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Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs

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Abstract

Interspecies transfer of mitochondrial (mt) DNA is a common phenomenon in plants, invertebrates and vertebrates, normally linked with hybridization of closely related species in zones of sympatry or parapatry. In central Europe, in an area north of 48°N latitude and between 8° and 22°E longitude, western Palaearctic water frogs show massive unidirectional introgression of mtDNA: 33.7% of 407 *Rana ridibunda* possessed mtDNA specific for *Rana lessonae*. By contrast, no *R. lessonae* with *R. ridibunda* mtDNA was observed. That *R. ridibunda* with introgressed mitochondrial genomes were found exclusively within the range of the hybrid *Rana esculenta* and that most hybrids had *lessonae* mtDNA (90.4% of 335 individuals investigated) is evidence that *R. esculenta* serves as a vehicle for transfer of *lessonae* mtDNA into *R. ridibunda*. Such introgression has occurred several times independently. The abundance and wide distribution of individuals with introgressed mitochondrial genomes show that *R. lessonae* mt genomes work successfully in a *R. ridibunda* chromosomal background despite their high sequence divergence from *R. ridibunda* mtDNAs (14.2–15.2% in the ND2/ND3 genes). Greater effectiveness of enzymes encoded by *R. lessonae* mtDNA may be advantageous to individuals of *R. ridibunda* and probably *R. esculenta* in the northern parts of their ranges.

Introduction

Interspecies transfer of mitochondrial (mt) DNA has been detected in a variety of organisms, for example in flowering plants (e.g. Bergthorsson *et al.*, 2003), invertebrates (e.g. Croucher *et al.*, 2004; Abe *et al.*, 2005; Bachtrog *et al.*, 2006), and among vertebrates in fish (e.g. Yamada *et al.*, 2001; Sullivan *et al.*, 2004), amphibians (e.g. Babik *et al.*, 2005), birds (e.g. Dabrowski *et al.*, 2005) and mammals (e.g. Ruedi *et al.*, 1997; Fredsted *et al.*, 2006). In most cases, introgressed mtDNA was found in very closely related species (especially in sister species) and was directly linked to interspecies hybridization in zones of sympatry or parapatry. Such genome transfer is always mediated by fertile or at least partially fertile hybrids that transmit their maternal mtDNA to the paternal gene pool via backcrosses with males of the paternal parental species. In some populations, introduced foreign mtDNA can completely replace the resident mtDNA either through genetic drift or natural selection (Ballard & Whitlock, 2004). The detection and analysis of introgressive hybridization is not only important for taxonomic purposes and phylogenetic inference, it may also provide fundamental insights into evolutionary processes, especially for studying the role of nuclear/mitochondrial co-adaptation in speciation, including hybrid breakdown and reproductive isolation (e.g. Ballard & Whitlock, 2004; Burton *et al.*, 2006).

The western Palaearctic water frog group offers significant opportunities to study these problems because it

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comprises several evolutionary species that are not reproductively isolated. There are large areas of sympatry in which fertile hybrids are formed (reviewed by Plötner, 2005). At least three of the known hybrid forms reproduce hemiclonally by a hybridogenetic (Schultz, 1969) gametogenesis: in their germ line one parental genome is excluded, whereas the remaining one is endoreduplicated prior to meiosis (reviewed by Graf & Polls Pelaz, 1989; Plötner, 2005).

The best studied hybrid form is *Rana esculenta* (genomic composition LR), which originated from natural crosses between *Rana lessonae* (LL) and *Rana ridibunda* (RR). In the germ line of this hybrid, one parental genome (usually L, sometimes R) is excluded and haploid gametes (ova or sperm) are produced that contain an intact, unrecombined genome of the other parental species (reviewed by Graf & Polls Pelaz, 1989; Plötner, 2005). Such hybrid lineages in most cases coexist with and reproductively depend on one parental species. Matings with that species replace the genome lost during gametogenesis and restore somatic hybridity in each hybrid generation. In the widespread and abundant *lessonae–esculenta* (L–E) system, in which both male and female hybrids occur, *R. esculenta* usually coexist, as sexual parasites, with *R. lessonae* and exclude the L genome prior to meiosis. In populations of the less frequent *ridibunda–esculenta* (R–E) system, in which hybrids are only males, they live together with *R. ridibunda* and usually exclude the R genome (Uzzell & Berger, 1975; Uzzell *et al.*, 1977; Günther & Plötner, 1988). In addition to diploid LR hybrids, some water frog populations, especially in north-eastern Germany and north-western Poland, contain triploid (3N) hybrids (Günther, 1975, 1983; Rybacki & Berger, 2001; Plötner, 2005) that normally have either two *R. lessonae* genomes and one *R. ridibunda* genome (genotype LLR) or one *R. lessonae* genome and two *R. ridibunda* genomes (genotype RRL). They usually exclude the rarer genome and form unrecombined haploid gametes (Günther *et al.*, 1979; Günther, 1983; Berger & Günther, 1988; Vinogradov *et al.*, 1991).

For size-related behavioural reasons, natural primary hybridizations between *R. ridibunda* and *R. lessonae* occur between the large females of *R. ridibunda* and the small males of *R. lessonae* rather than the reciprocal (Berger, 1970; Borkin *et al.*, 1979). As in most vertebrates, frog mtDNA is inherited strictly from the mother, so all newly formed *R. esculenta* lineages contain *R. ridibunda* mtDNA. In the L–E population system, most matings take place between *R. esculenta* females and *R. lessonae* males, but successful matings occur also in the reciprocal combination (Blankenhorn, 1977; Lengagne *et al.*, 2006). Each such mating between an *R. lessonae* female and an *R. esculenta* male generates *R. esculenta* lineages with *R. lessonae* mtDNA. In the absence of the parental species *R. ridibunda*, such a transfer of *R. lessonae* mtDNA into a hybridogenetic lineage is irreversible, and indeed, most hemiclonal hybrids of the L–E system contain mtDNA of their local host parental species rather than *R. ridibunda* mtDNA, a result of such occasional matings of host females with hybrid males (Spolsky & Uzzell, 1986). With repeated formation of *R. esculenta* lineages containing *R. lessonae* mtDNA and chance losses of lineages containing *R. ridibunda* mtDNA, all hybrid individuals in L–E system populations come to have *R. lessonae* mtDNA. When such hemiclonally reproducing hybrid lineages occur within the range of *R. ridibunda*, however, they provide a vehicle for interspecific introgression of *R. lessonae* mtDNA into *R. ridibunda*. Matings between female *R. esculenta* with *R. lessonae* mtDNA and male *R. ridibunda* (or *R. esculenta*) lead to *R. ridibunda* progeny of both sexes (or females only) with *R. lessonae* mtDNA (Spolsky & Uzzell, 1984; Hotz *et al.*, 1992).

Three geographical ranges are relevant to the introgression of *R. lessonae* mtDNA into *R. ridibunda*: (1) the area of sympatry of *R. ridibunda* and *R. lessonae*, because it defines the region where primary hybridizations between them can occur. Such sympatry presently exists from northern Ukraine and western Russia to eastern Austria and central Germany (Günther, 1990, 1997a, b). (2) The area in which *R. ridibunda* genomes in hybrids between *R. lessonae* and *R. ridibunda* lead to germ line exclusion of the L genome and thus found hemiclonally reproducing hybrid lineages rather than nonhybridogenetic sterile F1 hybrids. Here, *R. esculenta* lineages can constitute vehicles for the introgression of *R. lessonae* mtDNA into *R. ridibunda*. This area currently comprises most parts of the sympatry region (central and eastern Europe), but excludes an eastern zone from northern Ukraine to the Danube delta in eastern Romania. In this zone and in more southern parts of the *R. ridibunda* range, the *R. ridibunda* genome does not induce hemiclonal gametogenesis in hybrids with *R. lessonae* (cf. Hotz *et al.*, 1985; Bucci *et al.*, 1990; Günther *et al.*, 1991; Berger *et al.*, 1994; H. Hotz, unpublished data). (3) Areas in which the sex ratio in *R. esculenta* hinders regular matings between female *R. lessonae* and male *R. esculenta*; such matings are necessary to transfer *R. lessonae* mtDNA into *R. esculenta* lineages and via these lineages into *R. ridibunda*. Whereas *R. esculenta* lineages in most parts of central Europe contain both sexes, usually with a slight excess of females, they consist almost exclusively (~97%) of females in many populations of the Pannonian Basin (eastern Austria, northern Bosnia and Herzegovina, north-eastern Croatia, Hungary, northern Serbia, southern lowlands of Slovakia, north-eastern Slovenia, western Romania and Ukraine west of the Carpathian Mountains; Berger *et al.*, 1988; Gubányi, 1992; Morozov-Leonov *et al.*, 2003; H. Hotz, unpublished data) and in the Rostov Province of Russia (Borkin *et al.*, 2006).

We examined mtDNA of water frogs from 194 localities throughout and beyond the sympatry area of *R. ridibunda* and *R. lessonae*, determining species origin and haplotype of mtDNA. We asked how often and where the introgression of *R. lessonae* mtDNA into *R. ridibunda* occurred,

and addressed the geographic relation of the region of *R. lessonae* mtDNA introgressed into *R. ridibunda* with: (1) the sympatry area of *R. ridibunda* and *R. lessonae*; (2) the area in which hybrids reproduce hemiclonally; and (3) the area in which hybrids are almost exclusively females.

Material and methods

Sampling, taxon designation and ploidy determination

Localities of 407 *R. ridibunda*, 117 *R. lessonae*, 335 *R. esculenta* and two individuals of uncertain identification (*R. lessonae* or *R. esculenta*) are listed in Appendix S1 (Supplementary material online). We use the name *R. ridibunda* for individuals distributed in Europe, and include frogs currently known as *Rana kurtmuelleri*. Unpublished molecular data (J. Plötner, S.N. Litvinchuk & T. Ohst) suggest that individuals from the type locality of *R. ridibunda* (north coast of the Caspian Sea) may not be conspecific with individuals from Europe, in which case the oldest available name for the European frogs is *Rana fortis* Boulenger, 1884 (Dubois & Ohler, 1994). Except for two individuals from France (localities 31 and 33), for which we used DNA sequences for haplotype comparisons and sequence statistics, *R. ridibunda* from France and Switzerland were not considered because all lake frog populations in these countries trace back to individuals introduced not only from other European regions, but also from Anatolia (Grossenbacher, 1988; Pagano *et al.*, 2003; J. Plötner & T. Ohst, unpublished results).

Specimens were assigned to taxa on the basis of their external morphology; Günther (1990) and Plötner (2005) described diagnostic characters. Ploidy was determined by measurements of erythrocytes based on the finding that triploid (3N) erythrocytes are on average 30% larger than diploid (2N) ones (Uzzell & Berger, 1975; Günther, 1977). The kind of mtDNA present in all individuals in our sample was determined by one or more methods: sequencing of ND2, ND3 and parts of 12S rRNA gene, endonuclease restriction of whole mtDNA, *Alu*I restriction of a PCR-amplified segment of the ND2 gene. Our sequence data are supplemented by published restriction fragment analyses (Spolsky & Uzzell, 1984, 1986).

DNA extraction, PCR conditions, sequencing

Genomic DNA was isolated from blood, muscle and liver tissue using QIAmp blood and tissue kits (Qiagen GmbH, Hilden, Germany) according to the company's protocols. Before DNA isolation, ethanol-preserved material was soaked in three changes of distilled water over a 48-h period. Double-stranded amplification and cycle sequencing of mtDNA (1038 bp of ND2, 340 bp of ND3, 374 bp of 12S rDNA) were carried out as described in Appendix S2 (Supplementary material online). The sequenced sites correspond to positions 6546–7583 (ND2), 12089–12428 (ND3) and 3156–3528 (12S) in the genome of *Rana nigromaculata* (AB043889, Sumida *et al.*, 2001). Amplified DNA was purified using QIAquick PCR purification kits (Qiagen). A Big Dye Ready Reaction Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used to perform the sequencing reaction. Electrophoresis and detection of fluorescent dye-labelled nucleotide fragments was carried out with an automated DNA sequencer (ABI Prism 377, Applied Biosystems). All sequences were deposited in the EMBL Nucleotide Sequence Database under the accession numbers listed in Appendix S1 (Supplementary material online).

Restriction fragment analysis

Isolation and purification of mtDNA followed standard methods (Spolsky & Uzzell, 1984, 1986). Approximately 5–10 ng of each DNA was digested to completion with 19 different restriction enzymes (*Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hae*II, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Sst*I, *Xba*I and *Xho*I) under conditions recommended by the supplier (Boehringer Mannheim Biochemicals, Mannheim, Germany, Bethesda Research Laboratories, Rockville, Maryland, USA or New England Biolabs, Ipswich, MA, USA). Resulting DNA fragments were end-labelled with a mixture of four α-^{32}P-triphosphate deoxynucleosides (adenine, cytosine, guanine and tyrosine). Restriction enzyme fragment patterns were determined from autoradiographs after electrophoresis through horizontal 1% agarose or vertical 3.5–4% polyacrylamide gels. For each gel, fragment sizes were estimated from mobilities of size standards (λ and PM2 DNAs, each cut with *Hin*dIII for agarose gels; φX174 cut with *Hin*cII and pBR322 cut with *Alu*I for polyacrylamide gels).

*Alu*I restriction of ND2 PCR products

Mitochondrial ND2 sequences of 287 western Palaearctic water frogs representing at least 10 evolutionary species were analysed with the online tool WEB CUTTER (http://www.medkem.gu.se/cutter) to find specific restriction sites that allow differentiation between *R. lessonae* and *R. ridibunda* mtDNAs. One *Alu*I restriction site (AG↓CT) spanning positions 633–636 was detected in each of the 44 *R. ridibunda* ND2 sequences, in seven ND2 sequences of *Rana bedriagae* from the Near East (western Syria, Jordan, Nile delta) and in nine of *Rana cretensis,* but not in the ND2 sequences of 56 *R. lessonae* or any of other western Palaearctic water frogs (J. Plötner, unpublished results). To distinguish between *R. ridibunda*- and *R. lessonae*-specific mitochondrial genomes only a 770-bp-long mtDNA segment that contained this restriction site was amplified using the primers L2 and H2 (Appendix S2,

Supplementary material online). Two microlitres (20 units) of *Alu*I (Boehringer Mannheim [BM] or Angewandte Gentechnologie Systeme GmbH [AGS], Heidelberg, Germany), 2 μl of supplied incubation buffer (BM incubation buffer A or AGS buffer violet) and 1 μl of double distilled water were added to 15 μl of the solution that contained the PCR product. After incubation for 2 h at 37 °C, the fragments were separated by electrophoresis on a horizontal 2% agarose gel. *Rana ridibunda*-specific mtDNA was cut into two fragments with lengths of 319 and 451 bp, whereas mtDNA of *R. lessonae* remained undigested as evidenced by a single band in the gel.

Data analysis

Mitochondrial DNA sequences were initially aligned using CLUSTAL V (Higgins *et al.*, 1992). Subsequently, alignments were improved manually. They are available from the EMBL align database under the accession number ALIGN_001211. For sequence statistics the programs MEGA 3.1 (Kumar *et al.*, 2004) and DNASP 4.10.9 (Rozas *et al.*, 2003) were used. We calculated the total number of transitions, transversions and amino acid substitutions, the nucleotide diversity (Pi) and uncorrected *p* distances (a simple measure of sequence divergence). We applied Tajima's *D*-test (Tajima, 1989) to investigate departures from selective neutrality at the ND2 and ND3 genes in both *R. ridibunda* and *R. lessonae*, and a McDonald–Kreitman (MK) test (McDonald & Kreitman, 1991) to test for neutrality in interspecies sequence divergence. The neutrality index (NI) was calculated using Rand & Kann's (1996) formula. The restriction profiles were analysed by calculating the amount of sequence divergence from the fraction of restriction fragments shared by a pair of mtDNAs (Nei & Li, 1979), using Upholt's (1977) formula.

Mitochondrial haplotype genealogies were estimated from complete ND2 and ND3 sequences with maximum likelihood (PAUP 4.0b10: Swofford, 2003) and Bayesian algorithms (MRBAYES 3.1.2: Ronquist & Huelsenbeck, 2003; Huelsenbeck & Ronquist, 2005). For both maximum-likelihood and Bayesian inferences, the general time reversible model with corrections for invariant characters and gamma-distributed rate heterogeneity (GTR + I + G) was applied as recommended by Huelsenbeck & Rannala (2004). Model parameters were estimated from the data set. For MRBAYES, the posterior probabilities of phylogenetic trees were calculated by employing a 10^7-generation Metropolis-coupled Markov chain Monte Carlo (four chains, chain temperature parameter = 0.2) as implemented in the program. Trees were sampled every 1000 generations; the first 1000 recorded trees were discarded; a total of 9000 recorded trees were examined. The autocorrelation behaviour of MRBAYES was explored with program internal statistics and the tool AWTY (http://ceb.csit.fsu.edu/awty; Nylander *et al.*, 2007). Both analyses suggested convergence of the split frequencies (standard deviation after 3500 recorded trees was lower than 0.01). The convergence of the model parameter estimates was checked using the potential scale reduction factor calculated in MRBAYES: the limit of the statistic of 1.0 was reached for all model parameters.

For the phylogenetic analysis, we augmented the *R. ridibunda* and *R. lessonae* sequences with sequences of *Rana epeirotica* and *R. cretensis* as an outgroup, and *Rana shqiperica*, the presumptive sister species of *R. lessonae sensu lato*. This allowed a consistent rooting of the haplotypes for *R. ridibunda* and *R. lessonae* on the tree.

To analyse the effects of location (longitude and latitude) on haplotype composition in *R. esculenta* and on the introgression rate of *R. lessonae*-specific mtDNA into *R. ridibunda*, we calculated the relative frequency (f_l) of *R. lessonae*-specific haplotypes (including haplotypes of the Italian *R. lessonae* stock) for each population with sample size larger than one. We performed simple and multiple regression analyses with f_l as the dependent variable and latitude and longitude as independent variables using the software packet Statgraphics Plus 4.1 (Statistical Graphics Corp., StatPoint Inc., Herndon, Virginia, USA). To judge the relative magnitude of the residuals with respect to the explanatory power of the selected independent variables, a component-plus-residuals plot (Wood, 1973) was made. In the regression analyses, we also incorporated data published by Spolsky & Uzzell (1984, 1986) and Monnerot *et al.* (1985) (Appendix S1, Supplementary material online).

Results

Inter- and intraspecific variation of mtDNA

Both mitochondrial sequences (ND2, ND3, 12S rDNA) and digest profiles of whole mtDNAs revealed clear differences between *R. ridibunda* and *R. lessonae*. Interspecific comparisons of the 12S rRNA gene segments yielded substitution numbers between 9 and 12, which correspond to sequence divergences of 2.4% and 3.2% respectively (Plötner & Ohst, 2001). The interspecific differences in the less conserved ND2 + ND3 sequences are about five to six times higher than in the 12S rRNA gene segment. Nevertheless, because there are at least nine substitutions, the species specificity of mtDNA could be clearly determined on the basis of this gene segment.

The *R. ridibunda* ND2 + ND3 sequences contained 38 (2.8%) variable sites (31 in ND2 and seven in ND3) defining 19 haplotypes (Table 1, Fig. 1); 31 (2.3%) sites were parsimony informative. In both genes, almost all nucleotide substitutions were transitions, most of them at the third codon position; only three were found in the first position (two in ND2 and one in ND3) and two in the second position (both in ND2). Only five substitutions resulted in amino acid replacements (four in ND2

Table 1 Variable positions and nucleotide composition in the mt ND2 and ND3 gene defining 19 different haplotypes (HT) across 29 populations of *Rana ridibunda*.

	Site (position)																																					
	ND2																														ND3							
	093	141	220	221	273	321	342	345	405	435	474	489	498	518	531	561	579	588	591	624	652	747	762	804	819	867	894	967	969	978	1008	075	168	234	246	276	277	303	
Sequence														*																	*								
No.	3	3	**1**	**2**	3	3	3	3	3	3	3	3	3	**2**	3	3	3	3	3	3	1	3	3	3	3	3	3	**1**	3	3	3	3	3	3	3	3	**1**	3	HT
1–3	T	C	A	G	C	T	G	C	C	T	T	A	A	T	T	G	A	T	A	T	C	A	A	A	C	A	C	A	T	T	A	C	G	G	T	C	G	C	**R1**
4–9	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R2**
10	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	**R3**
11	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R4**
12	.	.	.	.	.	.	.	.	.	C	.	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R5**
13–15	.	.	.	A	.	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R6**
16–17	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	**R7**
18	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	**R8**
19	.	.	G	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	**R9**
20	C	.	.	.	.	C	.	T	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	T	A	A	.	.	.	T	**R10**
21	C	.	.	.	.	.	.	.	.	.	.	.	.	A	C	A	.	.	.	.	T	.	G	.	T	.	.	.	.	C	.	T	.	A	.	.	.	T	**R11**
22	C	.	G	.	.	.	.	.	.	.	.	.	.	A	C	A	.	.	.	.	T	.	G	.	T	.	.	.	.	C	.	T	A	A	.	.	.	T	**R12**
23–29	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	G	.	T	.	.	.	.	C	.	T	A	.	.	.	.	.	**R13**
30–31	C	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	C	.	.	T	.	G	.	T	.	.	.	.	C	.	T	A	.	.	.	.	.	**R14**
32	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	G	.	T	.	T	.	.	.	.	.	A	.	.	.	.	.	**R15**
33	C	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	G	.	T	.	T	.	.	.	.	.	A	.	.	.	.	.	**R16**
34–42	C	.	.	.	T	.	A	.	.	.	C	.	.	A	.	.	G	.	G	C	T	G	G	.	T	.	T	G	C	.	.	.	A	.	C	T	.	.	**R17**
43	C	.	.	.	T	.	A	.	.	.	C	.	.	A	.	.	G	.	G	C	T	G	G	G	T	.	T	G	C	.	.	.	A	.	C	T	.	.	**R18**
44	C	.	.	.	T	.	A	.	.	.	C	.	.	A	.	.	G	.	G	C	T	G	G	.	T	.	T	G	C	.	T	.	A	.	C	T	.	.	**R19**

Transversions are indicated by asterisks. The numbers below nucleotide positions indicate the codon position. Bold face numbers indicate amino acid replacement substitutions.

and one in ND3). Nucleotide diversity (Pi) amounted to 0.007 ± 0.0005. Genetic distance ranged from 0.07% to 1.6% among *R. ridibunda*-specific haplotypes with an overall nucleotide diversity of 0.74%.

In the ND2 + ND3 sequences of *R. lessonae*, the intraspecific variability is about 2.5 times lower than in *R. ridibunda*. Intraspecific *p* distances ranged between 0.1% and 0.7% with an average of 0.3%. Only 24 variable sites (1.7%) were found (21 in ND2 and three in ND3); almost all substitutions were transitions at the first (10) or third (11) codon position (Table 2). Seven sites (0.5%) were parsimony informative. Twelve amino acid replacements were observed caused by 11 substitutions in the first codon position and one substitution in the second codon position. A Pi-value of 0.00187 ± 0.00033 was calculated.

A total of 17 *R. lessonae*-specific haplotypes could be distinguished (Table 2), which represent three main clusters as supported by the results of maximum-likelihood (data not shown) and Bayesian analyses (Fig. 1). Cluster 1 comprises the very closely related haplotypes L1–L12; cluster 2 consists of only one haplotype (L13), which perhaps represents an ancestral state within the *R. lessonae* clade; and cluster 3 includes haplotypes L14–L17, which differ from the other *R. lessonae*-specific haplotypes by at least four substitutions (three in ND2 and one in ND3).

The digest profiles of *R. ridibunda* and *R. lessonae* mtDNAs were described in detail by Spolsky & Uzzell (1984, 1986). The proportion of fragments shared by both species ranged between 0.256 and 0.258, which corresponds to sequence differences of 8.1 ± 1.0% and 8.2 ± 1.0% respectively. In the ND2 gene, *R. ridibunda* differed from *R. lessonae* in at least 134 sites (12.9%); in the ND3 gene a minimum of 48 substitutions (14.1%) were detected. The sequence divergence (*p* distance) obtained as a result of interspecific comparisons of the complete ND2 + ND3 sequences ranged between 14.2% and 15.2%.

Compared with the 36 mitochondrial haplotypes differentiated on the basis of ND2 + ND3 sequences (Table 1 and 2), only three types of mtDNAs (A–C) were detected by restriction fragment patterns of total mtDNA (Spolsky & Uzzell, 1984). Type A was found in *R. ridibunda* and *R. esculenta* but not in *R. lessonae* and thus was considered as *R. ridibunda*-specific. Type A probably contains several of the 19 ND2 + ND3 haplotypes; because there are no differences in the recognition sites in the ND2 and ND3 genes of *R. ridibunda* for the restriction enzymes used by Spolsky and Uzzell, the

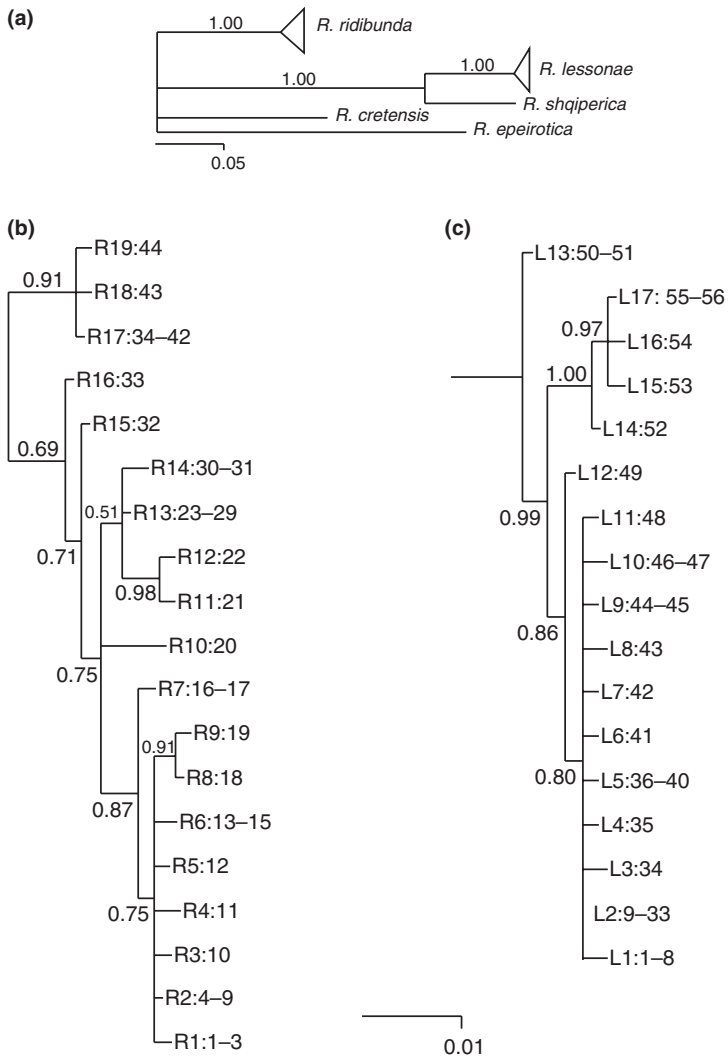

Fig. 1 Phylogenetic relationships of *Rana lessonae* and *Rana ridibunda* haplotypes. (a) Complete MrBayes maximum posterior tree of all haplotypes with *R. shqiperica* included as the sister taxon of *R. lessonae* and two presumptive outgroups of *R. ridibunda* (*Rana cretensis* and *Rana epeirotica*). (b) *R. ridibunda* clade: labels mark the *ridibunda* haplotype (R1–R19) and corresponding sequence numbers (Table 1); (c) *R. lessonae* clade: labels mark the *lessonae* haplotype (L1–L17) and sequence number (Table 2). The values at the branches are posterior probabilities for the clade to the right of that branch. The units of the scale bar in A, B and C are the expected mutations per site; B and C are on the same scale. Sequence numbers are specified in Supplementary material online.

ND2 + ND3 sequence diversity would not have been revealed in their study.

Types B and C, which were considered as *R. lessonae* specific, showed a sequence divergence of only 0.3%, consistent with the *p* distances obtained from intraspecific comparisons of the ND2 and ND3 sequences (0.1–0.7%). One *Bam*HI restriction site (G↓GATCC) was found in the ND3 gene (positions 1165–1170) of haplotypes L1–L12 and L13, but not in haplotypes L14–L17. An assignment of single haplotypes to a *R. lessonae*-specific fragment pattern type, however, is not possible. *Bam*HI cuts mtDNA of types B and C into three fragments with sizes of 8000, 6400 and 5600 bp (type B) and 11 500, 8000 and 470 bp (type C) respectively. Because a *Bam*HI restriction site exists in all the *R. lessonae* ND3 sequences except for haplotypes L14–L17, these cleavage patterns suggest that one of the restriction sites responsible for the 8000 bp fragment in types B and C patterns is in the ND3 gene. If so, haplotypes L14–L17 represent neither type B nor C, but an additional, less frequent, *R. lessonae*-specific haplotype that is probably identical to type LC described by Monnerot *et al.* (1985) on the basis of the digest profiles of *Bam*HI, *Eco*RI and *Pst*I.

Spolsky & Uzzell (1986) also described pattern type D, which is characteristic of Italian water frogs, but was also found in *R. lessonae* and *R. esculenta* from Switzerland (Hotz *et al.*, 1992) and southern Germany (T. Ohst & J. Plötner, unpublished results). The sequence divergence, calculated from shared restriction fragments between the mtDNAs of *R. lessonae* (B and C) and type D (3.4%), is consistent with distance values obtained on the basis of the ND2 + ND3 sequences (3.8–5.0%, mean: 4.4 ± 0.28%).

Tests of neutrality

Tajima's *D*-test, which compares the number of segregating sites per site with the nucleotide diversity,

Table 2 Variable positions and nucleotide composition in the mt ND2 and ND3 gene defining 17 different haplotypes (HT) across 39 populations of *R. lessonae*.

	Site (position)																								
	ND2																					ND3			
	0	1	2	2	3	3	4	4	4	4	6	6	7	7	8	8	8	8	9	9	1 0	0	1	2	
	7	1	0	0	6	6	1	6	7	8	9	9	3	6	0	3	3	9	6	9	1	8	2	5	
	8	5	1	8	6	9	5	3	2	9	1	2	8	2	7	2	5	2	4	6	1	5	9	5	
Sequence			*	*																					
No.	3	**1**	3	**1**	3	3	**1**	**1**	**1**	3	**1**	**2**	3	3	3	**1**	**1**	**1**	**1**	3	3	**1**	3	3	HT
1–8	G	G	C	C	C	A	A	A	C	G	G	C	T	G	A	G	G	G	C	A	T	A	A	T	**L1**
9–33	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**L2**
34	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	C	**L3**
35	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**L4**
36–40	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**L5**
41	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	**L6**
42	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**L7**
43	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	**L8**
44–45	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**L9**
46–47	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	**L10**
48	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	**L11**
49	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	**L12**
50–51	A	.	.	G	.	G	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	C	**L13**
52	.	.	.	.	T	G	G	.	.	.	A	.	.	.	G	A	.	.	.	.	.	.	G	C	**L14**
53	.	.	.	.	T	G	G	.	.	.	A	.	C	.	G	A	.	.	T	.	.	.	G	C	**L15**
54	.	.	.	.	T	G	G	.	.	.	A	.	C	A	G	A	.	.	.	.	.	.	G	C	**L16**
55–56	.	.	.	.	T	G	G	.	.	.	A	.	C	.	G	A	.	.	.	.	.	.	G	C	**L17**

Transversions are indicated by asterisks. The numbers below nucleotide positions indicate the codon position. Bold face numbers indicate amino acid replacement substitutions.

revealed no departure from a neutral model of sequence evolution within either *R. ridibunda* ($D = -1.62$; $0.10 > P > 0.05$) or *R. lessonae* ($D = 0.42$; $P > 0.10$). For ND2 and ND3 together, we observed 43 synonymous substitutions and 17 nonsynonymous substitutions within the two species, and 26 amino acid replacements and 156 silent fixed differences between them. The ratios of nonsynonymous to synonymous substitutions within and between species are significantly different from each other (Fisher's exact test, two tailed: $P = 0.02$, G-test: $G = 5.64$; $P = 0.02$). An NI of 2.37 indicates an excess of amino acid polymorphism, which is consistent with the hypothesis that most segregating functional variants are deleterious and thus subject to purifying selection. If ND2 and ND3 are considered separately, the NI values are 1.96 and 5.75 respectively. There is, however, no significant departure from neutrality (ND2: Fisher's exact test, two tailed: $P = 0.10$, $G = 3.03$, $P = 0.08$; ND3: Fisher's exact test, two tailed: $P = 0.13$, $G = 2.48$; $P = 0.12$).

Geographical distribution of haplotypes

In *R. ridibunda*, 16 specific haplotypes were found at single localities; only three (R2, R13 and R17) were found at two or more localities (Appendix S1, Supplementary material online). Haplotypes R1–R11 occur in *R. ridibunda* from the Balkan peninsular (Greece and Albania). Haplotype R13 was found in southern, south-eastern and central Europe. Haplotype R17 is the most common and characteristic haplotype of central European *R. ridibunda*.

Among the *R. lessonae*-specific haplotypes, three (L1, L2 and L17) were found at different localities (Appendix S1, Supplementary material online). L2, the most common haplotype of *R. lessonae*, is distributed from south-eastern Europe (Danube delta), over central Europe to Scandinavia. The closely related haplotypes L1 and L3–L12 occur in central and northern Europe. The less frequent haplotypes L13–L17 were found in northern Italy (L13), in south-eastern Europe (L14) and in central and eastern Europe (L15–L17).

Interspecific genome transfer and haplotype composition in *R. esculenta*

Of 407 *R. ridibunda,* 137 (33.7%) possessed mtDNA specific for *R. lessonae*. The introgressed genomes represent at least six different haplotypes (L1, L2, L8, L10, L12 and L15), three of them (L8, L12 and L15) observed only in *R. ridibunda*, two (L1 and L10) in both *R. ridibunda* and

R. esculenta and one (L2) in all three water frog forms. In contrast to *R. ridibunda*, introgression of *R. ridibunda*-specific mtDNA has not been detected in *R. lessonae*.

Of 335 *R. esculenta*, the majority (90.4%) possessed mtDNA characteristic of *R. lessonae* or the Italian taxon (*R. lessonae sensu lato*); haplotypes specific for the Italian *R. lessonae* stock represented 6.6% of these. Only 9.6% of the *R. esculenta* contained *R. ridibunda*-specific mtDNA, represented by haplotypes R13 and (according to ND3 data only) probably R17. All triploid individuals had only *R. lessonae*-specific mtDNA (Appendix S1, Supplementary material online).

Rana ridibunda individuals with *R. lessonae*-specific mtDNA are restricted to central Europe, whereas all individuals examined from southern and eastern Europe (south of 48°N latitude and east of 22°E longitude) possessed exclusively *R. ridibunda*-specific mtDNA (Fig. 2). This pattern is expressed in a statistically significant relationship between the frequency of individuals with *R. lessonae*-specific mtDNA (f_l) and the geographical coordinates at the 99% confidence level in both *R. ridibunda* and *R. esculenta* (Table 3 and Appendix S3, Supplementary material online). The fitted multiple linear regression models explain 46.3% of the variability in f_l for *R. ridibunda* and 44.5% for *R. esculenta*. There is a moderately strong negative correlation of f_l with longitude under a linear regression model in *R. ridibunda* ($r = -0.55$, ANOVA: F-ratio = 32.16, d.f. = 75, $P = 0$) and in *R. esculenta* ($r = -0.57$, ANOVA: F-ratio = 33.53, d.f. = 71, $P = 0$); and a significant positive correlation between f_l and latitude in both forms (*R. ridibunda*: $r = 0.56$, F-ratio = 33.72, d.f. = 75, $P = 0$; *R. esculenta*: $r = 0.41$, F-ratio = 13.81, d.f. = 71, $P = 0.0004$).

Discussion

Patterns of genome transfer

In western Palaearctic water frogs, introgression of *R. lessonae* mtDNA into *R. ridibunda* is closely related to the occurrence of the hybridogenetic hybrid *R. esculenta*. *Rana ridibunda* individuals with introgressed *R. lessonae* mtDNA are restricted to central Europe north of 48°N latitude and between 8° and 22°E longitude. In this area, hybridogenetic hybrids mainly live syntopically with a parental species in either *lessonae–esculenta* (L–E) or *ridibunda–esculenta* (R–E) populations (reviewed by Plötner, 2005). In eastern Europe (especially Russia and

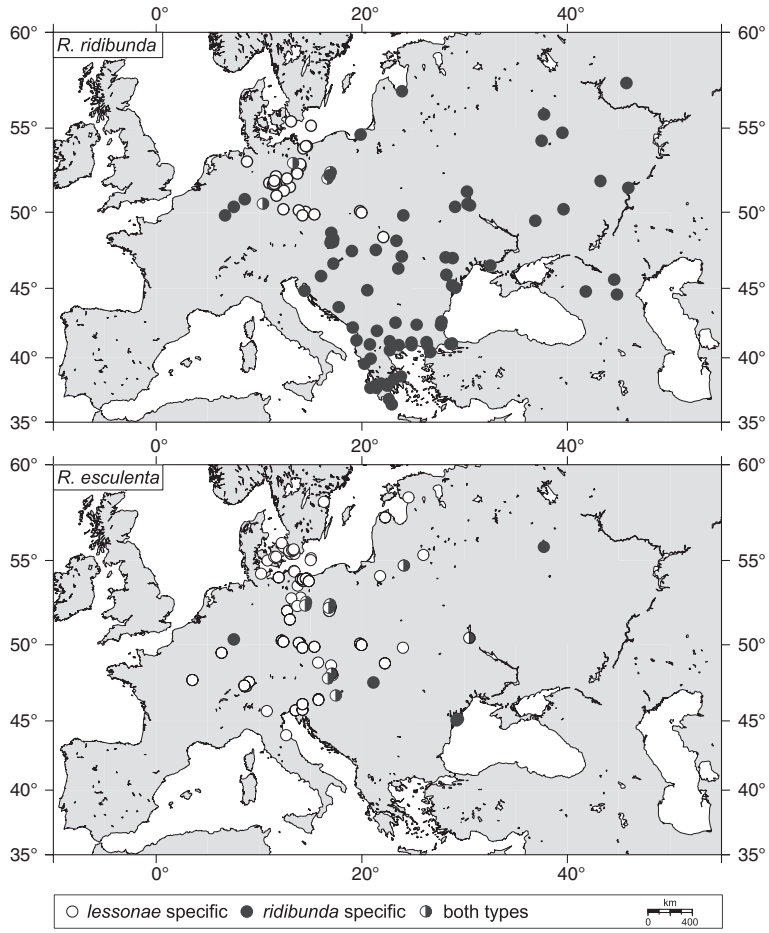

Fig. 2 Geographical distribution of haplotypes found in *Rana ridibunda* and *Rana esculenta*.

Table 3 Results of the multiple regression analysis with the relative frequency of *lessonae*-specific haplotypes (f_l) as the dependent variable and longitude and latitude as independent variables.

	Rana ridibunda		*Rana esculenta*	
Parameter	Model	Residual	Model	Residual
Sum of squares	7.01	8.11	1.78	2.08
Degrees of freedom	2	73	2	69
Mean square	3.50	0.11	0.89	0.03
F ratio	31.5		29.5	
P-value	0		0	
r^2	46.33		46.1	
Standard error of estimates	0.33		0.17	
Mean absolute error	0.26		0.12	
DW statistic	1.94		1.86	

The Durbin–Watson (DW) statistic tests (Durbin & Watson, 1950, 1951, 1971) the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. DW values > 1.4 indicate that there is not any serious autocorrelation in the residuals.

Ukraine), *R. ridibunda* also co-occurs with hybridogenetic *R. esculenta*, but, in this region, introgression of mitochondrial genomes has not been observed. In contrast to central European populations, where hybrids are both diploid (LR) and triploid (LLR and RRL), almost all *R. esculenta* in eastern Europe are diploid (Borkin *et al.*, 1986; Rybacki & Berger, 2001; Borkin *et al.*, 2004, 2006 described exceptions). The observed geographical patterns of mtDNA introgression, in concert with the uniform presence of *R. lessonae* mtDNA in triploid individuals and the geographical distribution patterns of triploids (Rybacki & Berger, 2001) suggest that the *R. lessonae* mitochondrial genome was, or is, transferred into *R. ridibunda* not only by LR females but also, at least in some populations, via triploid females. The occurrence in *R. ridibunda* of different *R. lessonae*-specific haplotypes that belong to distinct well-defined clades shows that the genome transfer traces back to several independent transmission events, although it is possible that each of these *R. lessonae* haplotypes seen in *R. ridibunda* was introgressed many times independently. Because some of the more divergent haplotypes have not been observed in *R. lessonae* itself, it is possible that they result from pre-Holocene introgression events.

Four of 38 *R. ridibunda* (10.5%) examined from the Pannonian Basin possessed *R. lessonae*-specific mtDNA, which shows that genome introgression takes place despite the scarcity of hybrid males (Berger *et al.*, 1988; Gubányi, 1992; Morozov-Leonov *et al.*, 2003). The unexpectedly high proportion of LR individuals with *R. lessonae*-specific mtDNA (11 of 23 *R. esculenta*; 48%) seems, however, to contradict the comparatively low introgression rate observed in Pannonian *R. ridibunda*. Crosses between diploid hybrid males (LR) and *R. lessonae* females are a precondition for the formation of LR females with *R. lessonae*-specific mtDNA that probably act as vehicles for the introgression of *R. lessonae* mtDNA into *R. ridibunda*. In contrast to our expectation, such hybrid females have been found, for example, in Hungary (locality 88: one of four individuals) and Slovakia (locality 154 and 156: four of five individuals; Appendix S1, Supplementary material online). Obviously, crosses between *R. ridibunda* males and LR females with *R. lessonae* mtDNA are rare, possibly because of specific mate recognition mechanisms, or do not result in fertile RR progeny.

Presumptive selective forces and mitochondrial–nuclear coadaptation

Whether sequence variation in mtDNA is selectively neutral or under selective constraints is still controversial. In our data set, the overall genetic variability in the ND2 + ND3 genes as measured by nucleotide substitutions is higher in *R. ridibunda*, but amino acid substitutions, caused mainly by mutations in the first codon position, are about two times higher in *R. lessonae*. As indicated by the results of the neutrality tests, this difference may reflect purifying selection rather than adaptation, supporting the hypothesis that most segregating functional variants are deleterious and rarely contribute to divergence (e.g. Meiklejohn *et al.*, 2007). Our data do not corroborate the assumption of Bazin *et al.* (2006) that population size does not influence genetic diversity in mtDNA. The higher genetic variability in *R. ridibunda* mtDNA is instead consistent with the hypothesis that mtDNA diversity and population size are positively related in animals (e.g. Mulligan *et al.*, 2006). *Rana ridibunda* has a distribution about 0.5 times larger than that of *R. lessonae* (e.g. Plötner, 2005) and based on empirical field data and measured heterozygosity of nuclear loci (S. N. Litvinchuk, unpublished data), probably has a larger effective population size. This problem needs further investigation, however, particularly because it is possible that the measured genetic variability is influenced by sample biases linked with our concept of species delineation.

Individuals of both *R. esculenta* and *R. ridibunda* with *R. lessonae* mtDNA may have an adaptive advantage rather than disadvantage, especially in their northern ranges, despite possible disruption of nuclear/mitochondrial protein coadaptation in *R. ridibunda* with introgressed *R. lessonae* mtDNA. In particular, the O_2/CO_2 metabolism of introgressed compared with nonintrogressed individuals, both as larvae and after metamorphosis, may be significant. Under hypoxic conditions, larvae of *R. ridibunda* develop less well than larvae of *R. lessonae* (Plenet *et al.*, 2000a, b). If *R. lessonae* mitochondria are more effective than *R. ridibunda* mitochondria under hypoxic conditions, and if this difference depends on enzymes that

are encoded in part by the mitochondrial genome, *R. ridibunda* larvae with *R. lessonae*-specific mtDNA may be less sensitive to oxygen deficiency caused by hypertrophic conditions or pollution than 'normal' *R. ridibunda* larvae. For metamorphosed *R. ridibunda*, the type of mitochondrial DNA may also play a role in their survival during hibernation. Because *R. ridibunda* hibernates exclusively beneath the water surface (Berger, 1982), it is exposed to an oxygen deficiency especially if the waters are covered with an ice sheet for a long period of time. Such a condition can lead to a high mortality or even extermination of whole populations (Berger, 1982). Under reduced oxygen pressure, the survival time of *R. lessonae* metamorphs was higher than those of *R. ridibunda* metamorphs (Tunner & Nopp, 1979; Lutschinger, 1988), which also demonstrates that *R. ridibunda* is more sensitive to oxygen deficiency than *R. lessonae*.

The large divergence between both the nuclear and the mt genomes of *R. lessonae* and *R. ridibunda* (the modified Nei distances calculated on the basis of 31 protein coding loci varied between 0.39 and 0.60; Beerli *et al.*, 1996) suggests that intergenomic coadaptation would be strongly disrupted when *R. lessonae* mtDNAs are introgressed into *R. ridibunda*. Because more than 90% of mitochondrial proteins are encoded in the nucleus, intergenomic protein coadaptation is assumed to be directly correlated with fitness (e.g. Burton *et al.*, 2006; Rand *et al.*, 2006; Gusdon *et al.*, 2007). The extent of transfer of *R. lessonae* mtDNA into *R. ridibunda* (33.7%) suggests that the presence of *R. lessonae* mtDNA does not significantly influence mitochondrial–nuclear interaction and hence, the fitness of this species, despite large divergence between the mitochondrial genomes of *R. lessonae* and *R. ridibunda*. It is possible, of course, that disruption of nuclear/mitochondrial protein coadaptation in water frogs with introgressed mtDNA is variable in its significance. The nature of this nuclear-mtDNA epistasis is not clear, however.

Although the possibility that introgressed *R. lessonae* mtDNA genomes provide an adaptive advantage to northern *R. ridibunda* (and perhaps also to northern *R. esculenta*) is a tempting hypothesis to be tested, there is, in clonal mole salamanders of the genus *Ambystoma*, a striking case of 'introgressed' mitochondrial genomes in which the phylogenetic divergence between the nuclear genomes of the normal host species and the phylogenetically closest possible source of the mitochondrial sequences is enormous. Here, an almost invariant mitochondrial genome (Spolsky *et al.*, 1992; C. Spolsky & T. Uzzell, unpublished data) exists independently for perhaps 5×10^5 years (Hedges *et al.*, 1992; Spolsky *et al.*, 1992) within the widespread triploid taxa *Ambystoma platineum* and *A. tremblayi* (Uzzell, 1964) of the subgenus *Ambystoma*, although the mitochondrial sequence itself apparently is derived from *Ambystoma barbouri* (Robertson *et al.*, 2006) of the distantly related subgenus *Linguaelapsus*. If strong coadaptation between proteins encoded by the nuclear and mitochondrial genomes of a taxon or population is a general rule and the disruption of this coadaptation by hybridization a frequent source of post-zygotic incompatibility between taxa (Burton *et al.*, 2006), the mitochondrial genomes of clonal A*mbystoma* must possess remarkable properties. What these might be for clonal *Ambystoma* is completely unknown, but at least for the frogs a plausible, although not yet tested, hypothesis can be proposed.

Biogeographic implications

Provided that the mt ND2 and ND3 genes of western Palaearctic water frogs diverge at about 2.0–2.5% per Myr (estimated from the sequence divergence in these two genes between *R. cretensis* and Anatolian water frogs, which are thought to have been separated for about 5 Myr, Beerli *et al.*, 1996), the divergence between different haplotypes within both *R. ridibunda* (maximum distance value 1.6%) and *R. lessonae* (maximum distance value 0.7%) indicates that much of the early intraspecific differentiation took place prior to the most recent glacial cycle. During the Würm glaciation, presumptive refuges are the southern Balkans (especially the Peloponnisos and adjacent areas) for *R. ridibunda* and the western (and probably northern) Black Sea region (for both *R. ridibunda* and *R. lessonae*). Haplotypes R1–R9 (the Greek *ridibunda* lineage *sensu stricto*) remained more or less confined to their potential refuge and nearby regions, resulting in a much smaller current range than other haplotypes (for example R13 and R17), which probably underwent rapid northward expansion with the advent of Holocene warming. Similar scenarios have been suggested for the European pond turtle, *Emys orbicularis* (Lenk *et al.*, 1999), the newt species *Triturus vulgaris* (Babik *et al.*, 2005), the fire-bellied toad *Bombina bombina* (Hofmann *et al.*, 2007) and the grasshopper *Chorthippus parallelus* (Hewitt, 2001).

Because of climatic oscillations, the post-Würm colonization of central Europe probably involved a sequence of range expansions followed by contractions and attendant reductions in population sizes (bottlenecks) that may have reduced genetic diversity in the northern populations. Such reductions might be lower in cold-tolerant taxa (Taberlet *et al.*, 1998; Hewitt, 2004). This hypothesis is supported by the immunological (Uzzell, 1979, 1983), mitochondrial and ecological data obtained in central and northern European water frog populations. Based on a comparison of the distribution ranges (*R. lessonae* goes farther north than *R. ridibunda*), the altitude distribution of water frog species (*R. lessonae* populates habitats up to 1,000 m above sea level while *R. ridibunda* is restricted to the lowlands; Günther, 1990, 1997a, b), and the results of preliminary ecological experiments that showed

a higher hatching success of *R. lessonae* larvae at temperatures below 13 °C (Günther, 1974), it seems probable that *R. ridibunda* is a rather cold sensitive species that recolonized the deglaciated northern parts of Europe later than *R. lessonae*. The amount of genetic variability observed in the mtDNA of *R. lessonae* (15 haplotypes) and *R. ridibunda* (only two haplotypes) from central and northern Europe corresponds well with this assumption.

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References

Abe, T.A., Spence, J.R. & Sperling, F.A.H. 2005. Mitochondrial introgression is restricted relative to nuclear markers in a water strider (Hemiptera: Gerridae) hybrid zone. *Can. J. Zool.* **83**: 432–444.

Babik, W., Branicki, W., Crnobrnja-Isailović, J., Cogălniceanu, D., Sas, I., Olgun, K., Poyarkov, H.A., Garcia-París, M. & Arntzen, J.W. 2005. Phylogeography of two European newt species – discordance between mtDNA and morphology. *Mol. Ecol.* **14**: 2475–2491.

Bachtrog, D., Thornton, K., Clark, A. & Andolfatto, P. 2006. Extensive introgression of mitochondrial DNA relative to nuclear genes in the *Drosophila yakuba* species group. *Evolution* **60**: 292–302.

Ballard, J.W.O. & Whitlock, M.C. 2004. The incomplete natural history of mitochondria. *Mol. Ecol.* **13**: 729–744.

Bazin, E., Glémin, S. & Galtier, N. 2006. Population size does not influence mitochondrial genetic diversity in animals. *Science* **312**: 570–572.

Beerli, P., Hotz, H. & Uzzell, T. 1996. Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. *Evolution* **50**: 1676–1687.

Berger, L. 1970. Some characteristics of the crosses within *Rana esculenta* complex in postlarval development. *Ann. Zool.* **27**: 373–416.

Berger, L. 1982. Hibernation of the European water frogs. *Zool. Pol.* **29**: 187–202.

Berger, L. & Günther, R. 1988. Genetic composition and reproduction of water frog populations (*Rana* kl. *esculenta* Synklepton) near nature reserve Serrahn, GDR. *Archiv Naturschutz u. Landschaftsf., Berlin* **28**: 265–280.

Berger, L., Uzzell, T. & Hotz, H. 1988. Sex determination and sex ratios in western Palearctic water frogs: XX and XY female hybrids in the Pannonian Basin? *Proc. Acad. Nat. Sci. Philadelphia* **140**: 220–239.

Berger, L., Uzzell, T. & Hotz, H. 1994. Postzygotic reproductive isolation between Mendelian species of European water frogs. *Zool. Pol.* **39**: 209–242.

Bergthorsson, U., Adams, K.L., Thomason, B. & Palmer, J.D. 2003. Widespread horizontal transfer of mitochondrial genes in flowering plants. *Nature* **424**: 197–201.

Blankenhorn, H.J. 1977. Reproduction and mating behavior in *Rana lessonae–Rana esculenta* mixed populations. In: *The Reproductive Biology of Amphibians* (D. H. Taylor & S. I. Guttman, eds), pp. 389–410. Plenum Press, New York & London.

Borkin, L.J., Garanin, W.I., Tichenko, N.T. & Zaune, I.A. 1979. Some results in the green frogs survey in the USSR. *Mitt. Zool. Mus. Berlin* **55**: 153–170.

Borkin, L.J., Zaune, I.A., Pikulik, M.M. & Sokolova, T.M. 1986. Distribution and structure of green frog complex in the USSR. In: *Studies in Herpetology* (Z. Roček, ed.), pp. 675–678. Charles University, Prague.

Borkin, L.J., Korshunov, A.V., Lada, G.A., Litvinchuk, S.N., Rosanov, J.M., Shabanov, D.A. & Zinenko, A.I. 2004. Mass occurrence of polyploid green frogs (*Rana esculenta* complex) in eastern Ukraine. *Russ. J. Herpetol.* **11**: 194–213.

Borkin, L.J., Lada, G.A., Litvinchuk, S.N., Melnikov, D.A. & Rosanov, J.M. 2006. The first record of mass triploidy in hybridogenic green frog *Rana esculenta* in Russia (Rostov Oblast). *Russ. J. Herpetol.* **13**: 77–82.

Bucci, S., Raggianti, M., Mancino, G., Berger, L., Hotz, H. & Uzzell, T. 1990. Lampbrush and mitotic chromosomes of the hemiclonally reproducing hybrid *Rana esculenta* and its parental species. *J. Exp. Zool.* **255**: 37–56.

Burton, R.S., Ellison, C.K. & Harrison, J.S. 2006. The sorry state of F2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am. Nat.* **168**: S14–S24.

Croucher, P.J.P., Oxford, G.S. & Searle, J.B. 2004. Mitochondrial differentiation, introgression and phylogeny of species in the *Tegenaria atrica* group (Araneae: Agelenidae). *Biol. J. Linn. Soc.* **81**: 79–89.

Dabrowski, A., Fraser, R., Confer, J.L. & Lovette, I.J. 2005. Geographic variability in mitochondrial introgression among hybridizing populations of Golden-winged (*Vermivora chrysoptera*) and Blue-winged (*V. pinus*) warblers. *Conserv. Genet.* **6**: 843–853.

Dubois, A. & Ohler, A. 1994. Frogs of the subgenus *Pelophylax* (Amphibia, Anura, Genus *Rana*): a catalogue of available scientific names, with comments on name-bearing types, complete synonymies, proposed common names, and maps showing all type localities. *Zool. Pol.* **39**: 139–204.

Durbin, J. & Watson, G.S. 1950. Testing for serial correlation in least squares regression. I. *Biometrika* **37**: 409–428.

Durbin, J. & Watson, G.S. 1951. Testing for serial correlation in least squares regression. 2, *Biometrika* **38**: 159–177

Durbin, J. & Watson, G.S. 1971. Testing for serial correlation in least squares regression. 3, *Biometrika* **58**: 1–19.

Fredsted, T., Wincentz, T. & Villesen, P. 2006. Introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into brown hares (*Lepus europaeus*) in Denmark. *BMC Ecol.* **6**: 17. Available at: http://www.biomedcentral.com/1472-6785/6/17.

Graf, J.-D. & Polls Pelaz, M. 1989. Evolutionary genetics of the *Rana esculenta* complex. In: *Evolution and Ecology of Unisexual Vertebrates* (R. M. Dawley & J. P. Bogart, eds), pp. 289–301. Bulletin 466. New York State Museum, New York.

Grossenbacher, K. 1988. Verbreitung der Wasserfrösche in der Schweiz. In: *Beiträge zur Biologie und Bibliographie (1960–1987) der europäischen Wasserfrösche* (R. Günther & R. Klewen, eds), Jb. Feldherp., Beiheft 1, pp. 129–134. Verlag für Ökologie u. Faunistik, Duisburg.

Gubányi, A. 1992. Distribution of green frogs (*Rana esculenta* complex, Anura: Ranidae) in Hungary. In: *Proc. Sixth Ord. Gen. Meet. S. E. H.* (Z. Korsós & I. Kiss, eds). pp. 205–210. Budapest, Hungary.

Günther, R. 1974. Neue Daten zur Verbreitung und Ökologie der Grünfrösche (Anura, Ranidae) in der DDR. *Mitt. Zool. Mus. Berlin* **50**: 287–298.

Günther, R. 1975. Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche, ''*Rana esculenta*'', L., in der DDR (Anura, Ranidae). *Mitt. Zool. Mus. Berlin* **51**: 145–158.

Günther, R. 1977. Die Erythrozytengröße als Kriterium zur Unterscheidung diploider und triploider Teichfrösche, *Rana* ''*esculenta*'' L. (Anura). *Biol. Zentralblatt* **96**: 457–466.

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

Günther, R. 1990. *Die Wasserfrösche Europas*. Die Neue Brehm-Bücherei 600, A. Ziemsen, Wittenberg–Lutherstadt.

Günther, R. 1997a. *Rana lessonae* Camerano, 1882. In: *Atlas of Amphibians and Reptiles in Europe* (J. P. Gasc, A. Cabela, J. Crnobrnja-Isailović, D. Dolmen, K. Grossenbacher, P. Haffner, J. Lescure, H. Martens, J.P. Martínez Rica, H. Maurin, M.E. Oliveira, T.S. Sofianidou, M. Veith, M. & A. Zuiderwijk, eds), pp. 148–149. Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle, Paris.

Günther, R. 1997b. *Rana ridibunda* Pallas, 1771. In: *Atlas of Amphibians and Reptiles in Europe* (J. P. Gasc, A. Cabela, J. Crnobrnja-Isailović, D. Dolmen, K. Grossenbacher, P. Haffner, J. Lescure, H. Martens, J.P. Martínez Rica, H. Maurin, M.E. Oliveira, T.S. Sofianidou, M. Veith, M. & A. Zuiderwijk, eds), pp. 154–155. Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle, Paris.

Günther, R. & Plötner, J. 1988. Zur Problematik der klonalen Vererbung bei *Rana* kl. *esculenta* (Anura). In: *Beiträge zur Biologie und Bibliographie (1960–1987) der europäischen Wasserfrösche* (R. Günther & R. Klewen, eds), Jb. Feldherp., Beiheft 1, pp. 23–46. Verlag für Ökologie u. Faunistik, Duisburg.

Günther, R., Uzzell, T. & Berger, L. 1979. Inheritance patterns in triploid *Rana* ''*esculenta*'' (Amphibia, Salientia). *Mitt. Zool. Mus. Berlin* **55**: 35–57.

Günther, R., Plötner, J. & Tetzlaff, I. 1991. Zu einigen Merkmalen der Wasserfrösche (*Rana* synkl. *esculenta*) des Donau-Deltas. *Salamandra* **27**: 246–265.

Gusdon, A.M., Votyakova, T.V., Reynolds, I.J. & Mathews, C.E. 2007. Nuclear and mitochondrial interaction involving mt-ND2 leads to increased mitochondrial reactive oxygen species production. *J. Biol. Chem.* **282**: 5171–5179.

Hedges, S.B., Bogart, J.P. & Maxson, L.R. 1992. Ancestry of unisexual salamanders. *Nature* **356**: 708–710.

Hewitt, G.M. 2001. Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Mol. Ecol.* **10**: 537–549.

Hewitt, G.M. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philos. Trans. R. Soc. Lond. B* **359**: 83–195.

Higgins, D.G., Bleasby, A.J. & Fuchs, R. 1992. CLUSTAL V: improved software for multiple sequence alignment. *CABIOS* **8**: 189–191.

Hofmann, S., Spolsky, C., Uzzell, T., Cogălniceanu, D., Babik, W. & Szymura, J.M. 2007. Phylogeography of the fire-bellied toads *Bombina*: independent Pleistocene histories inferred from mitochondrial genomes. *Mol. Ecol.* **16**: 2301–2316.

Hotz, H., Mancino, G., Bucci-Innocenti, S., Raggianti, M., Berger, L. & Uzzell, T. 1985. *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecies hybrids. *J. Exp. Zool.* **236**: 199–210.

Hotz, H., Beerli, P. & Spolsky, C. 1992. Mitochondrial DNA reveals formation of nonhybrid frogs by natural matings between hemiclonal hybrids. *Mol. Biol. Evol.* **9**: 610–620.

Huelsenbeck, J.P. & Rannala, B. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Syst. Biol.* **53**: 904–913.

Huelsenbeck, J.P. & Ronquist, F. 2005. Bayesian analysis of molecular evolution using MRBAYES. In: *Statistical Methods in Molecular Evolution* (R. Nielsen, ed.), pp. 183–232. Springer, New York.

Kumar, S., Tamura, K. & Nei, M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**: 150–163.

Lengagne, T., Grolet, O. & Joly, P. 2006. Male mating speed promote hybridization in the *Rana lessonae–Rana esculenta* waterfrog system. *Behav. Ecol. Sociobiol.* **60**: 123–130.

Lenk, P., Fritz, U., Joger, U. & Wink, M. 1999. Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Mol. Ecol.* **8**: 1911–1922.

Lutschinger, G. 1988. Zur Überwinterung der Wasserfrösche in den Donau- und Marchauen. In: *Beiträge zur Biologie und Bibliographie (1960–1987) der europäischen Wasserfrösche* (R. Günther & R. Klewen, eds), Jb. Feldherp. Beiheft 1, pp. 153–160. Verlag für Ökologie u. Faunistik, Duisburg.

McDonald, J.H.M. & Kreitman, M. 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. *Nature* **351**: 652–654.

Meiklejohn, C.D., Montooth, K.L. & Rand, D.M. 2007. Positive and negative selection on the mitochondrial genome. *Trends Genet.* **23**: 259–263.

Monnerot, M., Dubois, A. & Tunner, H. 1985. Mitochondrial DNA polymorphism among *Rana ridibunda*, *Rana lessonae* and *Rana* kl. *esculenta*: preliminary study. *Alytes* **4**: 101–112.

Morozov-Leonov, S.Y., Mezhzherin, S.V. & Kurtyak, F.F. 2003. The genetic structure of unisexual hybrid populations of frogs *Rana esculenta* complex from the Transcarpathian lowlands. *Cytol. Genet.* **37**: 43–47.

Mulligan, C.J., Kitchen, A. & Miyamoto, M.M. 2006. Comment on ''Population size does not influence mitochondrial genetic diversity in animals''. *Science* **314**: 1390a.

Nei, M. & Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl Acad. Sci. USA* **76**: 5269–5273.

Nylander, J.A.A., Wilgenbusch, J.C., Warren, D.L. & Swofford, D.L. 2007. AWTY (Are We There Yet?): a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. *Bioinformatics* **10**: 1–3. Available at: http://bioinformatics.oxfordjournals.org/cgi/reprint/btm388v1.pdf.

Pagano, A., Dubois, A., Lesbarrères, D. & Lodé, T. 2003. Frog alien species: a way for genetic invasion. *C. R. Biol.* **326**: 85–92.

Plenet, S., Hervant, F. & Joly, P. 2000a. Ecology of the hybridogenetic *Rana esculenta* complex: differential oxygen requirements of tadpoles. *Evol. Ecol.* **14**: 13–23.

Plenet, S., Pagano, A., Joly, P. & Fouillet, P. 2000b. Variation of plastic responses to oxygen availability within the hybridogenetic *Rana esculenta* complex. *J. Evol. Biol.* **13**: 20–28.

Plötner, J. 2005. Die westpaläarktischen Wasserfrösche. *Von Märtyrern der Wissenschaft zur biologischen Sensation*. Laurenti Verlag, Bielefeld.

Plötner, J. & Ohst, T. 2001. New hypotheses on the systematics of the western Palearctic water frog complex (Anura, Ranidae). *Mitt. Mus. Nat.kd. Berl., Zool. Reihe* **77**: 5–21.

Rand, D.M. & Kann, L.M. 1996. Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from *Drosophila*, mice, and humans. *Mol. Biol. Evol.* **13**: 735–748.

Rand, D.M., Fry, A. & Sheldahl, L. 2006. Nuclear-mitochondrial epistasis and *Drosophila* aging: introgression of *Drosophila simulans* mtDNA modifies longevity in *D. melanogaster* nuclear backgrounds. *Genetics* **172**: 329–341.

Robertson, A.V., Ramsden, C., Niedzwiecki, J., Fu, J. & Bogart, J.P. 2006. An unexpected recent ancestor of unisexual *Ambystoma*. *Mol. Ecol.* **15**: 3339–3351.

Ronquist, F. & Huelsenbeck, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rozas, J., Sánches, J.C., DelBarrio, X. & Rozas, R. 2003. DNASP, DNA polymorphism analysis by the coalescent and other methods. *Bioinformatics* **19**: 2496–2497.

Ruedi, M., Smith, M.F. & Patton, J.L. 1997. Phylogenetic evidence of mitochondrial DNA introgression among pocket gophers in New Mexico (family Geomyidae). *Mol. Ecol.* **6**: 453–462.

Rybacki, M. & Berger, L. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. *Mitt. Mus. Nat.kd. Berl., Zool. Reihe* **77**: 51–57.

Schultz, R.J. 1969. Hybridization, unisexuality and polyploidy in the teleost *Poeciliopsis* (Poecilidae) and other vertebrates. *Am. Nat.* **103**: 605–619.

Spolsky, C. & Uzzell, T. 1984. Natural interspecies transfer of mitochondrial DNA in amphibians. *Proc. Natl Acad. Sci. USA* **81**: 5802–5805.

Spolsky, C. & Uzzell, T. 1986. Evolutionary history of the hybridogenetic hybrid frog *Rana esculenta* as deduced from mtDNA analyses. *Mol. Biol. Evol.* **3**: 44–56.

Spolsky, C.M., Phillips, C.A. & Uzzell, T. 1992. Antiquity of clonal salamander lineages revealed by mitochondrial DNA. *Nature* **356**: 706–708.

Sullivan, J.P., Lavoué, S., Arnegard, M.E. & Hopkins, C.D. 2004. AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution* **58**: 825–841.

Sumida, M., Kanamori, Y., Kaneda, H., Kato, Y., Nishioka, M., Hasegawa, M. & Yonekawa, H. 2001. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. *Genes Genet. Syst.* **76**: 311–325.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G. & Cosson, J.-F. 1998. Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.* **7**: 453–464.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585–595.

Tunner, H.-G. & Nopp, H. 1979. Heterosis in the common European water frog. *Naturwissenschaften, Berlin* **66**: 268–269.

Upholt, W.B. 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. *Nucleic Acids Res.* **4**: 1257–1265.

Uzzell, T. 1964. Relations of the diploid and triploid species of the *Ambystoma jeffersonianum* complex (Amphibia, Caudata). *Copeia* **1964**: 257–300.

Uzzell, T. 1979. Immunological variation in *Rana lessonae* (Amphibia, Salientia). *Mitt. Zool. Mus. Berlin* **55**: 29–33.

Uzzell, T. 1983. An immunological survey of Italian water frogs (Salientia Ranidae). *Herpetologica* **39**: 225–234.

Uzzell, T. & Berger, L. 1975. Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae*, and their hybridogenetic associate, *Rana esculenta*. *Proc. Acad. Nat. Sci. Philadelphia* **127**: 13–24.

Uzzell, T., Günther, R. & Berger, L. 1977. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). *Proc. Acad. Nat. Sci. Philadelphia* **128**: 147–171.

Vinogradov, A.E., Borkin, L.J., Günther, R. & Rosanov, J.M. 1991. Genome elimination in diploid and triploid *Rana esculenta* males: cytological evidence from DNA flow cytometry. *Genome* **33**: 619–627.

Wood, F.S. 1973. The use of individual effects and residuals in fitting equation to data. *Technometrics* **15**: 677–695.

Yamada, M., Higuchi, M. & Goto, A. 2001. Extensive introgression of mitochondrial DNA found between two genetically divergent forms of threespine stickleback, *Gasterosteus aculeatus*, around Japan. *Environ. Biol. Fishes* **61**: 269–284.

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Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Origin, phenotype and number of individuals investigated, method applied for mtDNA typing and haplotypes detected.

Appendix S2. Protocols used for amplification and sequencing of the mtDNA.

Appendix S3. Component effects plot of latitude and longitude on the relative frequency of individuals with

R. lessonae-specific mtDNA (f_1) for *Rana ridibunda* and *Rana esculenta*.

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