MOLECULAR ECOLOGY

Molecular Ecology (2010) 19, 1814–1828 doi: 10.1111/j.1365-294X.2010.04603.x

Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location

MARTINA ARIOLI, CHRISTIAN JAKOB and HEINZ-ULRICH REYER

Institute for Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland

Abstract

***Pelophylax esculentus* is a hybridogenetic frog originating from matings between *P. ridibundus* (RR) and *P. lessonae* (LL). Typically, diploid hybrids (LR) live in sympatry with one of their parental species, upon which they depend for successful reproduction. In parts of their range, however, pure hybrid populations can be found. These hybrid populations have achieved reproductive independence from their parental species by using triploid hybrids (LLR, LRR) rather than LL and RR as their sexual hosts. These different breeding systems also entail differences in reproduction (clonal versus sexual) and hence offer the opportunity to study how genetic diversity is affected by reproductive mode, population structure and geographic location. We investigated 33 populations in the Scania region (South Sweden) and 18 additional populations from Northern and Central Europe. Within both genomes (L, R), genetic variability increases with the potential for recombination and declines from the main species distribution area southeast of the Baltic Sea to the fringe populations northwest of the Baltic Sea. Within the main study area in Scania, genetic diversity is low and decreases from a core area to the periphery. Genetic differentiation between Scania populations is small but significant and best explained by 'isolation by distance'. Despite the low genetic variability within the discrete genomes, all-hybrid *P. esculentus* populations in southern Sweden are apparently not suffering from direct negative fitness effects. This is probably because of its somatic hybrid status, which increases diversity through the combination of genomes from two species.**

Keywords: all-hybrid population, amphibia, microsatellites, ploidy, population structure, recombination.

Received 22 July 2009; revision received 28 January 2010; accepted 5 February 2010

Introduction

Genetic diversity is one of the key factors in evolution because it provides the genetic foundation for selection to act upon. The variation in genetic diversity among species or populations arises from a combination of genetic processes, such as mutation, genetic drift and gene flow, and ecological selection arising from spatial and temporal differences in the environment. High genetic variation can play a crucial role for the short- or long-term viability of a species or population because it offers the potential to persist and to adapt to changing or new environments (Lande & Shannon 1996). Conversely, the loss of genetic diversity may have a direct negative impact on the viability of the species or population (Amos & Balmford 2001). Since species are usually structured into more or less separate breeding populations, genetic diversity will depend on the size of and exchange between these subunits (Beebee & Rowe 2004). Natural or human caused fragmentation, for instance, can reduce population size drastically

Correspondence: Heinz-Ulrich Reyer, Fax: +41 44 635 68 21; E-mail: uli.reyer@ieu.uzh.ch

© 2010 Blackwell Publishing Ltd

which, in conjunction with the increasing probability of inbreeding in a small isolated population, decreases their genetic variability.

There are also mechanisms that can increase genetic variability. Although interspecific hybridization is mostly seen as maladaptive and viewed as a problem especially in conservation biology (Frankham *et al.* 2004), successful interspecific hybridization can instantly elevate the genetic variability in the offspring. In newly formed habitats, such suddenly increased diversity may even have played an important role in rapid adaptive radiation (Seehausen 2004). Once formed, however, many hybrids no longer reproduce sexually (Bullini 1994; Dowling & Secor 1997). Instead, they pass on their genome clonally, either without any sperm involved (parthenogenesis) or with sperm triggering egg development but no incorporation of the paternal genome (gynogenesis) or with the paternal genome being incorporated but later eliminated prior to the offspring's gamete production (hybridogenesis) (reviewed by Dawley 1989). For any clonal genome, its evolutionary capabilities are constrained because mutation remains the only source of genetic diversity, and the accumulation of deleterious mutations through Muller's ratchet (Muller 1964) can directly limit the longevity of a clone. In hybridogens low diversity in the clonally transmitted genome can be compensated by higher diversity in the second genome, as the latter comes from a sexual population. In some species, e.g. some fishes, genetic diversity of hybridogenetic individuals is enhanced by occasional incorporation of genetic material from the sympatric parental form (Pala & Coelho 2004). It is especially interesting to investigate the evolutionary potential of systems in which interspecific hybridization has elevated genetic diversity, but clonal reproduction limits genetic variability.

The water frog complex offers a study system where both these conditions occur. The hybrid taxon *Pelophylax esculentus* (named *Rana esculenta* until Frost *et al.* 2006) with the genome composition LR originates from hybridization between two water frog species, *Pelophylax ridibundus* (formerly *Rana ridibunda*) with the genome composition RR and *Pelophylax lessonae* (formerly *Rana lessonae*) with the genome composition LL. *Pelophylax esculentus* reproduces by hybridogenesis, in which only one genome is transferred to the gametes (usually clonally), while the other genome is discarded from the germ line before meiosis (Schultz 1969). In ponds where hybrids belong to the same hemiclone, i.e. carry and transmit the same clonal genome, offspring from hybrid × hybrid matings do not survive. This is due to the accumulation of deleterious mutations on the clonally inherited genome, which occur as homozygotes (Vorburger 2001). In cases where multiple clones with different mutations occur in one population, offspring from hybrid matings can be viable (Guex *et al.* 2002). Hybrid condition is restored in each generation by fusing the clonal gametes of the hybrid with gametes from the sexual parental species whose genome is discarded. This 'hemiclonal' reproduction (Dawley 1989) forces the hybrid into coexistence and mating with the parental species whose genome is discarded. Thus, the hybrid's existence depends on the presence of the parental species and stable coexistence is only achieved within certain boundary conditions for mating preferences, female fecundity and larval performance (Hellriegel & Reyer 2000).

Throughout Europe, different systems of mixed populations have been found. The most common one occurs in Central and Western Europe, where the hybrid *P. esculentus* lives in sympatry with the parental species *P. lessonae* (LE-system). Other common water frog systems consist of *Ridibundus/Esculentus* populations (RE) and *Ridibundus/Lessonae/Esculentus* populations (RLE) (reviewed by Günther 1991 and Plötner 2005). In these mixed populations, a certain degree of genetic variability can be maintained in hybrids by combining their clonal genome with recombined genetic material from the sexual parental species. However, in all-hybrid populations (EE-system), which mainly occur in the northern region of the distribution range (Ebendal 1979; Eikhorst 1987), the hybrid has become reproductively independent of the parental forms (Graf & Polls Pelaz 1989). This was achieved by polyploidization, more specifically by the emergence of triploid individuals. Polyploidy often results from malfunctioning gametogenesis in hybrids (Schultz 1969; Dufresne & Hebert 1994). Although polyploidization seems to be scarce in animals compared to plants, studies have shown that its role in animal speciation can no longer be neglected (Vrijenhoek 2006).

In the water frog system, two types of triploid animals exist, namely LLR and LRR. Triploid individuals are also found in some populations where hybrids occur together with one or both parental species, e.g. in Northern Germany (Günther 1975; Eikhorst 1984), Poland (Rybacki & Berger 2001) and the Ukraine (Borkin *et al.* 2004). It is the all-hybrid populations, however, where triploids play a key role because they take over the role as sexual hosts for the diploid that the parental species have in mixed populations: when mating with the diploid hybrids (the sexual parasite), the triploids provide them with the genome that was discarded during gametogenesis. Triploid individuals usually arise when diploid eggs produced by diploid females (LR) are fertilized by haploid sperm of diploid or triploid males (LR, LLR, LRR). Diploid individuals

© 2010 Blackwell Publishing Ltd

originate from haploid sperm of diploid or triploid males and haploid eggs of diploid or triploid females (Fig. 1) (Christiansen *et al*. 2005; Jakob 2007; Christiansen & Reyer 2009). Genomes are passed from diploids to triploids and vice versa and ploidy levels are therefore not genetically separated (Som & Reyer 2006). In several systems that comprise both diploid and triploid individuals, the triploids reproduce clonally (Poeciliidae) (Lampert *et al.* 2005) or apomictically (*Taraxacum* section *Ruderalia*) (Richards 1973). Hence, it is often assumed that the two ploidy types are reproductively isolated and no gene flow is present. Nevertheless, several studies have shown that such complete isolation is not always present in nature (Menken *et al.* 1995; Meirmans *et al.* 2003).

Although all-hybrid populations of *P. esculentus* have been reported from various areas (reviewed by Plötner 2005), they are often in close proximity to LE, RE or RLE systems. Hence, gene flow between hybrids and parental species cannot be excluded. Our main study area in Scania (southern Sweden) is geographically separated from the distribution of the parental species; therefore, the all-hybrid nature of the *P. esculentus* populations is guaranteed. In this study area, all three hybrid genotypes (LR, LLR, LRR) occur, but the ponds differ in their genotype composition (Jakob 2007). Our study pursued two main goals:

1) Compare genetic diversity of these all-hybrid populations at the northern border of the taxon distribution with that of mixed populations of hybrid and parental frogs in Central Europe.
2) Examine genetic structuring within a small and well defined area and, if found, test for any associations with genotype composition and sex ratio in the ponds.

Males / Females	LR (R)		LLR (L)		LRR (R)	
LR (LR) (R)	LRR	RR	LLR	LR	LRR	RR
LLR (L)	LR		LL		LR	
LRR (R)	RR		LR		RR	

Fig. 1 Gamete production in females and males for the three hybrid genotypes and offspring types arising from the nine potential mating combinations in an all-hybrid population of *P. esculentus*. Female LR can produce both diploid eggs and haploid eggs; triploids of both sexes produce haploid gametes that contain a copy of the genome present twice in the adult. Genotypes in grey boxes do not occur among the adults in the population although they are initially produced (Jakob 2007).

Materials and methods

Population samples

During a pilot study in 2001, one of us (HUR) visited more than 130 ponds all over the province of Scania (southern Sweden) and checked for the presence or absence of *P. esculentus*. Based on these results and additional information from local herpetologists, we identified the centre of the frog distribution (see star in Fig. 2b) and selected an area of 50 × 40 km around it for the actual study. Here, we caught a total of 1662 frogs in 33 ponds. We sampled between 19 and 130 frogs per pond per year. Eleven ponds were sampled in 2002, 13 in 2003 and 9 in 2004 (Fig. 2b, Appendix 1).

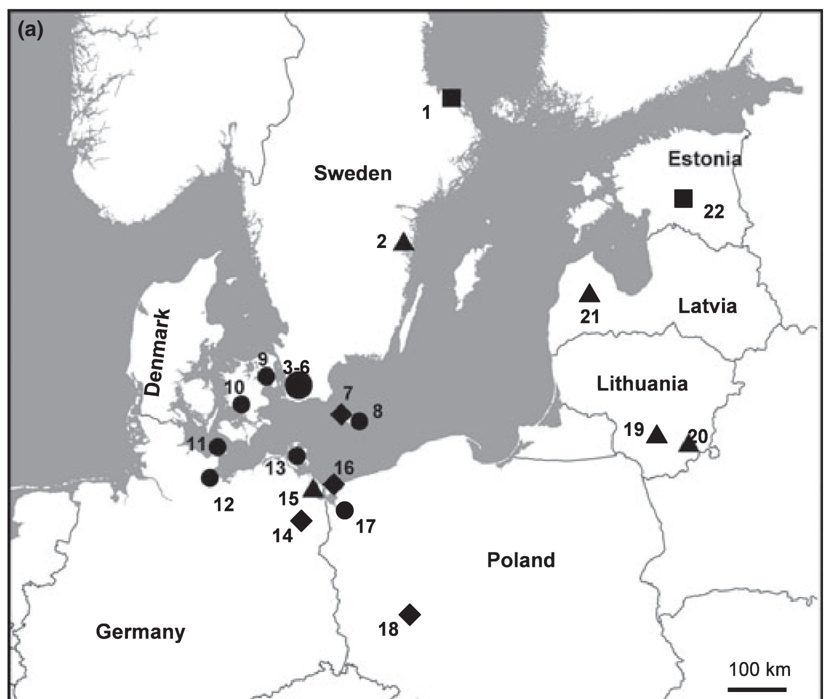
Frogs were caught at night by hand. The following day, we took morphological measurements (snout-vent length, tibia length, length of digitus primus and callus internus length) and collected a blood and tissue sample. Tissue samples (i.e. first segment of the forth toe) were stored in 70% EtOH until used for a microsatellite analysis (see below). Blood was collected by cutting the web in the hind leg, drawn off with a heparinized capillary and then stored in a sucrose buffer at –50 °C. The blood sample was used for flow cytometry analysis. This technique allowed us to distinguish diploid from triploid cells and also to discriminate the two types of triploids (LLR, LRR), because the R-genome contains 16% more DNA than the L-genome (Vinogradov *et al.* 1990; Sharbel *et al.* 1997). The blood samples (100 μL) were mixed with 220 μL of rainbow trout *Salmo gairdneri* red blood cells as internal standard cells, followed by 550 μL of propidium iodide nuclear isolation medium staining solution (PI-NIM), containing PBS buffer solution, octylphenylpolyethylene glycol, 50 μg/mL propidium iodide and 100 μg/mL RNAse A. Prior to flow cytometric analysis, blood samples were filtered. A FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) was used to measure fluorescence of sample blood cells excited with a 15 mW 488 nm Argon laser.

Morphology, microsatellite analyses and flow cytometry results were combined to determine the genotype of each individual as described by Jakob (2007). Additionally, we analysed genetic diversity on a larger European scale by comparing values from four populations in the core area of Scania with those from two more northern Swedish populations and 16 Central European populations (Fig. 2a, Appendix 2; see also Acknowledgements).

Pond measurements

We obtained geographical positions of the ponds in Scania using the program KARTEX2.10 (Lantmäteriverket

© 2010 Blackwell Publishing Ltd



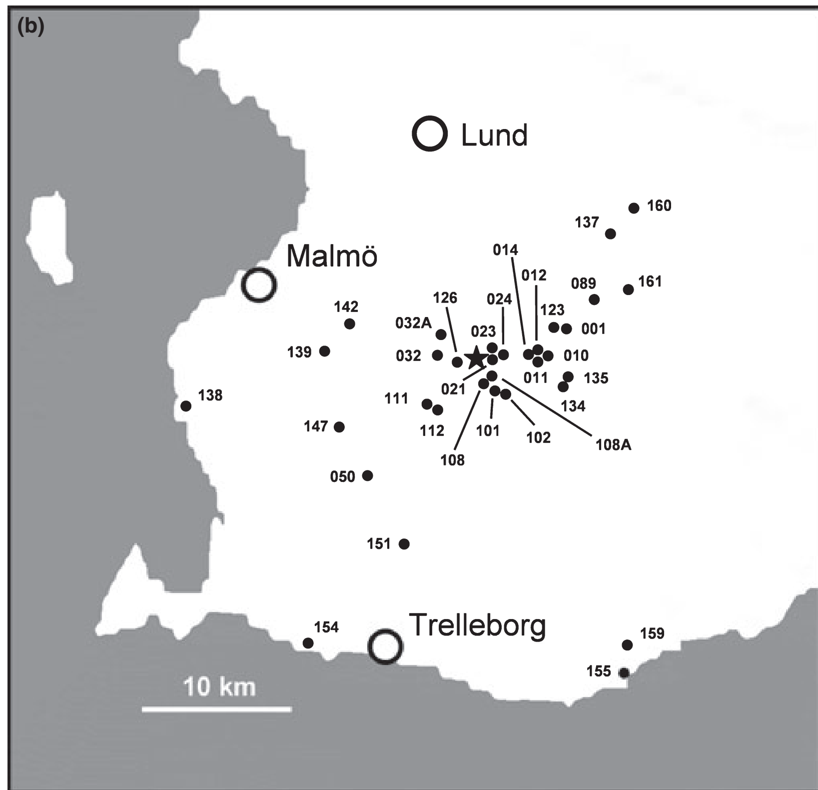


Fig. 2 Sampling sites in Europe (Fig. 2a) and in the main study area in southern Sweden (Fig. 2b). In Fig. 2a this area is shown by a larger dot, representing four ponds (localities 032, 112, 123, 134 in Fig. 2b). The star in Fig. 2b indicates the centre of the distribution from which pond distance within the area was calculated (cf. Fig. 4). Squares = pure *P. lessonae* (LL) populations; triangles = mixed populations of diploid hybrids (LR) and one or the other parental species (LL or RR); diamonds = mixed populations of diploid (LR) and triploid hybrids (LLR, LRR) and one or the other parental species (LL or RR); dots = pure hybrid populations with diploid (LR) and triploid individuals (LLR and/or LRR).

1996). Distances between ponds ranged from 80 m (ponds 108 and 108A) up to 39 km (ponds 154 and 160). Geographical positions for ponds outside Scania were determined using Google Earth.

Molecular methods

DNA of half a toe clip was extracted using QIAamp® DNA mini kit (Qiagen) (samples from 2002 and 2003)

© 2010 Blackwell Publishing Ltd

or BioSprint™ (Qiagen) (samples from 2004). In total, 105 microsatellite primer pairs were tested with a subsample of 15–34 individuals from five different populations in southern Sweden. Primer sets were chosen from Garner *et al.* (2000) (10 loci), Hotz *et al.* (2001) (four loci), Zeisset *et al.* (2000) (nine loci), and 12 primer sequences were kindly provided by H. Hotz and G.-D. Guex. Further primer pairs were developed after screening a mixed dinucleotide (CA, GA) and a tetranucleotide (CAGA) enriched library, following the procedures in Garner *et al.* (2000). Of the 105 loci, 39 did not amplify; among the 66 that did, 50 loci turned out to be monomorphic for the Scania subsample. These we did not use in our analyses because monomorphic loci do not provide insights into population genetics. For 9 of the tested primer pairs, the scored alleles were not unequivocally attributable to one of the two different genomes (L or R) and, therefore, they were also not applied in the analyses. Since we excluded the monomorphic loci for the computation of genetic diversity in Scania, our values overestimate diversity when compared to genetic diversity in other studies. Nevertheless, we can compare genetic diversity over a larger geographical scale, as the 18 populations outside Scania were tested with the same microsatellites.

We screened for variation in all individuals at seven polymorphic loci: RlCa1b5, RlCa5, RlCa18 (Garner *et al.* 2000), Ca1b6, Re1CAGA10, Re2CAGA3 (GenBank Accession nos: EF121548–50) and Ga1a19redesigned (EF121547, see also Christiansen 2009). With these loci, alleles could be unambiguously assigned to either the L- or the R-genome. We verified allele specificity by testing all primer pairs in 11 *P. lessonae*, 14 *P. ridibundus* and 4 *P. esculentus*, cf. Christiansen 2005). Loci RlCa5 and RlCa18 were species-specific for *P. lessonae*; locus Re2CAGA3 was species-specific for *P. ridibundus*. The other four microsatellite loci amplified in both the L- and R-genome (Table 1). Although genome specificity of loci was tested with frogs from Switzerland, we can be sure that the method yielded reliable results for the Swedish population, because the same microsatellite alleles occurred there, and genotypes and ploidies determined by peak height ratios of genome specific alleles (dosage effect) were always confirmed by flow cytometry (Jakob 2007). For the L-genome we found three polymorphic loci, whereas five loci were polymorphic for the R-genome (Table 1). We detected between 1 and 3 alleles for the L-genome (mean 1.67) and between 2 and 7 for the R-genome (mean 4.2).

PCR amplification and electrophoresis for loci RlCa1b5, RlCa18, RlCa5, Re1CAGA10 and Re2CAGA3 was done in a total 10 μL reaction volume containing 50–100 ng template DNA, 0.5 U Taq DNA Polymerase (Sigma) for loci RlCa1b5, RlCa18, and RlCa5 or 0.5 U Hot Start Taq Qiagen for loci Re1CAGA10 and Re2CAGA3, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (Sigma), 100 μM of each dNTP (Roche), 0.5 μM of both forward and reverse primers. PCR conditions for all loci are described in Appendix 3. PCR products of loci RlCa1b5, RlCa5, RlCa18, Re1CAGA10 and Re2CAGA3 were electrophoresed using the SEA 2000® Electrophoresis Apparatus with Spreadex® gels (Elchrom Scientific, Switzerland) and stained with SYBR® Gold nucleic acid stain (Molecular Probes, Inc.). Alleles were scored against the M3 Marker (Elchrom Scientific, Switzerland) using the Q-EL™ 330 Digital Recording and Analysis System (Elchrom Scientific, Switzerland). Because of heteroduplex formation, two of the selected primer pairs (Ca1b6 and Ga1a19redesigned) were amplified and genotyped using a single-stranded system. PCR amplification and genotyping of these two loci was therefore done using a single-stranded system (ABI Prism3100) as follows: for all 2002/2003 samples, PCR amplification was performed in a 10 μL reaction volume containing 10–20 ng of extracted DNA, 5 μL HotstarTaq master mix (Qiagen), double distilled water, and 0.5 μM of forward and reverse primers each. The forward primers were fluorescently labelled with FAM. For all 2004 samples, the 10 μL multiplex PCR reaction contained 10–20 ng of

Table 1 Polymorphic microsatellite loci used in this study

Locus	Electrophoresis system	L alleles	R alleles
RlCa18	Elchrom SEA 2000	183/189	—
RlCa5	Elchrom SEA 2000	258/262/266	—
Re2CAGA3	Elchrom SEA 2000	—	170/198/202/210/214/218/222
RlCa1b5	Elchrom SEA 2000	121	135/137
Re1CAGA10	Elchrom SEA 2000	92/98	96/108/110/114/120/124
Ca1b6	ABI Prism3100	79	86/93/98
Ga1a19redesigned	ABI Prism3100	197	201/203/207

Loci RlCa18 and RlCa5 were species-specific for the L-genome; Re2CAGA3 was species-specific for the R-genome and the other four loci amplified in both genomes.

© 2010 Blackwell Publishing Ltd

extracted DNA, 5 μL 2 × QIAGEN Multiplex PCR Master Mix (Qiagen), double-distilled water, and 0.75 μM of forward and reverse primers each. The forward primers were fluorescently labelled with FAM. The amplified products were diluted and mixed with formamide containing GENESCAN-500 (ROX) Size Standard (Applied Biosystems), and the genotype was determined on an ABI Prism3100 Genetic Analyzer using GeneScanAnalysis® Software3.7. PCR amplification for loci Ca1b6 and Ga1a19redesigned was carried out by Ecogenics GmbH (Zurich, Switzerland).

Data analysis

During hybridogenesis, one genome is discarded before meiosis and only one of the parental genomes is passed on to the gametes. Therefore, usually no recombination occurs between the L- and R-genome of any individual. Consequently, the parental genomes are considered to be independent, and all analyses were done on the basis of haplotypes, i.e. separately for each of the two genomes.

The genomes can occur in two different states; single-genomic if the individual has only one copy of this genome (i.e. LR, LRR for the L-genome and LR, LLR for the R-genome) or double-genomic, if the individual has two copies of this parental genome (i.e. LLR for the L-genome, and LRR for the R-genome). In the double-genome state, a locus can have two different alleles. Null alleles would have been detected in the single-genomic state as missing alleles; but we did not find any null alleles in our sample.

For each population we calculated genetic diversity (H_L, H_R) over all analysed loci (Nei 1987) based on haplotypes with ARLEQUIN 2.0 (Schneider *et al.* 2000). H_L and H_R were then regressed against the proportion of LLR individuals and of L-genomes per population and against LRR individiuals and of R-genomes per population, respectively, using SAS 9.1.3 (SAS Institute Inc. 2002–2003). This analysis was based upon the recently confirmed assumption, that in double-genomic frogs recombination occurs among the homotypic chromosomes (Christiansen & Reyer 2009). As a result, diversity should be elevated in populations with a high proportion of double-genomic frogs. Additionally, we used general linear models (GLM) to test if genetic diversity is related to the geographic position (longitude, latitude) of the pond and/or the possible ways of recombination for each of the two genomes in the particular population type (no recombination, recombination via parental species, recombination via triploid hybrids).

For the Scania frogs, we analysed genetic variation among sexes, genotypes and populations with separate analyses of molecular variance (AMOVA) (Excoffier *et al.* 1992) as implemented in ARLEQUIN 2.0. Pairwise F_{ST} values (Wright 1978) between all pairs of populations were calculated and tested for isolation by distance by comparing the $F_{ST}/(1 - F_{ST})$ matrix with the matrix of the natural logarithm of the geographical distance (Rousset 1997) in a Mantel test (10 000 permutations) within ARLEQUIN 2.0. For the analysis comparing populations from Scania, Denmark and Central Europe we determined allele frequencies for each of the populations and calculated genetic diversity with ARLEQUIN 2.0 (Schneider *et al.* 2000).

Results

Overall genetic diversity

European scale. On the Northern and Central European scale, average genetic diversity in the L-genome was lower than in the R-genome for all population systems (mean H_L = 0.127, range 0.000–0.303; mean H_R = 0.324, range 0.000–0.620) (Appendix 2). In both genomes diversity decreased significantly from south to north and east to west (Table 2). Moreover, diversity was significantly higher in populations where the presence of the parental species (LL or RR) allowed recombination of the respective genome than in those where the genome was transmitted clonally via diploid hybrids (Table 2; Fig. 3). With respect to the L-genome, genetic diversity in populations where recombination can occur

Table 2 General linear model for genetic diversity in (a) the *Lessonae* (H_L and (b) the *Ridibundus* H_R) genome in relation to latitude, longitude and three categories of recombination. Recombination is possible in parental species (LL in a, RR in b) and in triploid hybrids (LLR in a, LRR in b); no recombination is possible for the L-genome in RR/LR populations and for the R-genome in LL/LR populations

(a) H_L	df	*F*	*P*
Recombination	2	4.903	0.021
Latitude	1	5.019	0.039
Longitude	1	11.404	0.004
(b) H_R	df	*F*	*P*
Recombination	2	12.563	0.001
Latitude	1	35.744	<0.001
Longitude	1	5.892	0.028

The analysis is based on data from the 22 populations listed in Appendix 2. Each population was assigned to one of the three recombination categories on the basis of the population composition. When recombination was possible via both triploid hybrids and parental individuals, the population was assigned to the latter.

© 2010 Blackwell Publishing Ltd

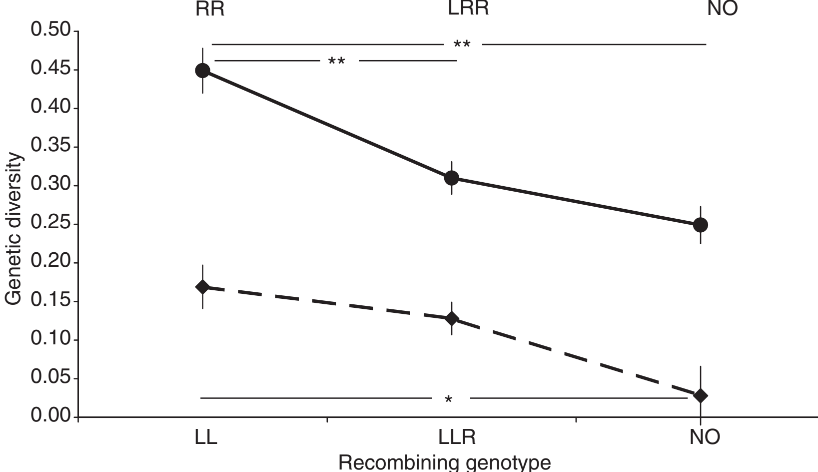


Fig. 3 Genetic diversity in the *lessonae*-genome (H_L, broken line) and the *ridibundus*-genome (H_R, solid line) in relation to whether the respective genomes are recombined in the parental species (LL, RR), in triploid hybrids (LLR, LRR) or not at all (NO). Significant differences in pairwise comparisons are shown by horizontal lines with * indicating $P < 0.05$ and ** indicating $P < 0.01$.

only via triploid LLR-hybrids was not different from diversity where recombination can occur via parental LL ($P = 0.597$, Scheffe test). For the R-genome, however, recombination via triploid LRR-hybrids alone resulted in significantly lower diversity than recombination via parental RR ($P = 0.008$, Scheffe test) (Fig. 3).

Scania scale. On the smaller geographical scale of our main study area in Scania, genetic diversity in the L-genome was also lower than in the R-genome (mean $H_L = 0.089$, range 0.000–0.151; mean $H_R = 0.241$, range 0.015–0.366) (Appendix 1). In order to see whether the lower genetic diversity in the L-genome might merely reflect the fact that we had only three polymorphic L- but five polymorphic R-primers, we repeated the comparison for Scania with a sub-sample of three R-primers (RlCa1b5, Ca1b6 and Ga1a19redesigned) that expressed about the same number of alleles as the L-genome. Based on these three primers, mean genetic diversity in the R-genome was similar to that in the L-genome ($H_{R_Sub} = 0.093$) (*t*-test, $P = 0.804$). Genetic diversity was not related to the proportion of triploid individuals (LLR, LRR) or haploid genomes (L, R) in the populations for either the L- or the R-genome ($N = 33$, all $r^2 \leq 0.074$, all $P \geq 0.124$).

We did find, however, that genetic diversity in the R-genome depended on the location of the pond within the distribution area: it decreased significantly from the centre to the periphery ($N = 33$, $r^2 = 0.512$, $P \leq 0.001$; Fig. 4). For the L-genome, the corresponding regression

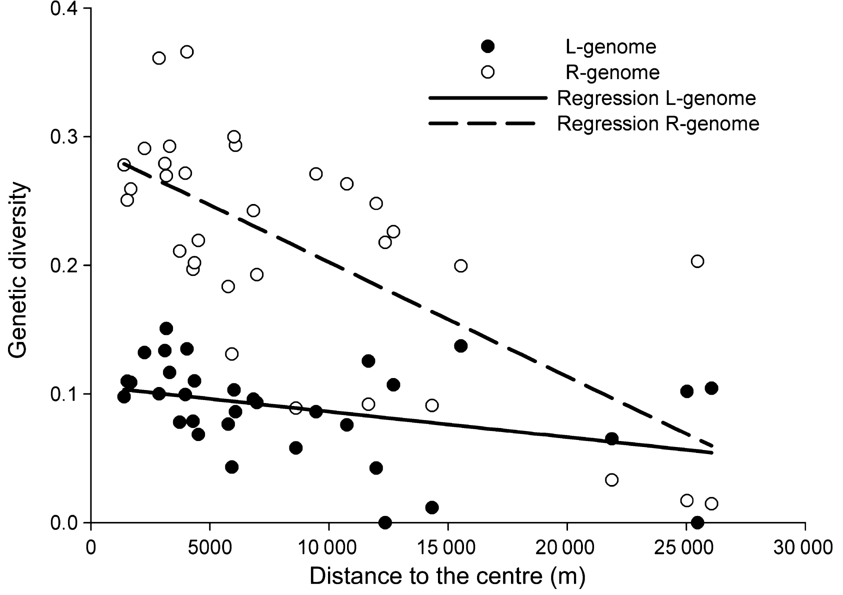


Fig. 4 Relationship between genetic diversity (Nei 1987) and geographic location of ponds within the distribution in southern Sweden. Pond location is expressed by its distance from the centre of the study area (see Fig. 2b).

© 2010 Blackwell Publishing Ltd

is not significant (r^2 = 0.076 P = 0.120), although ponds closer to the centre also tended to have a higher genetic diversity (Fig. 4).

Genetic population structure within Scania

Pooled over loci that amplified the L-genome, genetic differentiation between populations was highly significant (5.86%; P < 0.001, F_{ST} = 0.059; Table 3) and correlated positively with geographic distance between ponds (Mantel test, R^2 = 0.344, P < 0.001; Fig. 5a). Pairwise F_{ST} values ranged from 0 to 0.564. In the R-genome, 18.53% of the variation was assigned to between-population variation (P < 0.001, F_{ST} = 0.185) and pairwise F_{ST} values ranged from 0 to 0.842. When tested for isolation by distance, we also found a significant positive correlation between genetic differentiation of the R-genome and geographic distance (Mantel test, R^2 = 0.356, P < 0.001; Fig. 5b). Given that there were no real spatial clusters of ponds, and even ponds further away from the centre had other ponds nearby (e.g. 155/159 and 137/160; cf. Figs 2a and 4), the significant isolation by distance pattern in Fig. 5 cannot be attributed to a few remote ponds.

If genotypes (LR, LLR, LRR) are different in microsatellite genetics and the genotype composition follows a geographic pattern, it is possible that the above result of isolation by distance is influenced by the genotype composition in a pond. Consequently, we also tested three matrices, containing the differences in the genotype proportions of LR, LLR and LRR between ponds against the genetic differences in these ponds (Mantel test). For the genotype matrices of LLR and LR we found no correlation (all r^2 < 0.103 and P > 0.102), but in the case of LRR there was a marginally significant correlation for both genomes (L: P = 0.049, R: P = 0.040): the larger the difference in the proportion of LRR, the larger the genetic difference between the ponds.

Genetic differentiation between genotypes and sexes

To investigate whether allele transfer is unrestricted, we analysed whether any of the genetic variation can be explained by differences between sexes and/or genotypes (LR, LLR and LRR). Males and females did not differ genetically, although for the L-genome the difference was close to significance (Table 3); gene flow seems to be restricted between sexes (0.19%; P = 0.054). Genotype groups, however, differed significantly for both genomes. Subsequent pairwise comparisons show that, with respect to the L-genome, LRR individuals are mainly different from the LLR and LR individuals, whereas with respect to the R-genome the diploid LR individuals are different from the two triploids (LLR/LRR). In both the sex and the genotype comparison, however, the percentage of genetic variation explained by group differences was extremely low (<1.05%; Table 3). Hence, gene flow seems to be largely unrestricted.

Discussion

Our study yielded four major results: (i) For both genomes (L and R), the extent of genetic diversity varies with geographic location, both on a large northern European and a small southern Swedish scale. (ii) For each genome, genetic variability increases with the potential for recombination which, in turn, depends on population structure. (iii) In our main study area in

Table 3 Variance components from analysis of molecular variance (AMOVA) for the two genomes (L and R) between sexes (female/male), genotypes (LR/LLR/LRR) and among populations

Genome	Source of variation	df	Sum of squares	% Variation	F_{ST} value	P-value
L	Among sexes	1	1.245	0.19	0.002	0.054
R	Among sexes	1	0.861	−0.20	−0.002	0.877
L	Among genotypes	2	2.432	0.36	0.004	0.001
LRR–LR/LLR						<0.001
LLR–LR/LRR						0.674
LR–LRR/LLR						0.345
R	Among genotypes	2	19.361	1.05	0.011	<0.001
LRR–LR/LLR						0.319
LLR–LR/LRR						0.662
LR–LRR/LLR						<0.001
L	Among populations	32	36.703	5.86	0.059	<0.001
R	Among populations	32	310.283	18.53	0.185	<0.001

Negative variance components usually indicate an absence of genetic structure, although in some cases they can have a biological meaning (Excoffier, http://anthro.unige.ch/arlequin).

© 2010 Blackwell Publishing Ltd

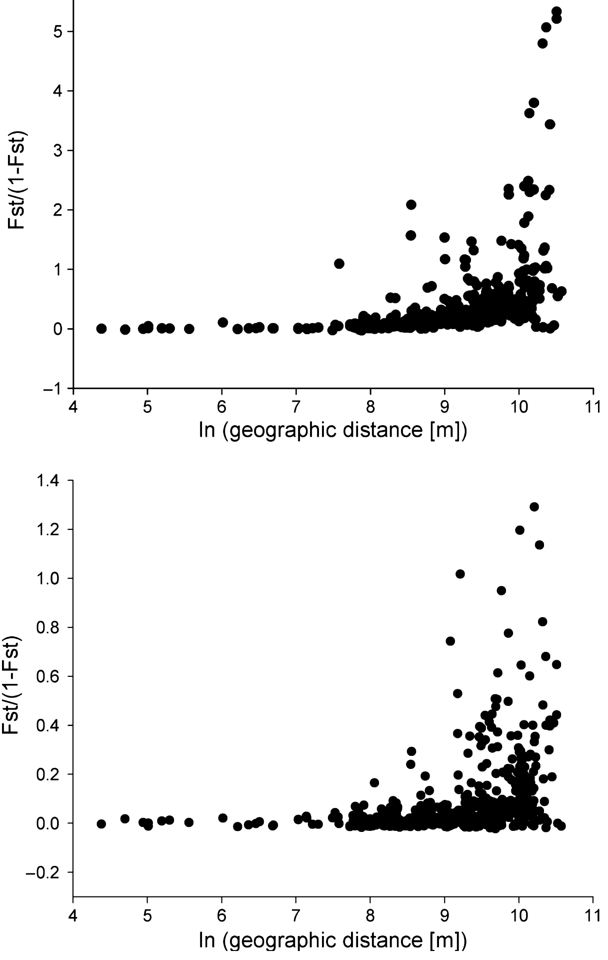


Fig. 5 Pairwise genetic distances, measured by $F_{ST}/(1 - F_{ST})$, plotted against geographic distance ln (m) for all localities in Scania reveal isolation by distance for both the L-genome (a) and the R-genome (b).

Scania (southern Sweden), genetic differentiation between populations is small but significant and best explained by 'isolation by distance'. (iv) Genetic variation in L- and R-genomes was not related to sex, but differed between genotype groups (LR, LLR, LRR), although only slightly. Below, we discuss each of these results.

Genetic diversity in relation to geography

The all-hybrid populations (EE-system) in our main study area in Scania showed low genetic diversity in both the L- and the R-genome. This is obvious from the fact that 76% of the primers that amplified were monomoprhic and most of the polymorphic ones had a low number of different alleles (Table 1). Slate & Pemberton (2002) caution that sample size and number of typed loci can have a strong effect on measuring genetic diversity and its association with fitness. Because we used the same markers for all populations, however, we can at least compare diversity among areas. On the European scale, diversity in *P. esculentus* decreased from southeast to northwest for both, the *lessonae*- and the *ridibundus*-genome. Similarly, Sjögren (1991) and Tegelström & Sjögren-Gulve (2004) found in an isolated metapopulation of *P. lessonae* in central Sweden that genetic diversity is extremely low compared with Central European populations. The decrease in genetic diversity with latitude and longitude is not surprising when we consider that the Swedish and the Danish island populations are located at the northwestern edge of the distribution range and are separated from the species' main distribution area by the Baltic Sea (Seppä & Laurila 1999). It has been shown in other anuran species that genetic variability decreases with increasing distance from the refugium during the Pleistocene (Merilä & Baker 1996; Beebee & Rowe 2000; Zeisset & Beebee 2001; Palo *et al.* 2004). Lower genetic diversity in peripheral compared to central populations is a regular, although not universal, phenomenon in plants and animals (reviewed by Eckert *et al.* 2008) and particularly common in organisms with low dispersal abilities, like anurans (Rowe *et al.* 1999; Garner *et al.* 2003, 2004).

The pattern from the larger European scale repeats itself at a smaller spatial scale in southern Sweden. There, we found diversity to be higher in the core area than at the periphery of the Scanian distribution, at least for the R-genome. It is known that water frogs have been present in this area at least since they were first described by Carl von Linné (1758), but nothing is known about their exact distribution then. Ebendal (1979) mentioned that the species in this area has extended its range eastwards during the 1970s, and current sampling in Scania attest to a wider distribution than has been described before (Jan Pröjts, personal communication). Our results support the hypothesis that the distribution of *P. esculentus* has been restricted to a core area in former times and has expanded its range in southern Sweden.

Low genetic diversity is usually assumed to decrease viability of genetically depleted populations, both because it reduces a population's ability to react to novel challenges and because it increases inbreeding effects and genetic drift, which in turn can increase the likelihood of extinction (Amos & Balmford 2001). In amphibians (and other species), however, direct tests for a positive correlation between genetic diversity and fitness measurements have yielded somewhat ambiguous results for both allozymes and neutral molecular markers. Some studies confirmed such a correlation (Hitchings & Beebee 1998; Rowe *et al.* 1999; Lesbarrères

© 2010 Blackwell Publishing Ltd

et al. 2005; Johansson *et al.* 2007), while others did not (Sjögren 1991; Rowe & Beebee 2001). This means that microsatellite diversity used in our and other studies may, in several cases, 'provide a valuable new approach in studying links between fitness and heterozygosity' (Rowe *et al.* 1999).

In the Swedish *P. esculentus* populations, negative effects of low genetic diversity within the L- and R-genomes may explain why the homotypic parental forms (LL or RR) resulting from hybrid × hybrid matings do not survive (Christiansen *et al.* 2005; Arioli 2007). The hybrids do, however, probably because the combination of two (LR) or three (LLR, LRR) parental genomes elevates somatic heterozygosity–even when each genome alone has low variability.

Genetic diversity in relation to recombination and population structure

It has long been suspected (e.g. Günther *et al.* 1979; Günther 1983; Eikhorst 1988; Vinogradov *et al.* 1990), and now conclusively been demonstrated (Christiansen & Reyer 2009), that in triploids the genome that is present only once is eliminated and the two remaining genomes recombine and segregate in a normal meiosis. This would predict that genetic diversity increases with the proportion of double-genomic individuals in a population and, hence, increasing likelihood of recombination. For the Scania population, our data do not seem to support this hypothesis: genetic diversity within each of the genomes was neither related to the proportion of the respective genome in the population (%L or %R) nor to the proportion of the heterozygote genotypes in these populations (%LLR or %LRR). If diversity was low at the beginning, however, detection of recombination becomes unlikely.

That genetic diversity is, indeed, correlated to recombination potential is illustrated by the comparison on the European scale (Fig. 3). Diversity in both the *lessonae*- and the *ridibundus*-genome was highest in populations where the genomes could be recombined via the parental species (LL, RR), lowest in those where transmission is clonal via diploid hybrids (LR) and intermediate when recombination can occur via triploid hybrids (LLR, LRR). L-genome diversity was independent of whether recombination occurred in LL and LLR, but R-genome diversity was higher when recombination was via parental RR than via hybrid LRR. There are two, not mutually exclusive, explanations for this difference. First, since the male determining Y factor is located on the L- genome in hybrids, R sperm usually produce only daughters (Christiansen 2009). As a result, the LLR sex ratio is balanced, whereas in LRR males are almost absent; thus, regular recombination can only occur via females (Christiansen *et al.* 2005; Jakob 2007) (cf. Fig. 1). Second, in all-hybrid populations, L gametes are only produced by recombining LLR, but R gametes can originate both from recombining LRR and from non-recombining LR frogs (cf. Fig. 1). This reduces the average recombination rate in the R-genome compared to that in the L-genome. In agreement with this is that in all-hybrid populations recombination rates in the L-genome are independent of the proportion of recombining triploids (i.e. independent of LLR/(LLR + LR)), whereas recombination in the R-genome increases with the proportion of recombining triploids (i.e. with LRR/(LRR+LR)) (Christiansen & Reyer 2009).

Spatial genetic differentiation in Scania

Most studies on genetic structuring within anuran populations have focused on large scale settings (Rowe *et al.* 1998). Only in the last decade has interest in fine scale studies increased in connection with dispersal barriers and possible inbreeding effects for amphibian populations. The findings of these studies are not always in agreement, but it has to be noted that they comprise a wide set of different species (Seppä & Laurila 1999; Rowe *et al.* 2000; Tallmon *et al.* 2000; Lampert *et al.* 2003). Most amphibians are thought to have limited mobility and to express high site fidelity, suggesting that they are bad dispersers (Beebee 2005). This argues for population structuring even over short distances. In our study populations of *P. esculentus*, we could show a significant population differentiation for both genomes on a small landscape scale. The best explanation for the structuring among these *P. esculentus* populations is the geographic distance between ponds. Such isolation by distance has also been demonstrated in other studies on a small scale (Lampert *et al.* 2003) and on a large scale (Palo *et al.* 2004), but distance effects were absent in a study by Seppä & Laurila (1999). The pattern of 'isolation by distance' needs time to evolve and arises through the balance of local genetic drift within populations and dispersal of individuals between populations. We know through Carl von Linné that *P. esculentus* has occurred in this region at least since 1758, so there was enough time for such a pattern to develop. Although the area of southern Sweden is quite populated and agriculture shapes the landscape, there are no apparent major barriers, such as roads or rivers that could influence dispersal of the frogs negatively over small distances.

Genetic differences between sexes and genotypes

In some Central European populations, *P. esculentus* is strongly sex-biased. At Neusiedlersee in Austria (Tunner 1974), for example hybrids are almost all female,

© 2010 Blackwell Publishing Ltd

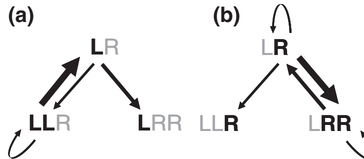


Fig. 6 Inheritance pathways of (a) an L allele and (b) an R allele in populations consisting of diploid (LR) and triploid (LLR, LRR) *P. esculentus* individuals. Width of the arrows indicates how often an allele travels within or between genotypes, based on the assumption of random mating and Fig. 1.

whereas in Latvia (Borkin *et al*. 1986) and near the Odra river in Germany (Uzzell *et al*. 1977) populations with only male hybrids were recorded. In the Swedish populations, we found almost no LRR males (Jakob 2007). This is probably because of the usual initial hybridization event between *P. lessonae* males and *P. ridibundus* females with the result that there are no male-determining factors on the R-genome. Haplotypes within genomes, however, do not seem to be sex-linked in the examined Swedish populations and there are apparently no barriers to gene flow between males and females. Therefore, both sexes were analysed together for the population structure.

Based on microsatellites, the three genotypes differed in their genetic composition in both genomes. At a first glance, this result is unexpected, because both genomes 'travel' between genotypes (Fig. 6). An L-genome is usually inherited from LR or LLR, but once it is in an LRR individual, it is not passed on. Conversely, the R-genome is passed on mainly between LR and LRR and reaches a dead end in a LLR frog. Our data show that, with respect to the L-genome, the LRR individuals are genetically slightly different from the LLR individuals. This might reflect unidirectional gene flow due to the fact that L-genomes get eliminated from the gene pool once they are in an LRR frog. With respect to the R-genome, LR individuals are genetically different from the other two genotype groups (LLR, LRR). This is surprising because LR individuals apparently form the link between the two triploid types. We have to consider though, that the proportion of genetic variation contributed by genotype composition is extremely low. Moreover the number of transmitting pathways strongly depends on the mating pattern or (if mating is random) on the exact proportion of each genotype in a particular pond which changes from year to year. Hence, it seems that gene flow between sexes and genotypes is basically unrestricted.

Acknowledgements

We would like to thank D. Christiansen, E. Embrechts, J. Loman, R. Muheim, J. Secondi, S. Gödderz, and M. Tobler for their help in the field in Sweden. The samples from Uppsala were kindly collected and made available by A. Laurila, those from the Baltic States and Poland by D. Christiansen, K. Fog and L. Iversen. We thank T. Garner, S. Röthlisberger and the people from Ecogenics for help in the laboratory. We also would like to thank B. Baldetorp, M. Johansson and E. Niederer for their invaluable assistance with flow cytometry analysis. Thanks also to the group of R. Zinkernagel at the University Hospital Zürich who kindly provided us access to their FACSCalibur and to K. Tschannen and K. Fink for their assistance. Many thanks also to L. Excoffier for help with the population genetic analysis. Comments by H. Hotz, P. Wandeler, Th. Uzzell and two anonymous reviewers greatly improved the manuscript. This work was supported by Swiss NF grant No. 31-64004.00 to H.-U. Reyer. Catching permits were provided by Länsstyrelsen i Skåne Län (552-18591-02, -522-9286-03,-552-6571-04).

References

Amos W, Balmford A (2001) When does conservation genetics matter? *Heredity*, **87**, 257–265.

Arioli M (2007) *Reproductive patterns and population genetics in pure hybridogenetic water frog populations of R. esculenta*. PhD Dissertation, University of Zurich.

Beebee TJC (2005) Conservation genetics of amphibians. *Heredity*, **95**, 423–427.

Beebee TJC, Rowe G (2000) Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from Pleistocene refugium. *Biological Journal of the Linnean Society*, **69**, 367–381.

Beebee TJC, Rowe G (2004) *An Introduction to Molecular Ecology*. Oxford University Press Inc., Oxford.

Borkin LJ, Caune IA, Pikulik MM, Sokolova TM (1986) Distribution and structure of the green frog complex in the USSR. In: *Studies of Herpetology* (ed. Rocek Z), pp. 675–678. Charles University, Prague.

Borkin LJ, Korshunov AV, Lada GA *et al*. (2004) Mass occurrence of polyploid green frogs (*Rana esculenta* complex) in eastern Ukraine. *Russian Journal of Herpetology*, **11**, 203–222.

Bullini L (1994) Origin and evolution of animal hybrid species. *Trends in Ecology and Evolution*, **9**, 422–426.

Christiansen D (2005) A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. *Molecular Ecology Notes*, **5**, 190–193.

Christiansen D (2009) Gamete types, sex determination and stable equilibria of all-hybrid populatins of diploid and triploid edible frogs (*Pelophylax esculentus*). *BMC Evolutionary Biology*, **9**, 135.

Christiansen D, Reyer H-U (2009) From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs. *Evolution*, **63**, 1754–1768.

Christiansen D, Fog K, Pedersen BV, Boomsma JJ (2005) Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. *Evolution*, **59**, 1348–1361.

Dawley RM (1989) An introduction to unisexual vertebrates. In: *Evolution and Ecology of Unisexual Vertebrates* (eds Dawley RM, Bogart JP), pp. 1–18. New York State Museum, Albany, New York.

© 2010 Blackwell Publishing Ltd

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593–619.

Dufresne F, Hebert PDN (1994) Hybridization and origins of polyploidy. *Proceedings of the Royal Society of London Series B*, **258**, 141–146.

Ebendal T (1979) Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). *Mitteilungen aus dem Zoologischen Museum in Berlin*, **55**, 143–152.

Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Molecular Ecology*, **17**, 1170–1188.

Eikhorst R (1984) *Untersuchungen zur Verwandtschaft der Grünfrösche: Verbreitung, Struktur und Stabilität von reinen Rana esculenta- Populationen*. PhD Thesis, University of Bremen, Bremen, Germany.

Eikhorst R (1987) Der Laich des Teichfrosches *Rana esculenta* Linnaeus, 1758 in einer reinen Bastardpopulation (Anura: Ranidae). *Salamandra*, **23**, 122–131.

Eikhorst R (1988) Die Verteilung von diploiden und triploiden Larven des Teichfrosches *Rana esculenta* in einer reinen Bastardpopulation (Anura, Ranidae). *Salamandra*, **24**, 59–68.

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Frankham R, Ballou JD, Briscoe DA (2004) *A Primer of Conservation Genetics*. Cambridge University Press, Cambridge.

Frost DR, Grant T, Faivovich J *et al.* (2006) The amphibian tree of life. *Bulletin of the American Museum of Natural History*, **297**, 8–370.

Garner TWJ, Gautschi B, Röthlisberger S, Reyer H-U (2000) A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology*, **9**, 2173–2175.

Garner TWJ, Angelone S, Pearman PB (2003) Genetic depletion in Swiss populations of *Rana latastei*: conservation implications. *Biological Conservation*, **114**, 371–376.

Garner TWJ, Pearman PB, Angelone S (2004) Genetic diversity across a vertebrate species' range: a test of the central-peripheral hypothesis. *Molecular Ecology*, **13**, 1047–1053.

Graf J-D, Polls Pelaz M (1989) Evolutionary genetics of the *Rana esculenta* complex. In:*Evolution and Ecology of Unisexual Vertebrate* (eds Dawley RM, Bogart JP). pp. 289–302, New York State Museum, Albany, New York.

Günther R (1975) Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche, ''*Rana esculenta*'',L., in der DDR (Anura, Ranidae). *Mitteilungen aus dem Zoologischen Museum in Berlin*, **51**, 145–158.

Günther R (1983) Zur Populationsgenetik der mitteleuropäischen Wasserfrösche des *Rana esculenta*-Synkleptons (Anura, Ranidae). *Zoologischer Anzeiger*, **211**, 43–54.

Günther R (1991) Europäische Wasserfrösche (Anura, Ranidae) und biologisches Artkonzept. *Mitteilungen aus dem Zoologischen Museum in Berlin*, **67**, 39–53.

Günther R, Uzzell T, Berger L (1979) Inheritance patterns in triploid *Rana ''esculenta''* (Amphibia, Salientia). *Mitteilungen aus dem Zoologischen Museum in Berlin*, **55**, 35–57.

Hellriegel B, Reyer H-U (2000) Factors influencing the composition of mixed populations of a hemiclonal hybrid and its sexual host. *Journal of Evolutionary Biology*, **13**, 906–918.

Hitchings SP, Beebee TJC (1998) Loss of genetic diversity and fitness in common toad (*Bufo bufo*) populations isolated by inimical habitat. *Journal of Evolutionary Biology*, **11**, 269–283.

Hotz H, Uzzell T, Guex GD *et al.* (2001) Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. *Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe*, **77**, 43–50.

Jakob C (2007) *Structure and Dynamics of Pure Hybridogenetic Water Frog Populations*. PhD Thesis, University of Zurich, Switzerland.

Johansson M, Primmer CR, Merilä J (2007) Does habitat fragmentation reduce fitness and adaptability? A case study of the common frog (*Rana temporaria*) *Molecular Ecology*, **16**, 2693–2700.

Lampert KP, Rand SA, Mueller UG, Ryan MJ (2003) Fine-scale genetic pattern and evidence for sex-biased dispersal in the tungara frog, *Physalaemus pustulosus*. *Molecular Ecology*, **12**, 3325–3334.

Lampert KP, Lamatsch DK, Epplen JT, Schartl M (2005) Evidence for a monophyletic origin of triploid clones of the amazon molly, *Poecilia formosa*. *Evolution*, **59**, 881–889.

Lande R, Shannon S (1996) The role of genetic variation in adaptation and population persistence in a changing environment. *Evolution*, **50**, 434–437.

Lantmäteriverket (1996) *Kartex 2.10*. Sverige.

Lesbarrères D, Primmer CR, Laurila A, Merilä J (2005) Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. *Molecular Ecology*, **14**, 311–323.

Meirmans PG, Vlot EC, Den Nijs JCM, Menken SBJ (2003) Spatial ecological and genetic structure of a mixed population of sexual diploid and apomictic triploid dandelions. *Journal of Evolutionary Biology*, **16**, 343–352.

Menken SBJ, Smit E, Den Nijs JCM (1995) Genetical population structure in plants: gene flow between diploid sexual and triploid asexual dandelions (*Taraxacum* section *ruderalia*). *Evolution*, **49**, 1108–1118.

Merilä J, Baker AJ (1996) Genetic population structure and gradual northward decline of genetic variability in the greenfinch (*Carduelis chloris*). *Evolution*, **50**, 2548–2557.

Muller HJ (1964) The relation of recombination to mutational advance. *Mutation Research*, **1**, 2–9.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Pala I, Coelho MM (2004) Contrasting views over a hybrid complex: between speciation and evolutionary ''dead-end''. *Gene*, **347**, 283–294.

Palo JU, Schmeller DS, Laurila A *et al.* (2004) High degree of population subdivision in a widespread amphibian. *Molecular Ecology*, **13**, 2631–2644.

Plötner J (2005) *Die westpaläarktischen Wasserfrösche*. Laurenti-Verlag, Bielefeld.

Richards AJ (1973) The origin of *Taraxacum* agamospecies. *Botanical Journal of Linnean Society*, **66**, 189–211.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.

© 2010 Blackwell Publishing Ltd

Rowe G, Beebee TJC (2001) Fitness and microsatellite diversity estimates were not correlated in two outbred anuran populations. *Heredity*, **87**, 558–565.

Rowe G, Beebee TJC, Burke T (1998) Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Molecular Ecology*, **7**, 751–760.

Rowe G, Beebee TJC, Burke T (1999) Microsatellite heterozygosity, fitness and demography in natterjack toads *Bufo calamita*. *Animal Conservation*, **2**, 85–92.

Rowe G, Beebee TJC, Burke T (2000) A microsatellite analysis of natterjack toad, *Bufo calamita*, metapopulations. *Oikos*, **88**, 641–651.

Rybacki M, Berger L (2001) Types of water frog populations (*Rana esculenta* complex) in Poland. *Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe*, **77**, 51–57.

SAS Institute Inc. (2002–2003) *Version 9.1.3 SP3 for Windows*. SAS Institute Inc., Cary, NC.

Schneider S, Roessli D, Excoffier L (2000) *Arlequin: a software for population genetics data analysis. Ver 2.000*. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva, Geneva.

Schultz RJ (1969) Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. *American Naturalist*, **103**, 605–619.

Seehausen O (2004) Hybridization and adaptive radiation. *Trends in Ecology and Evolution*, **19**, 198–207.

Seppä P, Laurila A (1999) Genetic structure of island populations of the anurans *Rana temporaria* and *Bufo bufo*. *Heredity*, **82**, 309–317.

Sharbel TF, Lowcock LA, Murphy RW (1997) Flow cytometry analysis of amphibian population composition. In: *Amphibians in Decline: Canadian Studies of a Global Problem, Herpetological Conservation* (ed. Green DM), pp 78–86. Society for the Study of Amphibians and Reptiles, Saint Louis, Missouri.

Sjögren P (1991) Genetic variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). *Evolutionary Ecology*, **5**, 248–271.

Slate J, Pemberton JM (2002) Comparing molecular measures for detecting inbreeding depression. *Journal of Evolutionary Biology*, **15**, 20–31.

Som C, Reyer H-U (2006) Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. *Evolutionary Ecology Research*, **8**, 1235–1248.

Tallmon DA, Funk WC, Dunlap WW, Allendorf FW (2000) Genetic differentiation among long-toed salamander (*Ambystoma macrodactylum*) populations. *Copeia*, **2000**, 27–35.

Tegelström H, Sjögren-Gulve P (2004) Genetic differentiation among northern European pool frog (*Rana lessonae*) populations. *Herpetological Journal*, **14**, 187–193.

Tunner HG (1974) Die klonale Struktur einer Wasserfroschpopulation. *Zeitschrift für zoologische Systematik und Evolutionsforschung*, **12**, 309–314.

Uzzell T, Günther R, Berger L (1977) *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). *Proceedings of the Academy of Natural Sciences of Philadelphia*, **128**, 147–171.

Vinogradov AE, Borkin LJ, Guenther R, Rosanov JM (1990) Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. *Genome*, **33**, 619–627.

Vorburger C (2001) Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. *Evolution*, **55**, 2319–2332.

Vrijenhoek RC (2006) Polyploid hybrids: multiple origins of a treefrog species. *Current Biology*, **16**, 245–247.

Wright S (1978) *Evolution and the Genetics of Populations. Volume 4. Variability Within and Among Natural Populations*. University of Chicago Press, Chicago.

Zeisset I, Beebee TJC (2001) Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. *Proceedings of the Royal Society Biological Sciences, Series B*, **268**, 933–938.

Zeisset I, Rowe G, Beebee TJC (2000) Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. *Molecular Ecology*, **9**, 1173–1174.

This work is part of the PhD theses of M. A. and C. J. on structure and dynamics of pure hybridogenetic frog populations. Their main interests are in population ecology and genetics as well as evolutionary ecology. H.-U. R. is a professor of zoology at the University of Zürich. His research focuses on evolutionary ecology, population structure and dynamics as well as behavioural ecology in vertebrates.

Appendix 1 Details on the 33 ponds with all-hybrid *P. esculentus* populations in Scania (southern Sweden)

Pond no.	Sample size	Coordinates (N,E)		Sampling year	Population composition	H_L	H_R
001	57	55° 35′ 17′	13° 21′ 15′	2003	LR/LLR/LRR	0.043	0.131
010	28	55° 34′ 12′	13° 19′ 37″	2002	LR/LLR/LRR	0.079	0.197
011	90	55° 34′ 06″	13° 19′ 47″	2003	LR/LLR/LRR	0.068	0.219
012	28	55° 34′ 09″	13° 19′ 38″	2002	LR/LLR/LRR	0.110	0.202
014	83	55° 34′ 08′	13° 19′ 01′	2003	LR/LLR/LRR	0.078	0.211
021	39	55° 34′ 09″	13° 16′ 42″	2002	LR/LLR/LRR	0.109	0.259
023	41	55° 34′ 23″	13° 16′ 55″	2002	LR/LLR/LRR	0.110	0.251
024	30	55° 34′ 27″	13° 16′ 49″	2002	LR/LLR/LRR	0.098	0.278

© 2010 Blackwell Publishing Ltd

Appendix 1 *(Continued)*

032	86	55° 34′ 03″	13° 12′ 53″	2003	LR/LLR/LRR	0.117	0.292
032A	56	55° 34′ 27′	13° 13′ 03′	2003	LR/LLR/LRR	0.100	0.361
050	38	55° 29′ 33′	13° 08′ 02′	2004	LR/LLR/LRR	0.107	0.226
089	130	55° 36′ 34′	13° 23′ 19′	2003	LR/LLR/LRR	0.058	0.089
101	42	55° 32′ 51″	13° 17′ 04″	2002	LR/LLR/LRR	0.099	0.272
102	61	55° 32′ 51′	13° 17′ 13′	2003	LR/LLR/LRR	0.135	0.366
108	76	55° 33′ 09″	13° 16′ 08″	2003	LR/LLR/LRR	0.151	0.270
108A	37	55° 33′ 11″	13° 16′ 09″	2002	LR/LLR/LRR	0.133	0.279
111	68	55° 32′ 06″	13° 12′ 33″	2003	LR/LLR/LRR	0.086	0.293
112	36	55° 32′ 05″	13° 12′ 44″	2002	LR/LLR/LRR	0.103	0.300
123	19	55° 35′ 17″	13° 21′ 07″	2002	LR/LLR	0.077	0.184
126	127	55° 33′ 59″	13° 14′ 12″	2003	LR/LLR/LRR	0.132	0.291
134	89	55° 33′ 03″	13° 21′ 22″	2003	LR/LLR/LRR	0.096	0.242
135	34	55° 33′ 12″	13° 21′ 39″	2002	LR/LLR/LRR	0.093	0.193
137	24	55° 39′ 14″	13° 24′ 32″	2002	LR/LLR	0.000	0.218
138	66	55° 31′ 32′	12° 55′ 45′	2003	LR/LLR/LRR	0.065	0.033
139	36	55° 34′ 06″	13° 05′ 35″	2003	LR/LLR/LRR	0.076	0.263
142	31	55° 35′ 08″	13° 06′ 42″	2004	LR/LLR/LRR	0.086	0.271
147	31	55° 31′ 12″	13° 06′ 18″	2004	LR/LLR/LRR	0.042	0.248
151	30	55° 27′ 03″	13° 10′ 17″	2004	LR/LLR/LRR	0.137	0.199
154	29	55° 22′ 24′	13° 05′ 32′	2004	LR/LLR/LRR	0.000	0.203
155	30	55° 22′ 08″	13° 26′ 14″	2004	LR/LLR/LRR	0.104	0.015
159	30	55° 22′ 59′	13° 27′ 01′	2004	LR/LLR/LRR	0.102	0.017
160	30	55° 40′ 01″	13° 25′ 48″	2004	LR/LLR/LRR	0.012	0.091
161	30	55° 36′ 40″	13° 26′ 18″	2004	LR/LLR/LRR	0.126	0.092

Pond numbers and locations according to the map in Fig. 2b. Ponds 032A and 108A, which were included in the sampling only after all ponds had been numbered, are separate from but close to ponds 032 and 108, respectively. H = genetic diversity per population for the L- and the R-genome.

Appendix 2 Details on the 22 study populations used for the European comparison. The 4 Scania ponds were chosen from the 33 in Appendix 2 to represent eastern, western, northern and southern locations within the core area (cf. Fig. 2b)

Pond no.	Pond locality	Country	Sample size	Coordinates (N, E)		Population composition	H_L	H_R
1	Uppsala	Sweden	29	60° 32′ 56′	17° 53′ 58′	LL	0.142	–
2	Östergötland	Sweden	41	58° 06′ 57′	16° 24′ 15′	LR/LL	0.090	0.000
3	Scania 032	Sweden	86	55° 34′ 03′	13° 12′ 53′	LR/LLR/LRR	0.117	0.292
4	Scania 112	Sweden	36	55° 32′ 05′	13° 12′ 44′	LR/LLR/LRR	0.103	0.300
5	Scania 123	Sweden	19	55° 35′ 17′	13° 21′ 07′	LR/LLR	0.077	0.184
6	Scania 134	Sweden	89	55° 33′ 03′	13° 21′ 22′	LR/LLR/LRR	0.096	0.242
7	Bornholm3/4	Denmark	22	55° 08′ 39′	15° 03′ 42′	LR/LLR/RR	0.062	0.440
8	Bornholm 014	Denmark	44	55° 07′ 23′	15° 09′ 10′	LR/LLR/LRR	0.107	0.343
9	N-Seeland 001	Denmark	24	55° 46′ 14′	12° 23′ 23′	LR/LLR	0.000	0.164
10	S-Seeland 001	Denmark	27	55° 12′ 08′	11° 39′ 55′	LR/LLR/LRR	0.092	0.176
11	Fehmarn 011	Germany	26	54° 32′ 13′	11° 03′ 17′	LR/LLR	0.011	0.256
12	Klützler Winkel, Wismar	Germany	30	53° 59′ 29′	11° 00′ 47′	LR/LLR/LRR	0.113	0.280
13	Rügen 011	Germany	25	54° 25′ 02′	13° 23′ 49′	LR/LLR	0.096	0.315
14	Rothemühl 04	Germany	29	53° 34′ 26′	13° 46′ 04′	LR/LLR/LRR/LL	0.265	0.342
15	Karsibor	Poland	20	53° 51′ 07′	14° 18′ 58′	LR/RR	0.000	0.620
16	Wiselka	Poland	23	53° 57′ 39′	14° 33′ 48′	LR/LRR/RR	0.000	0.456
17	Wysoka Kamineska	Poland	30	53° 49′ 12′	14° 50′ 27′	LR/LLR/LRR	0.256	0.441
18	Rogaczewo Wielkie	Poland	44	52° 03′ 22′	16° 49′ 07′	LR/LRR/LL/RR	0.198	0.599
19	Dasunikeskes	Lithuania	29	54° 42′ 49′	24° 05′ 54′	LR/RR	0.230	0.561

© 2010 Blackwell Publishing Ltd

Appendix 2 *(Continued)*

20	Baltoji Voke	Lithuania	33	54° 28′ 44′	25° 07′ 59′	LR/LL	0.232	0.373
21	Stikli	Latvia	33	57° 19′ 41′	22° 15′ 22′	LR/LL	0.303	0.089
22	Laeva	Estonia	27	58° 25′ 42′	26° 19′ 08′	LL	0.209	–

Pond numbers and localities according to the map in Fig. 2a. Where several ponds have been sampled in the same locality (e.g. Scania), the ones chosen for this analysis have additional number designations. H = genetic diversity per population for the L- and the R-genome.

Appendix 3 Details on PCR conditions for all loci used in the microsatellite analysis. Cycle temperatures are for denaturation (D), annealing (A) and extension (E), respectively

Locus	Initial denaturation	Cycles	Cycle temperatures D	A	E	Final extension
RlCa1b5	94 °C, 3 min	29	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min
RlCa18	94 °C, 3 min	29	94 °C, 30 s	58 °C, 30 s	72 °C, 30 s	72 °C, 5 min
RlCa5	94 °C, 3 min	29	94 °C, 30 s	58 °C, 30 s	72 °C, 30 s	72 °C, 5 min
Re1CAGA10	94 °C, 10 min	35	94 °C, 30 s	58 °C, 30 s	72 °C, 30 s	72 °C, 10 min
Re2CAGA3	94 °C, 15 min	Touchdown: 2 cycles each	94 °C, 30 s	60 °C, 58 °C, 56 °C, 54 °C, 52 °C, 50 °C, 25 cycles at 48 °C	72 °C, 30 s	72 °C, 10 min
Ga1a19redesigned (2002/03)	95 °C, 15 min	35	95 °C, 30 s	56 °C, 30 s	72 °C, 30 s	72 °C, 8 min
Ga1a19redesigned (2004)	95 °C, 15 min	35	95 °C, 30 s	53 °C, 90 s	72 °C, 60 s	60 °C, 30 min
Ca1b6 (2002/03)	95 °C, 15 min	35	95 °C, 30 s	56 °C, 30 s	72 °C, 30 s	72 °C, 8 min
Ca1b6 (2004)	95 °C, 15 min	35	95 °C, 30 s	53 °C, 90 s	72 °C, 60 s	60 °C, 30 min

© 2010 Blackwell Publishing Ltd