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Conserv Genet (2014) 15:869–878
DOI 10.1007/s10592-014-0585-0

RESEARCH ARTICLE

Are invasive marsh frogs (*Pelophylax ridibundus*) replacing the native *P. lessonae/P. esculentus* hybridogenetic complex in Western Europe? Genetic evidence from a field study

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Received: 2 December 2013 / Accepted: 10 February 2014 / Published online: 20 February 2014
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Abstract The water-frog L–E system, widespread in Western Europe, comprises the pool frog *Pelophylax lessonae* and the hybridogenetic edible frog *P. esculentus*, which originated from hybridization between pool frogs and marsh frogs (*P. ridibundus*). In *P. esculentus*, the *lessonae* (L) genome is eliminated during meiosis and has to be gained anew each generation from a *P. lessonae* partner, while the *ridibundus* (R′) genome is transmitted clonally. It therefore accumulates deleterious mutations, so that R′R′ offspring from *P. esculentus*×*P. esculentus* crosses are normally unviable. This system is now threatened by invasive *P. ridibundus* (RR) imported from Eastern Europe and the Balkans. We investigated the genetic interactions between invasive marsh frogs and native water frogs in a Swiss wetland area, and used genetic data collected in the field to validate several components of a recently postulated mechanism of species replacement. We identified neo-*ridibundus* individuals derived from crosses between invasive *ridibundus* and native *esculentus*, as well as newly arisen hybridogenetic *esculentus* lineages stemming from crosses between invasive *ridibundus* (RR) and native *lessonae* (LL). As their *ridibundus* genomes are likely to carry less deleterious mutations, such lineages are expected to produce viable *ridibundus* offspring, contributing to species replacement. However, such crosses with invasive *ridibundus* only occurred at a limited scale; moreover, RR×LL crosses did not induce any introgression from the *ridibundus* to the *lessonae* genome. We did not find any *ridibundus* stemming from crosses between ancient *esculentus* lineages. Despite several decades of presence on the site, introduced *ridibundus* individuals only represent 15 % of sampled frogs, and their spatial distribution seems shaped by specific ecological requirements rather than history of colonization. We therefore expect the three taxa to coexist stably in this area.

Keywords Amphibian · Hybridogenesis · Invasion · Microsatellites · Water frog

Electronic supplementary material The online version of this article (doi:10.1007/s10592-014-0585-0) contains supplementary material, which is available to authorized users.

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Introduction

Accidental or deliberate introductions of exotic species into non-native ranges are now occurring at unprecedented rates, partly due to the intensive development of international trade (Westphal et al. 2008). Some introduced species become invasive, which poses important threats to local biodiversity (Wilcove et al. 1998; Vorburger and Ribi 1999). Potential impacts on native species are both ecological and genetic (Williamson and Fitter 1966; Manchester and Bullock 2000).

Ecological effects are mediated by competition for food or space, predation, parasitism, habitat modification, or spread of new diseases (Mooney and Cleland 2001). Genetic effects result from hybridization with indigenous species, such as documented between wild and domestic cats, or between introduced Mallards and native ducks (Rhymer and Simberloff 1996). If hybrids are fertile, backcrosses with parental species induce genetic introgression that may lead to the progressive elimination of native genomes ("genetic swamping"; Rhymer and Simberloff 1996; Huxel 1999), as documented e.g. between introduced and local trout in the USA, or between Red deer and introduced Sika deer in Scotland (Rhymer and Simberloff 1996).

Water frogs from the *Pelophylax esculentus* complex represent a particular situation. The so-called klepto-taxon *P. esculentus* (edible frog) is a permanent F_1 hybrid that originally emerged from a hybridization between the pool frog *P. lessonae* and the marsh frog *P. ridibundus* (Graf and Polls Pelaz 1989). Due to still unknown features of the ancestral *ridibundus* (R) genome, the *lessonae* (L) genome is eliminated from the *esculentus* germ line, so that all gametes transmit the same, non-recombined *ridibundus* (R′) genome (Tunner and Heppich 1981). Mating with a *lessonae* (LL) partner is thus required to restore *esculentus* (R′L) offspring—a process called hybridogenesis. Being always expressed in hemizygous state, R′ hemiclones are largely protected from purifying selection; in addition, the strong Hill–Robertson interferences resulting from clonal transmission favor the rapid accumulation of deleterious mutations. As a result, crosses between two *esculentus* bearing the same hemiclone normally produce unviable or unfertile R′R′ offspring (Vorburger 2001a), although evidence exists that these newly formed *P. ridibundus* can occasionally survive and even mature, provided the combining R′ have fixed distinct deleterious mutations (Hotz et al. 1985, 1992; Berger et al. 1988; Schmidt 1993; Vorburger 2001b; Guex et al. 2002; Schmeller et al. 2005; Luquet et al. 2011).

The so-called L–E system, consisting of syntopic populations of *P. lessonae* and its obligate genetic parasite *P. esculentus*, is widespread throughout Western and Central Europe, where *P. ridibundus* (the other parental species) is absent (Uzzell and Berger 1975). This system is now threatened by the ongoing invasion of marsh frogs; native to Eastern Europe, the Balkanic peninsula and Western Asia, *P. ridibundus* has been successfully introduced in Central and Western Europe for commercial purpose (Kuzmin et al. 2009). Its expansion has drastic consequences on native amphibian species via competitive exclusion and direct predation. Native pool frogs have already been replaced at several sites (Schmeller et al. 2007; Holsbeek and Jooris 2009). The Bois de Finges, a nature reserve in western Switzerland originally occupied by the L–E system, was colonized in the 1960s by *P. ridibundus* (Marchesi et al. 1999). The numbers of marsh frogs have increased until in 1999 *P. lessonae* represented only 3 % of water frogs (Marchesi et al. 1999), and has become completely extinct by 2013 (Leuenberger, personal observation).

Replacement of native pool frogs may occur through both ecological and genetic interactions. The latter have been suggested to take place via a mechanism inherent to the hybridogenetic nature of this hybrid complex (Vorburger and Reyer 2003). On one hand, mating between invasive *ridibundus* (RR) and native *esculentus* (R′L) is expected to produce viable *ridibundus* (RR′) offspring. On the other hand, mating between *ridibundus* (RR) and *lessonae* (LL) generates *esculentus* (RL) primary hybrids; if the latter are hybridogenetic, mating between RL hybrids should restore pure and viable *ridibundus* (RR) offspring. Thus, the *ridibundus* genome is expected to progressively replace the *lessonae* genome, and eventually eliminate the L–E complex (Vorburger and Reyer 2003).

Several assumptions underlying this genetic mechanism have been tested and validated in the laboratory via experimental crosses (Vorburger and Reyer 2003). These crosses furthermore detected primary hybrid females that were non-hybridogenetic (see also Hotz et al. 1985), producing offspring with recombined *ridibundus* and *lessonae* genomes; this suggests an additional (and more classical) mechanism of genetic replacement in which the *ridibundus* genome would replace the *lessonae* genome by progressive introgression (Rhymer and Simberloff 1996). It is not known, however, whether the different processes mentioned above actually take place in nature, and if they do, how frequently they occur. It is also possible that behavioral or ecological preferences may result in assortative mating at such a high rate that complete genome replacement is prevented and long-term coexistence possible (Plénet et al. 2001; Luquet et al. 2011).

The present study was aimed at investigating the genetic mechanisms of species replacement in a region originally occupied by the L–E system, where invading *P. ridibundus*, of unknown origin, have been observed for more than three decades (M. Antoniazza pers. comm.; Berthoud and Perret-Gentil 1976). We thus expected the process of replacement to be well advanced, possibly with *lessonae* and/or *esculentus* already eliminated locally. We based our investigations on nuclear and mitochondrial markers for several reasons: (i) there is a large overlap between the *esculentus* phenotype and both parental species, so that species identity cannot be diagnosed with certainty based on morphology alone (Nöllert and Nöllert 2003; Meyer et al. 2009; Holsbeek and Jooris 2009); (ii) genetic markers are expected to provide relevant information on the gene diversity of invading *ridibundus* and native *lessonae*, on

Table 1 Sampling sites and sample numbers of *Pelophylax* spp. along the southern shore of Lake Neuchâtel (western Switzerland)

Sampling site	Coordinates	*P. lessonae*			*P. esculentus*			*P. ridibundus*		
		M	F	J	M	F	J	M	F	J
Chevroux	06°54′27″E; 46°53′42″N	0	0	1	0	9	1	4	8	3
Gletterens 1	06°56′14″E; 46°54′32″N	3	2	0	17	4	2	0	0	0
Gletterens 2	06°56′20″E; 46°54′44″N	8	6	0	7	5	0	0	0	0
Motte 1	06°59′06″E; 46°56′30″N	2	5	0	5	13	0	1	0	0
Motte 2	06°58′50″E; 46°56′23″N	5	5	1	11	5	8	0	0	0

M male, *F* female, *J* juvenile

their possible introgression, as well as on the diversity of R′ hemiclones in ancient *esculentus* lineages; (iii) nuclear markers offer the potential to differentiate the native hemiclonal R′ genomes from the invading R genomes, thereby allowing identification of *esculentus* primary hybrids resulting from *ridibundus* (RR)×*lessonae* (LL) crosses, as well as neo-*ridibundus* resulting from *ridibundus* (RR)×*esculentus* (LR′) or *esculentus* (LR′)×*esculentus* (LR′) crosses, respectively; (iv) nuclear markers offer the potential to identify possible polyploid *esculentus*, which would complicate the analysis further; (v) mitochondrial information might contribute to identify primary hybrids and neo-*ridibundus* individuals, and deliver information on their paternal *versus* maternal ancestry. It has been suggested (e.g. Luquet et al. 2011) that introgression of *lessonae* mtDNA into the *ridibundus* genome (e.g. via mating of female *esculentus* with male *ridibundus*; Spolsky and Uzzell 1984) may facilitate the local adaptation of invasive *ridibundus*.

Materials and methods

Field sampling and laboratory work

Water frogs were collected from five sites (Table 1) in a nature reserve (la Grande Cariçaie) along the southern shore of the Lake of Neuchâtel (Switzerland) during the 2012 breeding season (May to early August). Individuals were sampled for buccal cells (two sterile cotton swabs per individual; Broquet et al. 2007), then released at the place of capture. Swabs were stored dry at −20 °C before analysis. DNA was extracted with the Qiagen DNeasy Blood and Tissue Kit, using the Spin-Column Protocol (manual extraction) and the BioSprint 96 workstation (Qiagen, Valencia; PA; USA), following the manufacturer's protocols with overnight sample incubation in proteinase K at 56 °C.

DNA samples were genotyped for a set of 18 microsatellite markers selected for their potential to differentiate the two genomes under study (Table 2), five of them considered specific to the R genome, five other to the L genomes, and the last eight markers expected to amplify in both (Zeisset et al. 2000; Garner et al. 2000; Hotz et al. 2001; Arioli 2007; Christiansen 2009; Christiansen and Reyer 2009). Polymerase chain reactions (PCRs) were performed in four multiplex PCR containing primers for 4–5 loci (Table 2). Reactions were performed in 10 µl, containing 3 µl DNA extraction, 3 µl Qiagen Multiplex PCR Master mix, and from 2.2 to 3 µl of primer mixes with labeled forward and reverse primers (Table 2). The thermal profile of PCR amplification included an initial denaturation step of 15 min at 95 °C followed by 35 cycles of 30 s denaturation at 94 °C, 90 s annealing at T_a (Table 2), 60 s extension at 72 °C, and a final extension of 30 min at 60 °C. PCR products derived from primer mix 1 were diluted five times and from all other primer mixes twice. Of the diluted PCR product, 3 µl were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) with an internal size standard (GeneScan-350 ROX). Alleles were scored with GeneMapper v4.0 (Applied Biosystems).

DNA samples were also analyzed for the mitochondrial *cytb* gene, using the primers F1: 5′CGA AGC CTA GAA GAT CTT TG3′ and L1: 5′CTC CTG GGA GTC TGC CTA AT3′ (Dubey et al. in prep.). PCRs were performed in a total volume of 25 µl with 2.5 µl of 10× buffer, 0.2 mM of each primer, 0.05 mM of dNTPs, 8 mM of MgCl2, 1 U of Taq DNA polymerase (Qiagen, Valencia, PA, USA) and 2 µl of extracted DNA, under the following conditions: initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 40 s annealing at 50 °C, 90 s extension at 72 °C and a final extension of 10 min at 72 °C. PCR fragments were then purified and sequenced at GATC sequencing service (Cologne, D; http://www.gatc-biotech.com). Sequences were aligned by ClustalW (Higgins et al. 1994) implemented in Mega v5.2 (Tamura et al. 2011), together with the published sequences of two *P. lessonae* from central and southern Italy (GenBank accession numbers EU047775 and AB029942), two *P. ridibundus* from Ukraine and Greece (AB029945 and DQ474163), and one *P. nigromaculatus* as an outgroup (AB043889). We used MrAIC v1.4.4 (Nylander 2004) to select the best model of DNA substitution for Maximum likelihood (ML) analyses. The HKY+G model best fitted our dataset according to AIC, AICc and BIC criteria. ML analyses were performed with PhyML v3.0.1 (Guindon and

Table 2 Amplification conditions for the 18 microsatellite markers used, distributed in four primers mixes

Primers mix	Marker	Genome specificity as published	References	Forward primers labels	Volume (µl) of each primers (10 mM)	Annealing temperature (°C)
Primers mix 1	Ga1a19 redesigned	L+R	1, 2	FAM	0.4	58
	RlCA1b5	L+R	3	ATTO550	0.2	58
	RlCA5	L	3	HEX	0.3	58
	RlCA1b6	L+R	1	FAM	0.2	58
	Rrid064A	R	4	Dyomics630	0.4	58
Primers mix 2	Re2Caga3	R	1	ATTO550	0.1	55
	Res16	L+R	5	FAM	0.2	55
	Res20	L	5	HEX	0.5	55
	RlCA2a34	L+R	4	Dyomics630	0.5	55
Primers mix 3	ReGA1a23	L*	4	ATTO550	0.2	58
	Rrid169A	R	4	HEX	0.4	58
	Res22	R	5	FAM	0.2	58
	Rrid013A	L+R	6	FAM	0.1	58
	Rrid059A	L+R	6	ATTO550	0.2	58
Primers mix 4	Re1Caga10	L+R	1	HEX	0.4	58
	RlCA1a27	L	4	Dyomics630	0.4	58
	Rrid135A	R*	4	ATTO550	0.3	58
	RlCA18	L	3	FAM	0.2	58

Provided are for each marker the genome specificity (with published reference: *1* Arioli 2007, *2* Christiansen 2009, *3* Garner et al. 2000; *4* Christiansen and Reyer 2009, *5* Zeisset et al. 2000; *6* Hotz et al. 2001), labels of forward primers, volume of primers used, and annealing temperature

* Difference in specificity between our samples and the references (see "Results" section)

Gascuel 2003) implemented in SeaView v4.0 (Gouy et al. 2010).

Statistical analyses

Genotypes were clustered using a Bayesian algorithm (Structure v.2.3.3; Pritchard et al. 2000). The most likely number of clusters (K) was calculated from the ΔK method (Evanno et al. 2005) implemented in Structure Harvester v0.6.93 (Earl and vonHoldt 2012). Analyses were carried out on the 18 nuclear loci using an admixture model without prior species information, with 10,000 burn-in steps and 100,000 iterations. All runs were replicated 10 times with K ranging from 1 to 8.

In parallel, we performed principal component analyses (PCAs) with Pcagen v2.0 (Goudet 2005), which extracts the factors (linear component of the 18 initial variables) that maximize overall differentiation (F_{ST}). A first PCA was applied on individual genotypes, and a second after dissociation of the L and R′ genomes in *esculentus* individuals (see Results); each *esculentus* individual was thus represented by two entries in the matrix, one expressing its *lessonae* genome and the other its *ridibundus* genome.

Gene diversity (i.e., expected heterozygosity under HW assumptions, given allelic frequencies) was calculated with Fstat v2.9.3 (Goudet 1995), based on the 10 markers that amplified products from both genomes (see Results), and independently so in the R′ and L genomes of *esculentus* individuals. Differences in gene diversity between genomes were tested with a Mann–Whitney U test in R (R Core Team 2012).

Results

Species identification

A total of 141 water frogs (adults and juveniles) were captured over 27 nights of sampling (Table 1). All 141 individuals could readily be assigned to three genetic clusters based on the patterns of microsatellite amplification; 16 individuals (five males, eight females, three non-sexed juveniles) yielded no amplicons for four markers (*Rl*CA5, *Res*20, *Rl*CA1a27 and *Rl*Ca18) considered specific to the *lessonae* genome and therefore were assigned to *P. ridibundus*. Another 38 individuals (18 males, 18 females, two non-assigned individuals) showed no amplification for four other markers (*Rrid*064A, *Re*2Caga3, *Rrid*169A and *Res*22) considered specific to the *ridibundus* genome and therefore were assigned to *P. lessonae*. The remaining 87 individuals (39 males, 36 females, 12 non-assigned) amplified for all 18 markers, and were hemizygous for the eight markers mentioned above; these were therefore assigned to *P. esculentus*. Contrasting to the results by Christiansen and Reyer (2009), *Re*GA1a23 and *Rrid*135A were not strictly specific to the *lessonae* and *ridibundus* genomes respectively: the former yielded amplicons in many *ridibundus* genomes, and the latter in a

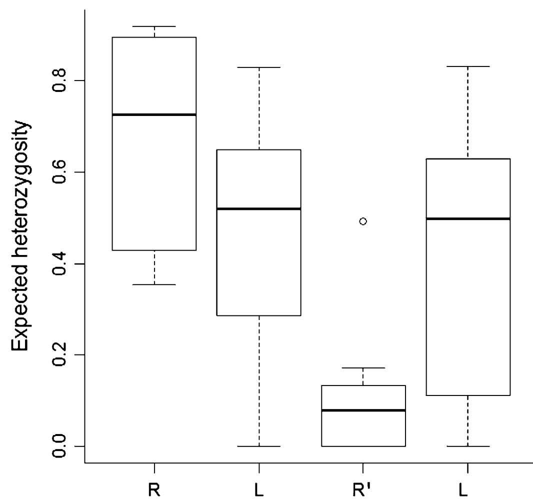

Fig. 1 Gene diversity of the R and L genomes from the two parental species (*left*; R = *P. ridibundus,* L = *P. lessonae*) and the R′ and L components of the *P. esculentus* genomes (*right*). *Boxes* represent interquartile ranges (50 % of the data) with the median given by the *bold line*. Whiskers identify values that do not exceed 1.5 times the interquartile range; the outlier (*empty dot*) is Re1Caga10

few *lessonae* genomes. Despite overall high levels of gene diversity, no individual displayed more than two alleles at any locus, pointing to all-diploid populations.

Gene diversity

The hybridogenetic nature of these 87 *P. esculentus* individuals was corroborated by allelic patterns: for the 10 loci in which two alleles could be amplified, one copy matched the size range and frequency distribution of *lessonae* alleles, while the other copy matched the *ridibundus* size range and was mostly monomorphic throughout. These patterns allowed straightforward identification of the L and R′ genomes in all *esculentus* individuals, and therefore independent estimation of gene diversity on both components (Fig. 1). Expected heterozygosity was highest in the R genomes of *P. ridibundus*, and lowest in the R′ genomes of *P. esculentus* (Mann–Whitney U test, U = 97, $p < 0.001$). The L genomes from *P. lessonae* and *P. esculentus* did not differ from each other (U = 51, $p = 0.970$), being slightly less diverse than the *P. ridibundus* R genomes (U = 28, $p = 0.104$).

Mitochondrial DNA

Species assignments were largely corroborated by mtDNA analyzes: all 38 individuals assigned to *P. lessonae* clustered in the same *cytb* clade, together with *lessonae* sequences from Italy, while all 16 *P. ridibundus* with one exception (3_EN_13) clustered in a distinct clade, together with *P. ridibundus* Genbank sequences (Fig. 2). Among the 87 *P. esculentus* individuals identified, 84 displayed a *lessonae* haplotype, while the remaining three (1_EN_40, 3_EN_02 and 2_EN_19) had a *ridibundus* haplotype.

Clustering of individuals

Structure analyses identified K = 3 as the most likely number of clusters. All individuals were assigned with high

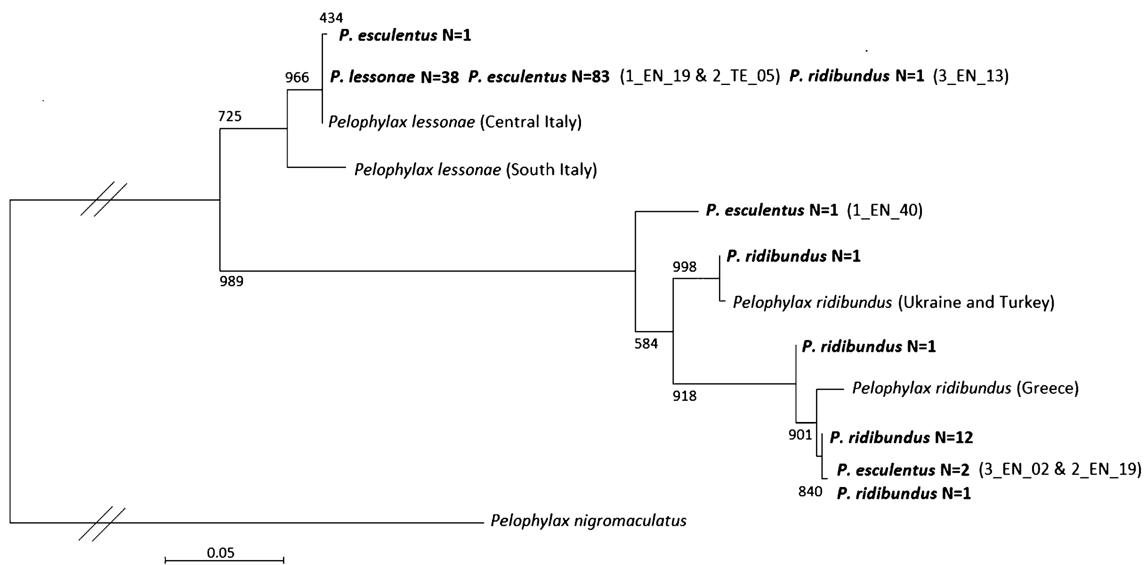

Fig. 2 *cyt-b* maximum-likelihood phylogeny of water frogs from our sampling site, with one outgroup (*P. nigromaculatus,* GenBank accession number AB043889*)* and four reference genomes (two *P. ridibundus* and two *P. lessonae*, respectively AB029945, DQ474163, EU047775 and AB029942). The names in *bold* correspond to the samples from our study, identified from the patterns of microsatellites amplification. Provided are bootstrap support values for the major branches (out of 1,000 replicates), as well as localization of the six outliers (one *ridibundus*, five *esculentus*)

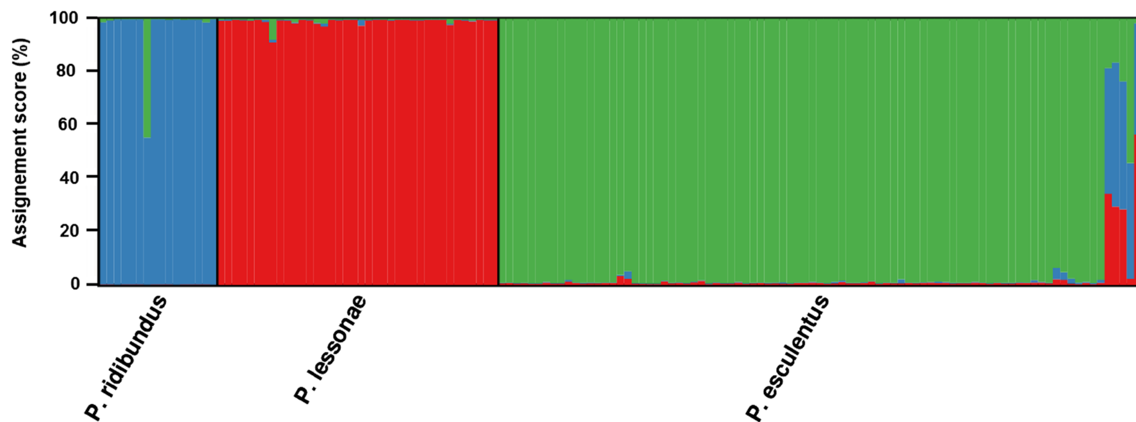

Fig. 3 Bayesian clustering analysis (STRUCTURE) for the most likely number of clusters (K = 3), with no prior information on taxonomic identity. All 141 individuals are assigned with high likelihood to either the *blue* (*ridibundus*), *red* (*lessonae*) or *green* (*esculentus*) cluster, with however six exceptions (one *ridibundus* and five *esculentus*)

likelihoods to one of these three clusters each, fully consistent with the patterns of marker amplification, however with six exceptions (Fig. 3): the *ridibundus* individual with a cytonuclear mismatch (3_EN_13) was assigned to a mix of *ridibundus* and *esculentus* with approximately equal probability, and five *esculentus* (from left to right in Fig. 3: 1_EN_40, 3_EN_02, 2_EN_19, 2_TE_05, and 1_EN_29) were assigned to a mix of *ridibundus* and *lessonae* with high probabilities. This subset includes the three *esculentus* individuals with *ridibundus* mtDNA haplotypes.

The PCAGEN analysis performed on individual genotypes identified the same three clusters and six outliers comprising five *P. esculentus* and one *P. ridibundus* (Fig. 4). The first factor (accounting for 31.22 % of the variance) differentiates *P. lessonae* from *P. ridibundus*, with *P. esculentus* in between. The second factor (9.26 % of the variance) differentiates *P. ridibundus* from *P. esculentus*. The *P. ridibundus* individual with a cytonuclear mismatch (3_EN_13), which was partly assigned to *esculentus* by STRUCTURE, takes an intermediate position between these two groups on the 2nd axis, while the five *P. esculentus* individuals assigned to a mix of *lessonae* and *ridibundus* (some of which with *P. ridibundus* mtDNA) also take intermediate positions between these clusters on both axes.

Clustering of genomes

As already pointed out, the assignment of *esculentus* genomes to either *lessonae* (L) or *ridibundus* (R′) origin was straightforward: four markers had a *ridibundus*-specific expression, four a *lessonae*-specific expression, and the ten others had parental-specific allelic sizes and/or frequency distributions. The PCAGEN analysis performed after discrimination of the *lessonae* (L) and *ridibundus* (R′) components of *P. esculentus* genomes also provides clear-cut results (Fig. 5). The first factor (accounting for 37.78 % of the variance) mostly differentiates the *lessonae* (L) from the *ridibundus* (R and R′) genomes, while the second axis (5.27 % of variance) discriminates between the R and the R′ genomes. All *esculentus* L genomes perfectly co-localize with the *lessonae* (LL) genomes (with however a larger variance in the distribution due to their haploid state), while their R′ genomes cluster in two groups. On one hand, 82 R′ genomes cluster into 11 haplotypes that are markedly diverged from the RR genomes and poorly diversified: 59 individuals share the exact same haplotype H1 (i.e., the same alleles at all of the 14 loci), while the 23 others have 1 of 10 other haplotypes that each differ from H1 by only 1 or at most 3 allelic substitutions. On the other hand, the 5 remaining R′ genomes cluster into 4 haplotypes partly overlapping the RR cluster, and diverging from H1 in at least 7 of the 14 loci. These diverged haplotypes characterize the five *esculentus* individuals that, according to STRUCTURE, share genetic features with the *ridibundus* and *lessonae* genomes (Fig. 3). The RR cluster, finally, is quite homogeneous, with the exception of the *ridibundus* individual with a cytonuclear mismatch (3_EN_13) that takes an intermediate position between the RR and R′ genomes.

Spatial distribution of genomes

The three taxa were not homogeneously distributed across our five sampling sites (Fig. S1): while the hybridogenetic *P. esculentus* occurred widespread at all sites, *P. ridibundus* was mostly limited to the westernmost site (Chevroux). *P. lessonae* was nearly absent from this *ridibundus*-dominated site, but constituted a significant part of the four other sites, and the majority in one of them (Gletterens 2). The *P.*

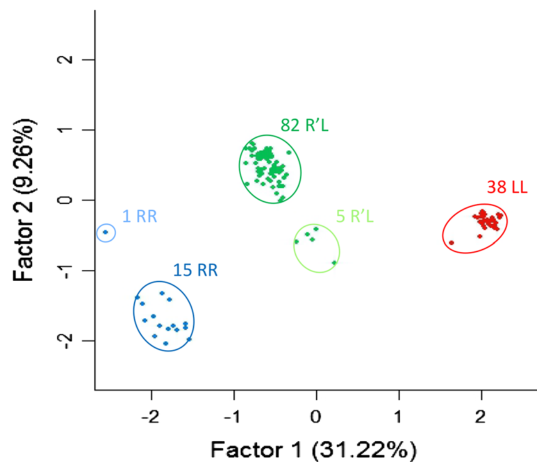

Fig. 4 Biplot of factor scores from the PCA performed on genotypes; RR *P. ridibundus* in *blue* (with the neo-*ridibundus* individual 3_EN_13 in *pale blue*), ancient R′L *P. esculentus* in *dark green*, neo R′L *esculentus* in *pale green*, and *P. lessonae* in *red*

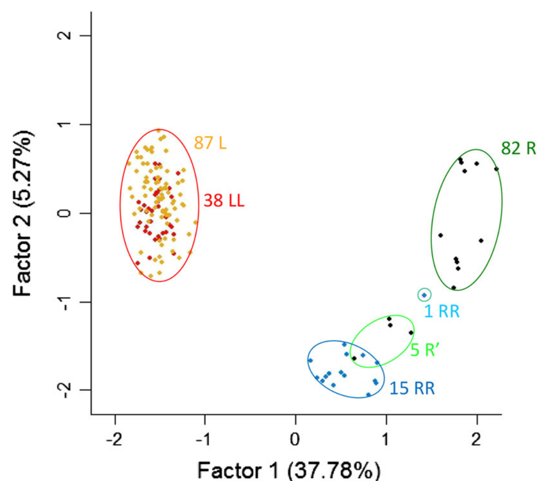

Fig. 5 Biplot of factor scores from the PCA performed on genomes; LL genomes of *lessonae* in *red*, L genomes of *esculentus* in *yellow*, RR genomes of *ridibundus* in *blue* (with the neo-*ridibundus* individual 3_EN_13 in *pale blue*), R′ genomes of *esculentus* in *black*

ridibundus with a cytonuclear mismatch (3_EN_13) was found at the site dominated by *ridibundus* and *esculentus* genomes (Chevroux), while the five *esculentus* with diverged hemiclones were spread among four sampling sites (Fig. S1).

Discussion

Our 18 microsatellite markers allowed straightforward species assignment: the patterns of amplification and allelic size ranges were highly specific to the three *Pelophylax* taxa, with no evidence of introgression between the *lessonae* and *ridibundus* genomes. Nuclear identification was corroborated by mtDNA analysis: the *cytb* tree consisted of two reciprocally monophyletic groups, with all *lessonae* clustering on one side, and all *ridibundus* except one on the other side (Fig. 2). Nuclear genetic diversity was high in *P. lessonae*, and even higher in the invading *P. ridibundus* (Fig. 1), suggesting multiple introductions (as also supported by the high *cytb* diversity).

The L genome from *P. esculentus* individuals was undistinguishable from that of pure *P. lessonae* (Fig. 5), as expected from ongoing hybridogenesis: the L genome is eliminated at each generation, and has to be obtained anew from a *P. lessonae* partner. In contrast, the *ridibundus* (R′) genome from most *P. esculentus* individuals (82 out of 87) differed drastically from the R genomes of invading *P. ridibundus* (Fig. 5), pointing to anciently diverged hemiclones. The clonal transmission of these frozen R′ genomes was confirmed by their very low genetic diversity (Fig. 1) and low number of distinct haplotypes. All these 82 ancient *esculentus* lineages harbored *lessonae* mtDNA, as expected from a long history of coexistence. For size and behavior-related reasons, L×E crosses often take place between *esculentus* females and *lessonae* males (Berger 1970), but reverse crosses also occur at some lower rate (Lengagne et al. 2006). Assuming *lessonae* females to contribute as few as 10 % of the crosses, the *ridibundus* mtDNA is expected to decline at a 10 % rate per generation, reaching frequencies below 5 % within 30 generations. Thus, occasional crosses between *P. esculentus* males and *P. lessonae* females are expected to progressively eliminate the original *ridibundus* mtDNA from L–E systems (Spolsky and Uzzell 1986; Plötner et al. 2008).

However, five *P. esculentus* displayed a very distinct pattern. Their R′ genomes cluster with those of *P. ridibundus* (RR), not those of other *P. esculentus* (Figs. 4, 5), pointing to recent hybridization events between invasive marsh frogs and native pool frogs. Three of them (3_EN_02, 2_EN_19, 1_EN_40) harbored *ridibundus* mtDNA, revealing their origin as a cross between a male *lessonae* and a female *ridibundus*. Interestingly, this includes two females (3_EN_02, 2_EN_19) coming from different localities (respectively Chevroux and Gletterens 1) that share the exact same R′ hemiclone and exact same *ridibundus cytb* haplotype. They therefore represent the 2nd generation (or more) of a new hybridogenetic lineage, created anew from a cross between a *ridibundus* mother and a *lessonae* father, with clonal transmission of the nuclear and mitochondrial DNA through R′L mother(s). In addition, one *ridibundus* female (3_EN_13) also displayed a distinct pattern, with a RR′ genome and a *lessonae cytb*, thus clearly resulting from a cross between a *P. esculentus* (R′L) mother and a *P. ridibundus* (RR) father. We did not find any R′R′ *ridibundus*, suggesting that, even if some

crosses between ancient *esculentus* lineages occasionally produce viable tadpoles (Hotz et al. 1992; Vorburger 2001a; Luquet et al. 2011), these offspring perform poorly at post-metamorphic stages.

As the six outliers clearly show, several components of the genetic mechanism of species replacement identified by Vorburger and Reyer (2003) are underway in the study site; invasive *P. ridibundus* are giving rise to neo-*ridibundus* lineages (RR′) by crossing with native *P. esculentus*, and to new hybridogenetic *esculentus* lineages by crossing with native *P. lessonae*. As these new *esculentus* lineages harbor *ridibundus* hemiclones devoid of deleterious mutations, further crosses between *esculentus* should produce viable *ridibundus* offspring. In theory, therefore, all ingredients of the genetic mechanism of species replacement envisioned by Vorburger and Reyer (2003) are in place. This raises the question of whether this mechanism will end up in the complete elimination of the native L–E system by invasive marsh frogs, as otherwise documented in several places throughout Western Europe (see "Introduction" section). Fully answering this question requires long-term monitoring of the study site; similar genetic sampling in approximately another decade would certainly provide useful insights on the dynamics of the process. However, the data already in hand suggest some reasons for optimism in this specific case. Although marsh frogs have been recorded for several decades at the site, the L–E system still represents 85 % of all water frogs, with 27 % of pure *P. lessonae* and 58 % of ancient *esculentus* lineages. The *lessonae* genome harbors high gene diversity, testifying to large effective populations sizes. We found no evidence for genetic introgression between the R and L genomes, expected if introduced *P. ridibundus* do not induce hybridogenesis (Hotz et al. 1985; Vorburger 2001b). This may suggest either that new *esculentus* are all hybridogenetic or that non-hybridogenetic hybrids are not fertile. Both mechanisms exclude a risk of genetic swamping (Rhymer and Simberloff 1996).

More importantly, the *ridibundus* and L–E complex show strikingly distinct distributions over the study sites (Fig. S1), with one single site heavily colonized by P. *ridibundus*, and four other sites dominated by the L–E system. This heterogeneity likely stems from ecological differences: *P. ridibundus* prefers open and well-oxygenated water bodies such as lakes and large ponds, whereas *P. lessonae* seems more adapted to small water bodies with low oxygen content and dense vegetation cover (Negovetic et al. 2001; Plénet et al. 2001, 2005; Holenweg Peter et al. 2002; Schmeller et al. 2007; Meyer et al. 2009). Accordingly, densely vegetated sites in the eastern part of our study area only harbored *P. lessonae* and *P. esculentus*, *P. ridibundus* being limited to the open site with low vegetation cover of Chevroux. That their distinct distributions across the study site stem from ecological differentiation rather than history of colonization is further corroborated by the distribution of the hybrids: the single neo-*ridibundus* (R′R) individual (with a *lessonae* mtDNA) was found at the site dominated by *P. ridibundus*, while the five neo-*esculentus* individuals were spread across all sites (including sites where no pure *ridibundus* was found), as were the native *esculentus* lineages. The complete absence of R′R′ neo-*ridibundus* individuals also excludes the risk that crosses between native *esculentus* lineages favors adaptation of invaders to pool frogs' habitat through hybrid-derived *ridibundus* genomes that are locally adapted (Luquet et al. 2011).

Thus, although our study provides direct evidence for genetic interactions between the invasive *P. ridibundus* and the native *P. lessonae* and *P. esculentus* that might, in theory, lead to the replacement of this native species complex, our data also suggest that ecological differentiation is sufficient to limit the extent of interbreeding, so that the three taxa can be expected to stably coexist in the study area over the coming decades. This rather optimistic statement obviously assumes long-term maintenance of important landscape features, including old ponds with a dense vegetation cover. This, however, may pose a dilemma in terms of wetland management. Due to intrinsic dynamics, any water body is bound to progressively disappear via the processes of alluviation, silting, and natural succession. A usual way to counter these dynamics is through pond restoration by re-digging and re-profiling. Pond restoration is certainly a highly successful option for pioneer species (e.g. Petranka et al. 2007; Rannap et al. 2009), not only among amphibians. However, this may also pose a threat for specialist species like *P. lessonae*, which require shallow stagnant water bodies with dense herbaceous vegetation. If space and resources allow, wetland management policies should in such cases focus on the creation of new ponds. Alternatively, old ponds should be restored in such a way that they match the ecological requirements of the native species (Plénet et al. 2001, 2005; Holenweg Peter et al. 2002; Schmeller et al. 2007).

Acknowledgments We thank M. Antoniazza and A. Ghiraldi for sharing their knowledge of the Grande Cariçaie; C. Benjemia, S. Biollay, O. Darbellay, L. Dutoit, F. Goetschi, N. Hazi, A. Jost, H. Lovis, T. Martignier, L. Megali, G. Mottaz, A. Murakozy, M. Podolak, E. Rapin, M. Ribaux, N. Rodrigues, P. Roelli, A. Rogivue, M. Stojiljkovic and Q. Theiler for their help during field work; S. Röthlisberger and N. Pruvost from Uli Reyer's lab in Zürich for sharing their experience with *Pelophylax* microsatellites; C. Aletti, C. Berney, C. Dufresnes, R. Savary and R. Sermier for their help in the lab; S. Dubey for his help in phylogenetic analyses; C. Betto-Colliard for discussions. We acknowledge financial support from the Association de la Grande Cariçaie, the School of Biology (University of Lausanne) and the Swiss National Science Foundation (Grant 31003A_129894 to NP). The samples were collected with the authorisation of the veterinary and nature offices of the Canton de Vaud (N°2012-24).

References

Arioli M (2007) Reproductive patterns and population genetics in pure hybridogenetic water frog populations of *Rana esculent*a. PhD thesis. (http://www.dissertationen.uzh.ch), University of Zurich

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

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 Natl Sci Phila 140:220–239

Berthoud G, Perret-Gentil C (1976) Les lieux humides et les batraciens du canton de Vaud. Mém Soc vaud Sci Nat 16:1–39

Broquet T, Berset-Braendli L, Emaresi G, Fumagalli L (2007) Buccal swabs allow efficient and reliable microsatellite genotyping in amphibians. Conserv Genet 8:509–511

Christiansen DG (2009) Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). BMC Evol Biol 9:135

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

Earl Dent A, VonHoldt Bridgett M (2012) STRUCTURE HARVESTER: a website and program for visualizing SRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

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

Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-statistics. J Hered 86:485–486

Goudet J (2005) PCAGEN. http://www2.unil.ch/popgen/softwares/pcagen.htm. Accessed 7 Nov 2012

Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221–224

Graf J-D, Polls Pelaz M (1989) Evolutionary genetics of the *Rana esculenta* complex. In: Dawley RM, Bogart JP (eds) Evolution and ecology of unisexual vertebrates. Bulletin 466, New York State Museum, pp 289–302

Guex GD, Hotz H, Semlitsch RD (2002) Deleterious alleles and differential viability in progeny of natural hemiclonal frogs. Evolution 56:1036–1044

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Higgins D, Thompson J, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Holenweg Peter A-K, Reyer H-U, Abt Tietje G (2002) Species and sex ratio differences in mixed populations of hybridogenetic water frogs: the influence of pond features. Ecoscience 9:1–11

Holsbeek G, Jooris R (2009) Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus* complex). Biol Invasions 12:1–13

Hotz H, Mancino G, Bucci-Innocenti S, Ragghianti 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

Hotz H, Uzzell T, Guex G et al (2001) Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. Mitt Mus Nat kd Berl Zool 77:43–50

Huxel GR (1999) Rapid displacement of native species by invasive species: effects of hybridization. Biol Conserv 89:143–152

Kuzmin S, Tarkhnishvili D, Ishchenko, Vladimir Dujsebayeva T, et al (2009) *Pelophylax ridibundus* IUCN red list of threatened species. http://www.iucnredlist.org/. Accessed 6 Jan 2013

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

Luquet E, Vorburger C, Hervant F et al (2011) Invasiveness of an introduced species: the role of hybridization and ecological constraints. Biol Invasions 13:1901–1915

Manchester SJ, Bullock JM (2000) The impacts of non-native species on UK biodiversity and the effectiveness of control. J Appl Ecol 37:845–864

Marchesi P, Fournier J, Rey A (1999) Etat des populations de "grenouilles vertes" *Rana lessonae*, *Rana* kl. *esculenta* du Bois de Finges (Salquenen, VS). Bull Murithienne 117:13–22

Meyer A, Zumbach S, Schmidt B, Monney J-C (2009) Amphibiens et reptiles de Suisse, Haupt Verlag, Bern, 336 pp

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. Proc Natl Acad Sci USA 98:5446–5451

Negovetic S, Anholt BR, Semlitsch RD, Reyer HU (2001) Specific responses of sexual and hybridogenetic European waterfrog tadpoles to temperature. Ecology 82:766–774

Nöllert A, Nöllert C (2003) Guide des amphibiens d'Europe, Delachaux & Niestlé, 383 pp

Nylander J (2004) MrAIC. pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

Petranka JW, Harp EM, Holbrook CT, Hamel JA (2007) Long-term persistence of amphibian populations in a restored wetlands complex. Biol Cons 138:371–380

Plénet S, Hervant F, Joly P (2001) Ecology of the hybridogenetic Rana esculenta complex: differential oxygen requirements of tadpoles. Evol Ecol 14:13–23

Plénet S, Joly P, Hervant F et al (2005) Are hybridogenetic complexes structured by habitat in water frogs? J Evol Biol 18:1575–1586

Plötner J, Uzzell T, Beerli P, Spolsky C, Ohst T, Litvinchuk SN, Guex G-D, Reyer H-U, Hotz H (2008) Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. J Evol Biol 21:668–681

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

R Core Team (2012) R: A Language and Environment for Statistical Computing

Rannap R, Lõhmus A, Briggs L (2009) Restoring ponds for amphibians: a success story. Hydrobiologia 634:87–95

Rhymer M, Simberloff D (1996) Extinction by hybridization and introgression. Ann Rev Ecol Evol Syst 27:83–109

Schmeller DS, Seitz A, Crivelli A, Veith M (2005) Crossing species' range borders: interspecies gene exchange mediated by hybridogenesis. Proc R Soc Biol Sci Ser B 272:1625–1631

Schmeller DS, Pagano A, Plénet S, Veith M (2007) Introducing water frogs—is there a risk for indigenous species in France? C R Biol 330:684–690

Schmidt BR (1993) Are hybridogenetic frogs cyclical parthenogens? Trends Ecol Evol 8:271–273

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 Ra*na esculenta* as deduced from mtDNA Analyses. Mol Biol Evol 3:44–56

Tamura K, Peterson D, Peterson N et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tunner HG, Heppich S (1981) Premeiotic genome exclusion during oogenesis in the common edible frog, *Rana esculenta*. Naturwissenschaften 68:207–208

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

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

Vorburger C (2001b) Non-hybrid offspring from matings between hemiclonal hybrid waterfrogs suggest occasional recombination between clonal genomes. Ecol Lett 4:628–636

Vorburger C, Reyer H (2003) A genetic mechanism of species replacement in European waterfrogs ? Conserv Genet 4:141–155

Vorburger C, Ribi G (1999) Aggression and competition for shelter between a native and an introduced crayfish in Europe. Freshw Biol 42:111–119

Westphal MI, Browne M, MacKinnon K, Noble I (2008) The link between international trade and the global distribution of invasive alien species. Biol Invasions 10:391–398

Wilcove DS, Rothstein D, Dubow J et al (1998) Quantifying threats to imperiled species in the United States. BioSciences 48:607–615

Williamson M, Fitter A (1966) The varying success of invaders. Ecology 77:1661–1666

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*. Mol Ecol 9:1173–1174