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GENETIC DIFFERENTIATION AMONG NORTHERN EUROPEAN POOL FROG (*RANA LESSONAE*) POPULATIONS

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The isolated Swedish metapopulation of pool frogs (*Rana lessonae*) was not discovered until the 1940s and is classified as "vulnerable" in conservation terms. Swedish pool frogs are now known from 96 localities along the Baltic coast of east-central Sweden, and differ from Central European conspecifics in terms of coloration and low allozyme heterozygosity. Using mini- and microsatellite DNA fingerprinting and allozyme electrophoresis, we studied genetic differentiation among pool frogs from Poland, Latvia, Russia, and Sweden. Allozyme variability was partitioned equally within and among the populations (F_{ST} = 0.50). Both allozyme and DNA fingerprint analyses indicated that Swedish frogs were most similar to Latvian ones. The average similarity in DNA fingerprints among Swedish populations was of the same order as the similarity within the Polish, Latvian or Russian populations. Pool frogs from opposite ends of the Swedish distribution, however, were as different from one another as they were from continental conspecifics. Our results complement and corroborate the evidence from other studies, suggesting that there is a "northern clade" of Swedish, Norwegian and British pool frogs, and that the Swedish pool frogs constitute a relict population rather than being descendants from a recent introduction by humans.

Key words: DNA fingerprinting, enzyme electrophoresis, green frogs

INTRODUCTION

The pool frog, (*Rana lessonae* Camerano), is a European water frog taxon that occurs from northern Italy, northwards through central Europe, and with the northernmost populations at 59 and 60 °N in Russia and Sweden, respectively (Fig. 1). In Scandinavia it is known from 96 localities along the Baltic coast of east-central Sweden and from two localities in southern Norway (Dolmen, 1997; Edenhamn & Sjögren-Gulve, 2000). The Swedish local populations form a metapopulation on the northern fringe of the species' distribution, isolated from other conspecific populations on the European continent. In Sweden, dispersing individuals connect central local populations, but there are also a number of isolated populations. Isolation-dependent extinction is common at distances > 1 km from the closest local pool frog population, and from the 1960s until 2001, 60 extinctions and 51 new populations have been recorded (Sjögren, 1991*a*; Sjögren-Gulve & Ray, 1996; Sjögren-Gulve, unpubl. data). Annual census size of an average Swedish local breeding population over five years ranged from 79 to 204 adults with an effective breeding population size of 35-60 (Sjögren, 1991*b*) – about 10% of the size of more central European populations (L. Borkin & M. Rybacki, personal communication).

In Sweden, the pool frog is classified as "vulnerable" according to IUCN criteria (Gärdenfors, 2000), mainly due to its restricted geographical occurrence and threats posed by large-scale forestry (Sjögren-Gulve and Ray, 1996). The Swedish population was discovered in the 1940s, and its origin is unknown and debated (Forselius, 1948; 1962; Waldén, 1955). Two main hypotheses have been put forward: (1) pool frogs from Central Europe were introduced by humans in the mid-18th century (Waldén, 1955); (2) the frogs constitute a relict from the warm Ancylus period about 7000-5500 BC (Forselius, 1962) when the average temperature was 2-2.5 °C higher than today. These two scenarios have different implications for the population's conservation value. If introduced by man, its conservation would merit low consideration, whereas if the population is a geographically peripheral and genetically distinct relict it implies a significantly different population history compared to continental populations, and substantial conservation value (e.g. Lesica & Allendorf, 1995). A similar issue concerns British pool frogs from an area in Norfolk that were examined by Zeisset & Beebee (2001) using genetic variation at microsatellite loci. Using six polymorphic loci, they found that pool frogs from Norfolk, Norway and Sweden clustered as a "northern clade", genetically distinct from conspecifics in the Netherlands, France, Poland, Switzerland, Hungary and Italy. Like the frogs from Norfolk and Norway, Swedish pool frogs differ from Central European conspecifics by having brown dorsal colour instead of green or greenish. They also differ by their pronounced sexual colour dimorphism with very dark brown females (Forselius, 1962; Berger, 1977; Sjögren, 1991*b*), which may gain thermal advantages in the northern climate (Gibbson &

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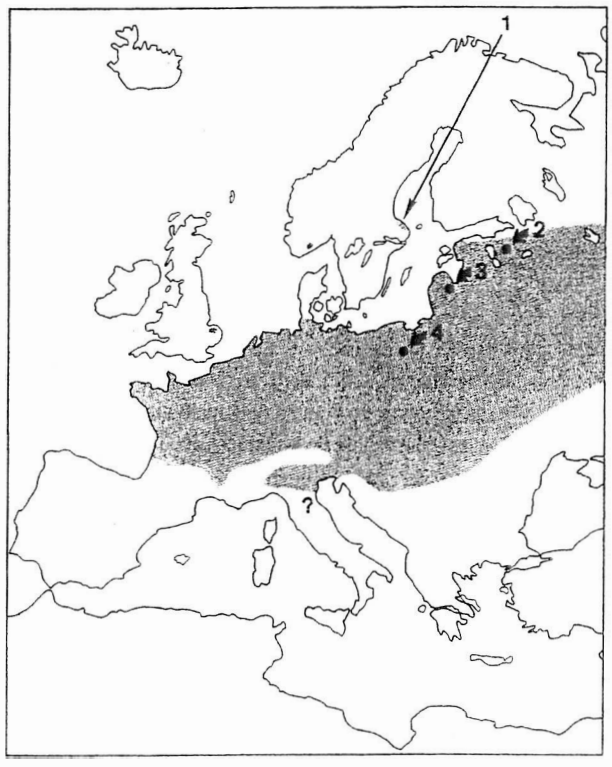

FIG. 1. The distribution of the pool frog (*Rana lessonae*) in Europe (modified from Sjögren 1991*b*). Sampling sites for the present study was: 1. Sweden (see also Fig. 2), 2. Luga (Russia), 3. Tukums (Latvia) and 4. Turew (Poland).

Falls, 1979). The results of Zeisset & Beebee (2001) and the potentially adaptive coloration of the "northern clade" of pool frogs support the relict hypothesis. However, so far, pool frogs from countries north of Poland and east of the Baltic Sea remain unexamined, and these are also potential source areas for introductions.

Fringe populations of widespread taxa often are less genetically variable than central populations (e.g. Lesica & Allendorf, 1995). A previous study (Sjögren, 1991*b*) found that Swedish pool frogs have low allozyme heterozygosity ($\bar{H}_t$ = 0.002, 31 loci) with one or two variable loci in two out of five populations. This low variability seemed more plausibly explained by long-term fluctuations in population size than by anthropogenic introduction in the mid-18th century (Sjögren, 1991*b*). However, because repeated bottlenecks may have the same effect as a single founder event, data on allozyme variability alone do not allow any firm conclusion regarding the population's origin. The process of restoring variation by mutation is slow for neutral genes like most allozymes, in the range of hundreds of thousands of generations (Lande & Barrowclough, 1987). In our present examination of pool frogs from Poland, Latvia, Russia, and Sweden representing "the northern clade", we have therefore used, in addition to allozymes markers, markers with a fast mutation recovery time, which potentially could reveal a higher level of genetic variability and detect a genetic structuring among Swedish local populations. Multilocus DNA fingerprinting using mini- or microsatellites detects many loci at the same time and because of their high mutation rate, in the order of 0.001 to 0.05 mutations per locus and generation (Jeffreys *et al.*, 1985*a*,*b*), the method may discern variability in populations depauperate in allozyme variation and may therefore also be sensitive to recent partitioning of populations.

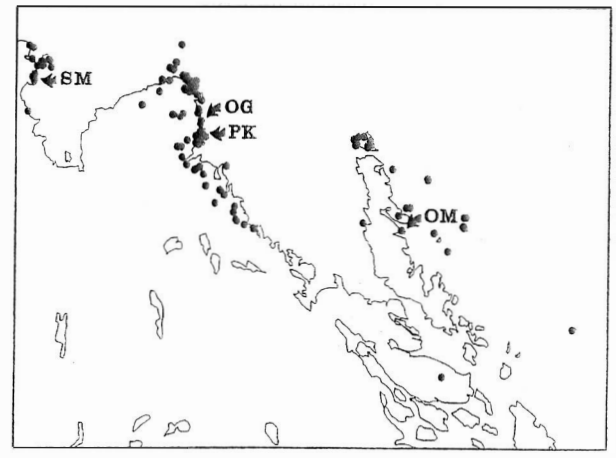

FIG. 2. The regional distribution of pool frog (*Rana lessonae*) localities along the Baltic coast of east-central Sweden (1962 – 1994) and the localities where Swedish samples were collected.

MATERIAL AND METHODS

Biological Material

Frogs were collected from three Swedish localities (Fig. 2, Södra Marörspussen = SM, Prästbäckskärret = PK and Östra Mörtarö = OM). These localities were chosen to represent the distribution of the Swedish population with the easternmost locality (OM) on an island separated from the mainland, one locality (PK) within the main distribution (about 30 km west and east from the other two investigated localities), and finally a western locality (SM). These populations are isolated from each other by sea, geographic distance and effective migration (Sjögren, 1991*a*,*b*). Populations nearby surround population PK. This set represents the centre of the Swedish distribution, whereas OM and SM represent the periphery. Eastern populations on the European continent are represented by one locality in Latvia (Tukums =TK), one locality in Russia (Luga = LU) and central European populations by one locality in Poland (Turew = TU, Fig. 1). The three samples from the European continent each are from one local population situated within a fairly continuous distribution and with population sizes about tenfold larger than the Swedish local populations (L. Borkin & M. Rybacki, personal communication). For the enzyme electrophoresis, one additional Swedish local population was used (Östra Granskärsdammen = OG) which is closely situated to locality PK (about 2 km) and these two localities are probably sparsely interconnected by migration. Some of the results of the enzyme electrophoresis have been published elsewhere (Sjögren, 1991*b*).

Different numbers of individuals have been used for enzyme electrophoresis and DNA fingerprinting. For

enzyme electrophoresis, the average sample size per locus for the four Swedish local populations was SM: 34.0, PK: 9.0, OM: 11.0, OG: 71.1 and for the three remaining eastern and southern localities on the European continent the sample sizes were TK: 23.2, LU: 9.0 and TU: 63.0. For the DNA fingerprinting, sample sizes were considerably lower due to the complexity of the technique, especially because comparisons between gels are not possible and only about 14 individuals could be used on one gel. Thus, comparisons between individuals from the same local population and between individuals from different populations often had to be performed on separate gels. Three Swedish local populations were used for DNA fingerprinting, and sample sizes were SM: 4 , PK: 10 and OM: 11. Sample sizes for the DNA fingerprinting of the populations on the European continent were six individuals each for populations TK, LU and TU.

Enzyme Electrophoresis

Starch-gel enzyme electrophoresis was performed as described by Sjögren (1991*b*) and scored 28 loci: AAT-1, AAT-2, ADH-1, ADH-2, AGP, CPK, DIA-1, DIA-2, EST-1, EST-2, EST-3, FDP-1, FDP-2, GAPDH, GUS, IDH-1, IDH-2, LDH-1, LDH-2, MDH-1, MDH-2, ME-1, ME-2, PGI-1, PGI-2, PGM, PMI and SOD. Analysis of genetic distance was performed using Rogers' modified distance (Rogers, 1972; Wright, 1978) and Nei's unbiased genetic distance (Nei, 1978) with the programme BIOSYS (Swofford & Selander, 1989).

Isolation of DNA

Blood samples were collected from the tarsal vein with a sterile syringe, transferred to Eppendorf tubes with SSC buffer (0.15 M NaCl, 0.15 mM trisodium citrate, 0.5 mM EDTA pH 7.0) and stored at -70°C. Genomic DNA was extracted from approximately 25 µl of blood by addition of 2.5 ml SET-buffer (0.15 M NaCl, 0.05 M Tris-HCl, 1 mM EDTA pH 8.0, autoclaved), 50 µl of 25% SDS w/v and 80 µl of proteinase K (10 mg/ml). The tubes were gently shaken for 2-4 hrs at 37°C, and DNA was purified with two extractions of phenol/chloroform and two with chloroform. DNA was precipitated with 0.1 volume 3 M sodium acetate and 2 volumes 99% -20°C ethanol, removed with a sterile glass hook, washed by dipping in 70% ethanol and dissolved in 0.4-1.5 ml sterile 0.01 M Tris-HCl, pH 8.0 for at least 24 hours. DNA (8-10 µg) was digested with 30 units of restriction enzyme *Alu* I for 4 hours at 37°C, extracted once with phenol/chloroform, once with chloroform and precipitated as above, pelleted at 12 000 g for 20 minutes, washed with 70% ethanol and vacuum dried. The digested DNA was dissolved in 25 µl 0.01 M Tris-HCl, pH 8.0. DNA-fragments were separated in 20 x 30 cm 0.8% agarose gels for about 48 hrs at 1.6 V/cm and transferred to Biodyne Nylon membranes by Southern blotting in 10 × SSC.

DNA Fingerprinting

50-75 ng of the insert of human minisatellite clone 33.15 (Jeffreys *et al.*, 1985*a*,*b*) was ^{32}P labelled by the random primer method (Feinberg & Vogelstein, 1983). Prehybridization and hybridization were performed according to Georges *et al.* (1988) using dried skimmed milk. Membranes were washed 2 × 15 min in 1.5 × SSC, 0.1% SDS at room temperature, 2 × 15 min in 1 × SSC, 0.1% SDS at 60°C and finally 10 min in 1 × SSC at room temperature and autoradiographed at -70°C for 1-6 days using Kodak X-omat AR and intensifying screens. Apart from the 33.15 DNA probe, that was used throughout our investigation, we initially also tested three other DNA probes (and two other restriction enzymes, *Hae III* and *Hinf I*) to improve resolution. The minisatellite probe M13 (Vassart *et al.*, 1987) was isolated directly from the phage and labelled, hybridised and washed from the membranes with the stringency conditions given above. The synthetic dinucleotide microsatellite repeats $(TC)_n$ and $(TG)_n$ (250ng, Pharmacia LKB Biotechnology) were labelled by standard nick translation (Promega), hybridised to the *Alu* I digested DNA according to Ellegren (1991) and membranes were washed in 0.1 × SSC, 0.1% SDS at 60 °C for 40 min and exposed to x-ray film as above. DNA probes were removed from membranes by washing in 0.4 M NaOH and 0.2 M Tris-HCl, pH 7.5. Most membranes were subjected to different exposure times to visualise bands of different intensities. DNA fragments in the range of 2-20 kilobases separated in the same gel were compared using acetate sheet overlays where all bands were marked and subsequently used to calculate genetic similarity. The band sharing statistics according to Wetton et al. (1987) were used to calculate the proportion of shared bands between two individuals (Similarity index $S = 2N_{AB}/(N_A+N_B)$). The significance of differences between average similarities was tested using two-tailed *t*-tests.

RESULTS

Enzyme Electrophoresis

Among the seven populations that were investigated for enzyme variation at 28 loci, there were seven polymorphic loci (AAT-2, EST-2, EST-3, GUS, IDH-2, LDH-2 and PGM). Among the four Swedish local populations, two loci were polymorphic (EST-2 and IDH-2), but two of the local populations (PK and OM with the smallest sample size, 9 and 11, respectively) were monomorphic at all 28 loci. The Russian sample (LU) was variable at one locus (EST-2) and fixed for a unique alternative allele at the GUS locus. The Latvian sample (TK) was polymorphic at two loci (AAT-2 and EST-2) and the Polish population (TU) at five loci (AAT-2, EST-2, EST-3, LDH-2 and PGM). No more than two alleles were found per locus. The average heterozygosity was low in the Swedish populations (0.0-0.5%; SE: 0.0 - 0.4%) and in no case did observed

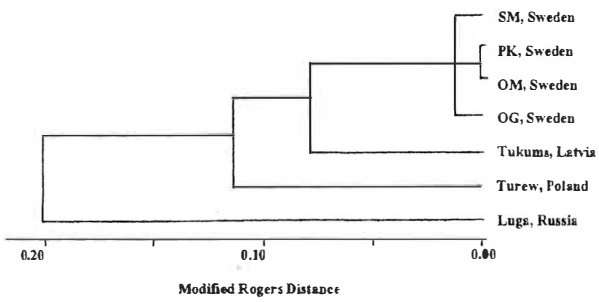

FIG. 3. Genetic similarity in allozymes (28 loci) among Swedish, Latvian, Polish and Russian pool frogs (*Rana lessonae*) illustrated by a UPGMA clustering phenogram based on Rogers (1972) modified distance.

heterozygosity deviate from the Hardy-Weinberg expectations in the Swedish or the other populations. The heterozygosity level was considerably higher in the Polish population (5.3 %±2.7) as well as in the Latvian population (2.3%±1.7) whereas the Russian population showed an average heterozygosity (0.8%±0.8) close to that of the Swedish local populations. Allele frequency data for all populations are available from the second author on request.

In the total sample and over all variable loci, the relative genetic diversity (F_{ST}) calculated according to Nei (1973) showed that approximately 50% of the variability was distributed within the populations, and the remaining 50% of the diversity was distributed between populations. The comparably high level of between-population variability indicates that they have diverged genetically. Based on the modified Rogers distance (Wright, 1978), a phenetic analysis showed that the four Swedish local populations cluster first with small distances (0.000-0.011, Fig. 3). The Latvian population is the genetically most similar continental population (distances of 0.077-0.079 versus the Swedish conspecifics), followed by the Polish population (distances of 0.104-0.105 vs. Swedish conspecifics) and the Russian population (distances of 0.189-0.190). The Russian population was homozygous for an alternative and unique GUS allele. If all loci except GUS are considered, the Russian frogs were most similar to the Swedish ones. The same clustering of populations emerged using Nei's (1978) unbiased distance, but with lower resolution (distances between 0.006 and 0.037 between Swedish and continental populations, and 0.000 among Swedish populations).

DNA FINGERPRINTING

The four different DNA probes we tested showed very different levels of variability within and differentiation between populations. M13 gave the lowest level of variability, sometimes with identical fingerprints for frogs from Swedish local populations (Tegelström & Sjögren, 1990) and fewer bands compared to the other probes tested. The 33.15 probe revealed an intermediate level of variability, a high number of well-resolved bands (Fig. 4a) and was chosen for further analysis.

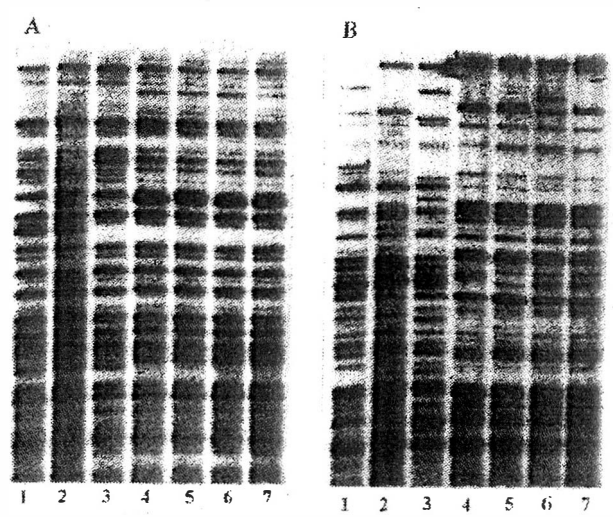

FIG. 4. DNA fingerprints of Swedish pool frogs (*Rana lessonae*) using the minisatellite probe 33.15 (A) and the dinucleotide microsatellite repeat $(TG)_n$ (B) on the same membrane. Fragment sizes are between 20 000 (top) and 2000 base pairs (bottom). Samples 1-3 are from locality PK and samples 4-7 from locality OM. Note the difference in variability and difference between localities revealed by the two DNA probes.

However, the two dinucleotide microsatellite repeats $(TC)_n$ and $(TG)_n$ gave well-resolved fingerprints with a high number of bands and with a potential to detect more variation than the minisatellite probes. Especially the $(TG)_n$ probe gave excellent resolution and detected a higher level of variation than the minisatellite probes (Fig. 4b), even within local Swedish populations.

The average number of bands detected by the $(TG)_n$ probe was 30.2±3.2 (mean±SE; based on 24 Swedish individuals. The level of similarity was consistent within the three Swedish populations (SM: 0.86±0.05, PK: 0.76±0.06, OM: 0.89±0.04, considerably lower than that obtained by DNA probe 33.15 (0.94, $t>100$, $P<0.0001$). When individuals from different Swedish local populations were compared using the $(TG)_n$ probe we found a similarity of 0.50±0.09 (12 comparisons), considerably lower than that found using the 33.15 probe (0.73; $t= 5.6$, $P<0.001$). This level of similarity within the Swedish population obtained by the $(TG)_n$ probe indicates that when more distantly related populations are compared the DNA fingerprinting similarity will approach zero and therefore will be insensitive for such estimates. Therefore, we chose the 33.15 probe for our further investigations to compare the similarities between samples from Sweden and the European continent.

The total number of scorable bands per individual detected by the 33.15 probe ranged from 29 to 43 with an average of 35.6±3.9 over all populations. The band sharing between individuals within the Swedish local populations was 0.94±0.05 (106 comparisons, range 0.84-1.00). The level of similarity was consistent within the three populations (SM: 0.99±0.01, PK: 0.91±0.05, OM: 0.97±0.02). When we compared individuals from

TABLE 1. Band sharing (mean and S.D.) among pool frog (*Rana lessonae*) individuals from the same locality (diagonal) or different localities using DNA probe 33.15. n= number of pairwise comparisons. Locality PK and OM are Swedish, LU is Russian, TK is from Latvia and TU from Poland

	PK	OM	LU	TK	TU
PK	0.81±0.08 *n*=4	0.74±0.07 *n*=6	0.56±0.07 *n*=5	0.62±0.09 *n*=5	0.47±0.07 *n*=5
OM		0.98±0.01 *n*=22	0.50±0.03 *n*=5	0.50±0.05 *n*=5	0.33±0.02 *n*=5
LU			0.74±0.08 *n*=5	0.54±0.05 *n*=8	0.37±0.08 *n*=7
TK				0.75±0.03 *n*=5	0.47±0.05 *n*=10
TU					0.71±0.07 *n*=3

the different Swedish local populations we found a mean band sharing similarity of 0.73±0.10 (11 comparisons, range 0.56-0.79) which is considerably lower than within the local populations (t=6.95, P<0.001). In no case did a value for a comparison of individuals from different local populations exceed the lowest value found for comparisons of individuals from the same population.

The average similarity found between individuals from the three Swedish populations was almost identical to that found within the other three European populations (0.74±0.06, range 0.71-0.75, Table 1). A lower similarity was found when we compared individuals from the Swedish local populations with individuals from Russia, Latvia and Poland (0.48±0.09). These between-population similarities were significantly lower than found for the within-population comparisons (t=11.10, P<0.001). The band sharing similarity for comparisons between the four geographical populations (Table 1) shows that the two Swedish local populations are most closely related to the Latvian (0.56±0.09) and the Russian population (0.53±0.07) and least similar to the Polish population (0.4±0.09). The Polish population compared to the Russian and Latvian population showed a low level of similarity (0.43±0.08) indicating that the sample of Polish pool frogs is the most dissimilar among the four geographical populations.

DISCUSSION

Genetic Variability in the Swedish Population

A previous investigation of allozyme variability of Swedish pool frogs (Sjögren, 1991*b*) showed a low level of variability, which was attributed to long-term strong fluctuations in effective population size. Of the 31 loci that were scored in five local populations, only two (EST-2 and IDH-2) showed variation with one common allele (frequency ≥0.95) and one rare allele. This variation was found in the population OG, while population SM was variable at one locus only (EST-2) and the other three populations (not investigated in the present report) were monomorphic at all 31 loci. These results were confirmed in the present study where two of the Swedish local populations (PK and OM) were monomorphic at all 28 loci and the other two were variable at one locus (EST-2, population SM) and two loci (EST-2 and IDH-2, population OG) respectively. Thus, the allozyme analyses show a very similar genetic constitution of the Swedish local populations.

In a pilot study, Tegelström & Sjögren (1990) used DNA fingerprinting (with the M13 and 33.15 DNA probes) to examine seven juvenile frogs and one adult male from the OG population and found no variation. The present investigation shows that the variability we can detect in single local populations is dependent on the characteristics of that population and which DNA probe is used. The most variable of the local Swedish populations (PK) showed an average similarity of 0.81 using the 33.15 probe and 0.76 with the $(TG)_n$ probe, corresponding to seven bands that differed between individuals, a high level of variation compared to the results using allozymes. Obviously, a single local population may harbour significant micro- or minisatellite variation, indicating that other parts of the genome that may be important for local adaptation also may contain a significant level of genetic variation. Among the three Swedish populations, the two most peripheral ones (SM and OM) showed the lowest level of variation and the most central (PK) the highest. This is an expected result considering both the effective size of the peripheral populations, that is less than that of the central population, and the level of gene flow which is higher among the more central populations (Sjögren, 1988, 1991*b*). By both these mechanisms, peripheral populations are more exposed to genetic drift than central populations and will lose alleles at higher rates than the more central populations.

Even though pool frogs from the same pond or local population are comparably similar in DNA fingerprints, the geographically separated Swedish local populations are genetically different. Using the most variable microsatellite DNA probe $(TG)_n$ we found an average similarity of 0.50 among Swedish populations. Thus, more than 50% of the bands were different comparing

individuals from different populations. Although we have investigated only one central and two peripheral populations, spanning the Swedish range, the large difference in the DNA fingerprints between individuals from different populations, and the unique IDH-2 allele of the OG population, indicate that the Swedish population has gone through a differentiation process. A significant part of the microsatellite variability has become distributed between populations. Pool frogs from the NW and SE ends of the Swedish distribution are as different from one another as they are from Latvian and Russian conspecifics more than 380 km away. Assuming that the neutral genetic markers we have used in our investigation are also good indicators for genetic differences in loci and characters exposed to selection, our results have significant conservation implications. Populations have their own genetic characteristics, rendering a marked genetic conservation value. In cases where translocation of individuals is necessary for rescue actions, actions should be preceded by a genetic survey of the population characteristics.

The Origin of the Swedish Pool Frogs

Two main hypotheses have been suggested for the occurrence of the Swedish pool frogs, isolated from their Baltic and Central-European conspecifics. Either the frogs were introduced from Central Europe by humans in the mid-18th century (Waldén, 1955) or they colonized part of present-day Sweden during the warm Ancylus period about 7000-5500 BC (Forselius, 1962). The importance of distinguishing between these two alternatives could be unimportant, were it not for arguments concerning their conservation value and costs or efforts involved to save these marginal populations. If they were introduced – possibly with a small founder population size – they would be just a sample of the original population and would have lost much of the original genetic variation

Zeisset & Beebee's (2001) study showed that Swedish pool frogs belong to the "northern clade", which also includes English (now extinct) and Norwegian conspecifics, and which is genetically dissimilar to Central European populations. At fingerprint loci, we found that frogs from opposite ends of the Swedish range differ as much from one another as they do from continental conspecifics. Considering both allozymes and microsatellites, we argue that there is sufficient genetic divergence to assign a high conservation value to the Swedish frogs, and frogs representing the "northern clade" (Zeisset & Beebee, 2001) – whether introduced by humans or being relicts. Both allozymes and microsatellite loci clearly show that Swedish pool frogs are more closely related to conspecifics from north-eastern Europe than from Poland indicating a closer ancestry among these marginal populations than to Central European populations. The distribution of the diversity among Swedish local populations could be explained by an introduction 250 years ago, but because of the unique GUS genotype of Russian frogs, we would have to assume that frogs were collected at a location in Latvia, Estonia or Lithuania. The number of introduced frogs would also have to be fairly high, and not just introduced at one Swedish locality, but at several. This scenario is inconsistent with what would be required to achieve the present-day allozyme constitution of the Swedish frogs (see Sjögren, 1991*b*). Inclusion of the alternative AAT-2 allele among founders would also be highly likely, and the Swedish IDH-2 polymorphism must have arisen since the hypothetical introduction. We conclude that the differentiation pattern demonstrated here and in Zeisset & Beebee (2001), together with the brown coloration of the "northern clade" of pool frogs, indicate that the relict hypothesis is the most likely to explain the unusual presence of the peripheral Swedish pool frog population.

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