify further pool frog cytochrome *b* sequences from across Europe.

RESULTS

Verification of species identification

The marsh frog (*P. ridibundus*) specific markers (res22 and the serum albumin intron marker) verified that all pool frog (*P. lessonae*) individuals had been correctly identified using morphological characters. In the hybrid edible frogs (*P. kl. esculentus*) the two markers failed to amplify any marsh frog DNA in one sample (a female from Piikkiö) and this is most likely a case of a misidentified pool frog. This result was supported by the microsatellite genotype for this individual, which was identical to that of the Swedish pool frogs.

Microsatellite DNA

Microsatellite diversity estimates for *P. lessonae* are presented in Table 1. Central and southern populations (e.g. Italy, Hungary, France, Poland, Netherlands and Switzerland) were all more polymorphic as measured by allelic richness (R), mean number of alleles (N_a), number of polymorphic loci as well as having higher observed and expected heterozygosities, than the northern populations (UK, Norway, Sweden, Finland and Estonia). All individuals from Norway, Sweden and UK (native) were invariant at all five loci and fixed for the same allele at four loci. Only the allele at locus RlCA18 distinguished these three populations (see also Zeisset & Beebee, 2001). Six of the Finnish pool frogs, as well as two edible frogs, had the same allele at this locus as the Swedish population. However, the Estonian and Finnish pool frogs also showed slightly more variation, RlCA18 was polymorphic in Estonia and three loci (RlCA18, res16 and res20) were polymorphic in Finland (in individuals from Raisio). The alleles found in the Finnish pool frogs generally also occurred in the Finnish *P. kl. esculentus*. For details of allele sizes and frequencies in northern clade pool frogs see Table 2.

We used allele frequencies from the five microsatellite loci to construct a phylogeographical tree for *P. lessonae*. The neighbour-joining tree (Saitou & Nei, 1987) in Figure 2 is based on Nei's D_A distance (Nei et al., 1983). D_A is generally considered best for correct tree topology (Takezaki & Nei, 2008), but similar tree topologies were obtained using Nei's standard genetic distance (D_{ST}), with or without sample size bias correction (Nei, 1972), as well as when using the UPGMA method for tree construction (Sneath & Sokal, 1973). There was strong support for the 'northern clade' group, previously consisting of UK, Sweden and Norway (see also Zeisset & Beebee, 2001) and now including the populations from Estonia and Finland with a bootstrap value of 83%. Due to the relatively small number of loci used not all relationships could be resolved, but there was strong support for two larger groups, a 'north-eastern' group consisting of UK, Sweden, Norway, Estonia, Finland, Hungary, Poland and Italy as well as another 'western' group, consisting Switzerland, the Netherlands and France (bootstrap value of 92%).

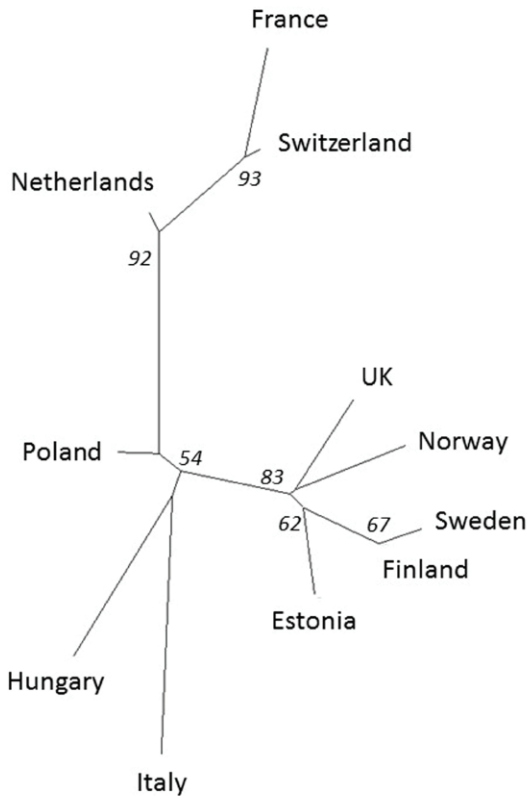

Figure 2. Phylogeographical tree of European *P. lessonae* populations based on D_A distances and the neighbour-joining method. The numbers are percentages of bootstraps (out of 1000); only bootstrap values >50% are shown.

Clustering of microsatellite genotypes

Bayesian clustering assignment of all European *P. lessonae* populations, including the Finnish *P. kl. esculentus*, using STRUCTURE, indicated highest support for the same two larger groups as the phylogeographical tree (ΔK= 429.4; Figure 3). Group one consisted of Norway, Sweden, native UK (prior to re-introduction), Estonia, Finland (*P. lessonae*; PL), Italy, Hungary and Poland (the 'north-eastern' group); group two encompassed the Netherlands, France and Switzerland (the 'western' group), as well as the Finnish *P. kl. esculentus* (PE). There were two individuals within the Finnish *P. lessonae* (both from Raisio) which were assigned to group two and two *P. kl. esculentus* (one from Rusko and one from Piikkiö), which were assigned to group one. One of these was most likely a misidentified pool frog (see 'verification of species identification' above).

Mitochondrial DNA sequences

We sequenced a fragment of 552bp of the mitochondrial DNA (mtDNA) cytochrome *b* gene of 18 individuals. We identified four haplotypes, NC-01 (MG214959), CC-01 (MG214960) and CC-02 (MG214961), which all differed between 1 and 3bp, and CC-03 (MG214962), which differed in 28 to 30bp from the other three. All individuals from northern clade populations (Finland (N=8), Sweden (N=4) and Norway (N=1)), as well as the Italian sample consisted of one genetic lineage (haplotype NC-01). In

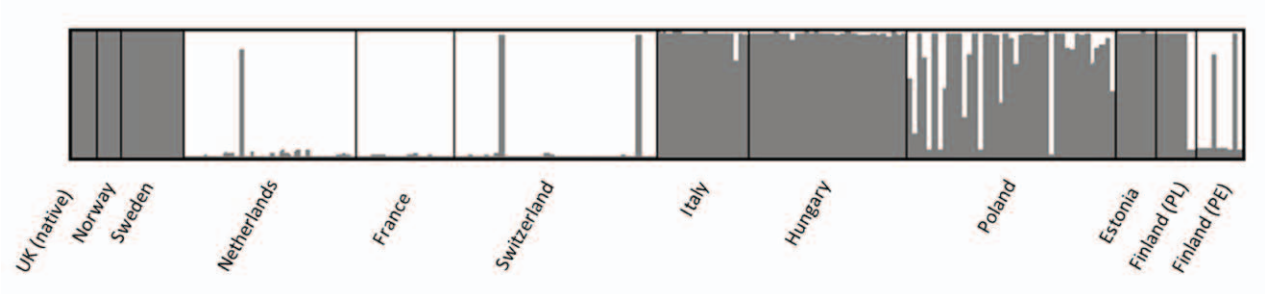

Figure 3. Assignment of European *P. lessonae* populations to genetic clusters using the STRUCTURE algorithm (K=2), assuming admixture and correlated allele frequencies. Vertical lines represent individuals, black lines separate different populations. Finland (PE) consist of *P. kl. esculentus* individuals.

France we detected two haplotypes (two individuals with haplotype CC-01 and one with haplotype CC-03; 534bp sequenced) and a fourth haplotype was found in the Hungarian sample (haplotype CC-02). A BLAST nucleotide search of GenBank revealed that the northern clade haplotype NC-01 is widely distributed in Europe and can be also found in central Sweden (LES17, MF094344), South Sweden (LES25, MF094352), Germany (LES25, MF094352), Italy (LES25, MF094352; LES21, MF094348), Ukraine (LES25, MF094352), Czech Republic (LES20, MF094347) , France (LES25, MF094352; LES23, MF094350), Austria (LES25, MF094352), and Switzerland (LES22, MF094349; LES20, MF094347) (Dufresnes et al., 2017). Haplotype CC-02, which we found in Hungary, can also be found in other central/eastern European countries, such as Poland (LES26, MF094353), the Czech Republic, Romania and the Ukraine (LES04, MF094331) and haplotype CC-01, which we detected in France, has been found in northern Germany (LES11, MF094338). Haplotype CC-03 differed greatly from the other three haplotypes, but only in 1 base pair to sequences identified by others as belonging to the Italian pool frog *P. bergeri* (e.g. BER21, MF94325; Dufresnes et al., 2017).

DISCUSSION

The molecular phylogeographical analysis based on five microsatellite loci strongly inferred that both the Finnish and Estonian *P. lessonae* belong to the distinct northern clade of pool frogs, which include populations in Norway, Sweden and the UK (Zeisset & Beebee, 2001). Although sample sizes were low, the similarity in the genetic profile between the Swedish and Finnish pool frog populations was notable. The five microsatellite loci are invariant within populations in Norway, Sweden and the UK and only one of the five (RlCA18) is polymorphic across these populations (Zeisset & Beebee, 2001). Many of the Finnish pool frogs, as well as some of the edible frogs, had an allele at locus RlCA18 which is found in Swedish pool frogs and only at very low frequency elsewhere (i.e. at a frequency of 0.013 in Poland). As per theoretical expectations the northern clade populations have markedly lower genetic diversity indices than other European populations. Although based on small sample sizes, the Estonian as well as the Finnish population both exhibited slightly higher diversity values than those from Norway, Sweden and UK (historic samples).

The phylogeographical analysis and the inclusion of the Estonian pool frogs in the northern clade supports the notion that the northern clade pool frogs originated from an easterly postglacial colonisation route (Zeisset & Beebee, 2001; Snell et al., 2005). Northward range expansion was generally faster along eastern routes than in western Europe for many species (Hewitt, 2000). There is also mounting evidence of more northerly 'cryptic' refugia for many species (Provan & Bennett, 2008; Schmitt & Varga, 2012; Stewart & Lister, 2001) and the moor frog *Rana arvalis*, for example, is thought to have survived several glacial cycles in a refugium in the Carpathian basin (Babik et al., 2004). The discovery of isolated pool frog populations in Romania, and higher genetic diversity in pool frogs in central and eastern Europe, also point towards the possibility of secondary glacial refugia in this geographic region, which may have been the main contributors during the northward colonisation after the last ice age (Covaciu-Marcov et al., 2008; Hoffmann et al., 2015; Dufresnes et al., 2017).

Intraspecific nucleotide polymorphism for mitochondrial DNA in pool frogs in the post-glacial expansion area of Europe is low, with for example four sequenced *P. lessonae* mtDNA genomes (15,376-78 bp without control region) differing on average by only 19 nucleotides in central and western Poland (Hofman et al., 2012). A recent study which sequenced 974bp of the cytochrome *b* in pool frogs across Europe identified 27 haplotypes, although many of these haplotypes differed in only 1bp and northern clade populations were not included (Dufresnes et al., 2017). In our study, all eastern and northern European *P. lessonae* had one of two haplotypes (NC-01 or CC-02) and all northern clade populations in this study had the same haplotype (NC-01), exhibiting no diversity at the section of cytochrome *b* we investigated. Haplotype NC-01 was the most wide-spread haplotype in Europe, found across much of central, eastern and northern Europe, as well as in northern Italy. Additionally we identified a haplotype (CC-03) in France which was highly similar to those commonly found in central Italy and markedly different from the other haplotypes. Pool frogs in Italy may in fact be comprised of two species or subspecies, the pool frog (*P. lessonae*) in the north, and the Italian pool frog (*P. bergeri*) in central and southern Italy. Central Italian mtDNA haplotypes have been documented by others in French and Swiss water frog populations (Dubey et al., 2014; Dufresnes et al., 2017).

There was clear evidence that the Finnish pool frog population was most closely related to the Swedish frogs and there are three possible explanations for this: the population could be (1) a longstanding relic of post-glacial migration patterns, (2) a recent natural colonisation from Sweden or (3) the result of a recent introduction.

Although there are no historical records of the species in Finland, the first explanation seems, according to our results and previously published work, the most likely. The Finnish population is within the natural range limit for northern clade pool frogs and the existence of a natural population in south-western Finland would concord with proposed postglacial recolonisation routes (see Zeisset & Beebee, 2001; Snell et al., 2005). The species is much harder to detect than other water frogs, and populations can easily remain unnoticed for long times. The population in Uppland, Sweden for example was only discovered in the 1940s (Gislén & Kauri 1959), the Norwegian population as late as 1986 (Dolmen, 1997; Dolmen, 2012) and the now extinct population near Norfolk, UK, had gone unnoticed for over a century before being rediscovered in the 1960s (Buckley & Foster, 2005). Moreover, according to some local residents in south-western Finland, water frogs had in fact been present in the area long before they were first reported in 2008 (Ari Karhilahti, pers. comm.). There has been a longstanding lack of records of the Finnish herpetofauna and for example the moor frog (*Rana arvalis*), which is a widespread and common species in Finland, was only known from very few locations before the 1960s (Haapanen & Salkio, 1966). The inland populations of the great crested newt (*Triturus cristatus*) also remained undiscovered until the 1990s (Terhivuo, 1993). The higher level of genetic variation in the Finnish and Estonian pool frogs, compared to the Swedish populations, indicates a possible expansion westwards from Finland and Estonia towards Sweden during postglacial recolonisation. The Swedish population would have lost diversity further during the postglacial colonisation process due to serial bottlenecking and random genetic drift, whilst the Finnish populations would have retained more.

However, a recent or indeed historic natural colonisation from Sweden is possible. We did not find any 'Finnish-specific' alleles at the microsatellite locus RlCA18, as can be found in other northern clade populations and the Swedish population is located on the coast of Uppland on the other side of the Baltic Sea at the same latitude as the Finnish population. The total distance between the populations is about 200 km, but the Åland islands and Turku archipelago form a continuum of islands between Sweden and Finland, with a maximum of about 12 km between islands. This has been a known colonisation route for several species, such as the adder (*Vipera berus*), smooth snake (*Coronella austriaca*) and grass snake (*Natrix natrix*) (Galarza et al., 2014; Kindler et al., 2014). Water frogs are known to be tolerant of brackish water (e.g. Milto, 2008; Litvinchuk et al., 2015) and pool frogs have been found even on distant islets off the Swedish coast in the Baltic Sea (Lindgren et al., 2014; Sjögren-Gulve, 1994). The possible natural expansion of the pool frog from Sweden to Finland via the archipelago has been suggested already by Kaisila (1949). There have been no records from the Åland islands and the Turku archipelago so far, but the presence of undiscovered populations here is possible.

A recent introduction by humans is another possible explanation that cannot be entirely eliminated. A couple of amphibian species traditionally not belonging to the Finnish fauna have been found near Turku in south-western Finland since 2008. Apart from *P. lessonae* and *P. kl. esculentus*, there is an established population of alpine newts (*Ichthyosaura alpestris*; Finnish Invasive Alien Species Portal, vieraslajit.fi) and one confirmed observation of yellow bellied toad (*Bombina variegata*; Ari Karhilahti, pers. comm.), which is strong evidence that introductions of amphibians have recently taken place in Finland. Human aided translocations of water frogs across Europe appear to have happened with some frequency, for example the existence of central Italian lineages of *P. bergeri* in Switzerland and France have been attributed to movement by humans (Dubey et al., 2014; Dufresnes et al., 2017) and a number of alien water frog species have been reported from Belgium (Holsbeek et al., 2010). The presence of the edible frog (*P. kl. esculentus*) in Finland can hardly be explained by other means than an introduction by humans, because there are no known populations within natural colonisation range and the occurrence of the edible frog at this latitude is highly unusual.

However, a recent natural or human aided colonisation should result in a loss of diversity, rather than a gain, and the higher diversity values, albeit based on a small sample size, indicate that the existence of relic populations may be a better explanation.

Our results also suggest that interbreeding between *P. lessonae* and *P. kl. esculentus* may have taken place in Finland, as northern clade specific alleles could be found in the edible frogs. Edible frogs are unlikely to have contributed to the genome and diversity of the Finnish pool frogs, as most matings between hybrids (or hybrid and pool frog) produce hybrid offspring. Matings between triploid hybrids can occasionally produce pool frogs, but these usually die during the larval stage (Christiansen et al., 2010; Christiansen & Reyer, 2011). However, there is a small possibility that there are some pool frogs, produced by hybrid matings, amongst the edible frogs in Finland, and they may have contributed to the genetic diversity of the northern clade pool frogs.

The origin of the Finnish hybrid water frogs *P. kl. esculentus* could not be established in this study and requires further investigation. In our study, their microsatellite genotypes clustered to the 'western' group along with those from the Netherlands, France and Switzerland, but further data from edible frog populations across Europe are needed to resolve this fully.

To conclude, the pool frog populations we investigated belong to the rare northern clade of this species. The Finnish populations appear to be most likely relicts of postglacial migration, but we cannot rule out a recent colonisation (natural or human aided) from Sweden, or indeed the possibility that more than one of the proposed scenarios acted together. The extent of interbreeding between the northern clade pool frogs and other water

frogs in Finland is currently unclear but warrants further investigation. Establishing the presence of northern clade pool frogs in Finland and Estonia presents a valuable addition to our efforts to preserve this unique clade of pool frogs. The protection of the species and its habitats in Finland is recommendable, whether these frogs are relic populations, or the result of a natural or human aided range expansion from Sweden, as currently both pool frogs and edible frogs are considered invasive alien species in Finland.

ACKNOWLEDGEMENTS

We would like to thank Ditte Guldager Christiansen for providing Estonian samples and water frog advice, Joel Rahkonen and Ari Karhilahi (Zoological museum, University of Turku) for providing Finnish samples, Maureen Berg for technical help and Trevor Beebee as well as two anonymous reviewers for advice on the manuscript.

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Accepted: 29 January 2018