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A genetic mechanism of species replacement in European waterfrogs?

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Abstract

Introduced *Rana ridibunda* currently replace the native waterfrogs *R. lessonae* and *R. esculenta* in several areas of central Europe. The unusual reproductive system in waterfrogs of the *Rana esculenta* complex suggests that this replacement may be driven by a genetic mechanism: *Rana esculenta*, a hybrid between *R. ridibunda* and *R. lessonae*, eliminates the *lessonae* genome from the germline and clonally transmits the *ridibunda* genome (hybridogenesis). Hybrids form mixed populations with *R. lessonae* (L-E-system) in which they persist by backcrossing with the parental species. Matings between hybrids are unsuccessful, because their *ridibunda* genomes contain fixed recessive deleterious mutations. When introduced into a L-E-system, *R. ridibunda* can mate with both native taxa, producing *R. ridibunda* offspring with *R. esculenta*, and *R. esculenta* offspring with *R. lessonae* (primary hybridizations). If primary hybrids are hybridogenetic, they produce viable *R. ridibunda* offspring in matings with other hybrids, because their clonal genomes are unlikely to share the deleterious alleles present in the ancient clones. Thus, *R. ridibunda* will increase in the population at the expense of both native taxa, eventually leaving a pure *R. ridibunda* population. We provide three lines of evidence for this process from a currently invaded population in Switzerland: (1) Primary hybridizations take place, as roughly 10% of hybrids in the population possess *ridibunda* genomes derived from the introduced frogs. (2) Hybridogenesis occurs in primary hybrids, although at a low frequency. (3) Many hybrid × hybrid matings in the population indeed produce viable offspring. Hence, the proposed genetic mechanism appears to contribute to the species replacement, although its importance may be limited.

Introduction

The spread of species into non-native habitats has been greatly increased by human transport (Williamson 1996). Most introduced species do not become established, but those that do can have dramatic impacts on the invaded communities, including the decline or even the extinction of native species. Mechanisms by which exotics affect native species include competition, predation, habitat modification, or the transmission of pathogens (e.g. Pimm 1987; Söderbäck 1995; Vorburger and Ribi 1999; Manchester and Bullock 2000). If the native community contains species that are closely related to the invader, hybridization with or without introgression may also genetically threaten the native species' existence (reviewed by Rhymer and Simberloff 1996). Here we investigate the possibility that a genetic mechanism drives the ongoing species replacement of the native waterfrogs *Rana lessonae* and *Rana esculenta* by introduced *Rana ridibunda* in Switzerland, and presumably other areas of central Europe.

Live imports of *R. ridibunda* into Switzerland are common, mainly for consumption of froglegs but also for scientific purposes. Since about the middle of the 20th century, *R. ridibunda* has repeatedly escaped or deliberately been released in several parts of the country. The first documented specimens were captured in western Switzerland in 1950 (Grossenbacher 1988). *Rana ridibunda* became established

and expanded its range ever since. Its distribution in Switzerland, according to the latest survey, is illustrated in Figure 1. In western Switzerland, *R. ridibunda* has largely replaced the native waterfrogs *R. lessonae* and *R. esculenta*, which are now at the edge of local extinction (Grossenbacher 1988; Hofer-Polit 1998; Marchesi et al. 1999). In northern and eastern Switzerland, invaded populations appear to still contain all three taxa (e.g. Holenweg Peter 2001; Holenweg Peter et al. 2002). This situation may be transient and *R. ridibunda* may prevail in the future, but it seems clear that the spread of *R. ridibunda* has occured more rapidly in western Switzerland than in other areas of the country (Grossenbacher 1988).

The factors promoting the ongoing species replacement have not yet been investigated. Ecological factors such as higher competitive ability or higher fecundity of *R. ridibunda* are entirely plausible, but the extraordinary reproductive mode of *R. esculenta* known as hybridogenesis (Schultz 1969) suggests a genetic mechanism may be especially important (Grossenbacher 1988). Hybridogens are hemiclonal interspecific hybrids that eliminate the genome of one parental species from the germline prior to meiosis and clonally transmit the genome of the other parental species. So far, hybridogenesis has been found in unisexual stick insects of the genus *Bacillus* (Mantovani and Scali 1992), teleost fish of the genera *Poeciliopsis* and *Tropidophoxinellus* (Schultz 1969; Carmona et al. 1997), and in bisexual waterfrogs of the genus *Rana* (reviewed by Graf and Polls Pelaz 1989). *Rana esculenta* is a natural hybrid between *R. ridibunda* and *R. lessonae* (Berger 1967, 1968). It excludes the *lessonae* genome from the germline and produces functional haploid gametes only containing the clonally transmitted *ridibunda* genome. Over large parts of central Europe, including Switzerland, *R. esculenta* originally coexisted with only one of its parental species, *R. lessonae*, forming the so called L-E-system (Uzzell and Berger 1975). It is assumed that hybrids have immigrated after the last glaciation from eastern Europe, where *R. ridibunda* and *R. lessonae* are syntopic, and recurrent hybridization is possible. In the L-E-system, *R. esculenta* persists by backcrossing with *R. lessonae*, producing *R. esculenta* offspring that again exclude the *lessonae* genome from their germline (Figure 2). Similar to clonal *monacha* genomes in the hybridogenetic fish *Poeciliopsis monacha-lucida* and *P. monacha-occidentalis* (Leslie and Vrijenhoek 1980), clonal *ridibunda* genomes in *R. esculenta* have accumulated and fixed recessive deleterious mutations due to their long history of clonal inheritance (Berger 1976; Graf and Müller 1979; Vorburger 2001a). As a consequence, *R. ridibunda* offspring from matings between hybrids are generally inviable and die at an early larval stage (Blankenhorn et al. 1971; Heusser and Blankenhorn 1973; Berger and Uzzell 1977; Binkert et al. 1982; Semlitsch and Reyer 1992). This inviability is essential for the persistence of the L-E-system (Som et al. 2000; Hellriegel and Reyer 2000).

Now suppose that a small number of *R. ridibunda* is introduced into a mixed population of *R. lessonae* and *R. esculenta*. Since their breeding seasons overlap, matings between all three taxa are possible and do actually occur (C. Vorburger, personal observation). Matings of *R. ridibunda inter se* and with *R. esculenta* both produce *R. ridibunda* offspring, the latter representing a form of "destabilizing hybridization" – i.e. a disruption of the reproductive isolation between a sexual and a (hemi)clonal taxon – as proposed by Lynch (1984). Matings of *R. ridibunda* with *R. lessonae* (primary hybridzations) produce *R. esculenta* offspring (Figure 2). If introduced *R. ridibunda* are capable of inducing hybridogenesis, newly formed hybrids will exclude the *lessonae* genome from their germlines and clonally transmit the *ridibunda* genomes just as the hybrids already present in the population do. However, the *ridibunda* genomes of newly formed hybrids should not share the deleterious mutations for which the ancient hemiclones are fixed. As a consequence, *R. esculenta* × *R. esculenta* matings will produce viable *R. ridibunda* offspring if at least one parent belongs to a recently formed hemiclone, and this will become more likely as more primary hybridizations have occurred in the population. Thus, the frequency of *R. ridibunda* will increase in the population, because all matings within *R. ridibunda*, all matings between *R. ridibunda* and *R. esculenta*, and an increasing fraction of matings within *R. esculenta* produce *R. ridibunda* offspring, whereas *R. lessonae* will decline, because only matings within *R. lessonae* produce *R. lessonae* offspring (Figure 2). Since their sexual host becomes less common, hybrids will also decline, finally resulting in a pure *R. ridibunda* population. The simple model presented in Figure 3 illustrates the expected dynamics of such a genetically driven species replacement. More sophisticated and biologically realistic models show that in populations containing all three taxa, a pure *R. ridibunda* population is indeed an almost inevi-

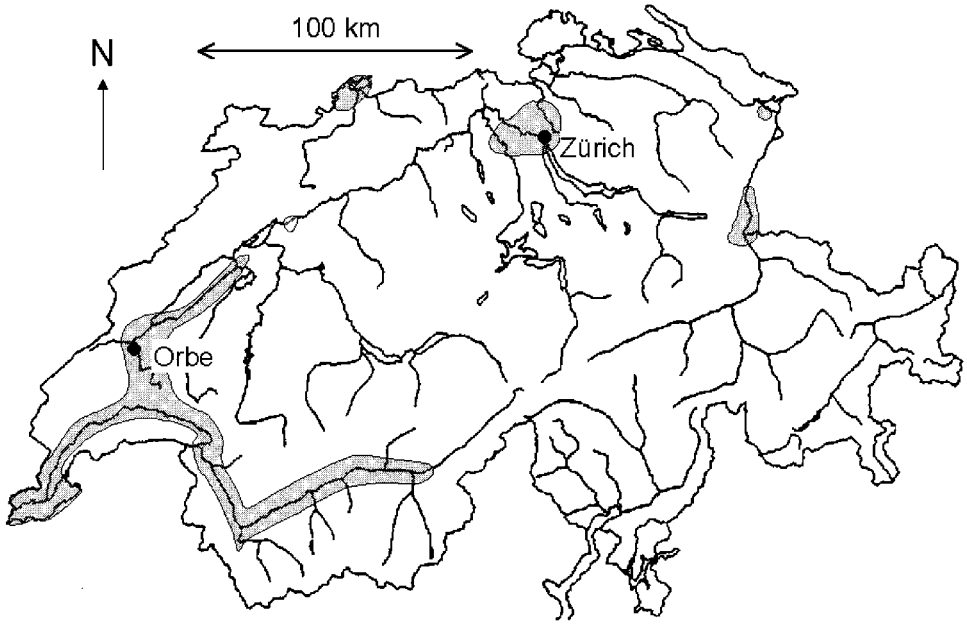

Figure 1. Distribution of the introduced waterfrog *R. ridibunda* in Switzerland (modified from Grossenbacher 1988). Our main study site is located in the vicinity of Zürich, additional *R. ridibunda* for primary hybridizations were collected near Orbe.

		Male		
		Rana lessonae (LL)	*Rana esculenta* (RL)	*Rana ridibunda* (RR)
Female	*Rana lessonae* (LL)	LL (*R. lessonae*)	RL (*R. esculenta*)	RL (*R. esculenta*)
	Rana esculenta (RL)	RL (*R. esculenta*)	RR (*R. ridibunda*)	RR (*R. ridibunda*)
	Rana ridibunda (RR)	RL (*R. esculenta*)	RR (*R. ridibunda*)	RR (*R. ridibunda*)

Figure 2. Possible mating combinations and resulting offspring in a native L-E-system (bold frames) and in one invaded by *R. ridibunda* (all frames). Genotypes of the three taxa are given in capital letters, with R representing a haploid *ridibunda* genome, and L representing a haploid *lessonae* genome. *Rana ridibunda* offspring from *R. esculenta* × *R. esculenta* matings in a native L-E-system are initially inviable, but an increasing fraction of these matings should produce viable offspring as more *R. esculenta* stem from primary hybridizations, i.e. from matings between introduced *R. ridibunda* and native *R. lessonae*.

table outcome unless very strong ecological selection against *R. ridibunda* is assumed (B. Hellriegel, personal communication).

If this mechanism plays a role in L-E-systems that are invaded by *R. ridibunda*, the following predictions should be fulfilled in such populations: First, primary hybridizations should produce hybridogenetic *R. esculenta*. Second, invaded populations should contain more different hemiclones than uninvaded populations, in which no primary hybridizations are possible. Third, at least some *R. esculenta* × *R. esculenta* matings should produce viable *R. ridibunda* offspring in invaded populations containing recently formed hemiclones. We tested these three predictions in a Swiss waterfrog population into which *R. ridibunda* has been released, and which presently contains all three taxa.

Methods

Study site and field sampling

Our main study site in the vicinity of Zürich Airport, Switzerland (Figure 1), comprises eight permanent ponds distributed over an area of slightly more than 1 km^2. Waterfrogs breed in and migrate between all ponds, and can thus be regarded as a single population (Holenweg Peter 2001). Originally, it consisted only of *R. lessonae* and *R. esculenta*, but *R. ridibunda* was introduced into the area about three decades ago (Grossenbacher 1988), and presently makes up about 10% of the population. All taxa may occur in every pond, but the taxon composition differs consistently with pond size and vegetation (Holenweg Peter et al. 2002).

We collected frogs on ten occasions during the 1999 breeding season. Animals needed for the experiments (see below) were taken to the University of Zürich, the rest were released at the capture sites. We took toe clips from all experimental frogs and from a subsample of the remaining individuals for cellulose acetate electrophoresis of enzymes following standard procedures (Hebert and Beaton 1993). A total of 47 *R. lessonae*, 41 *R. ridibunda*, and 171 *R. esculenta* were screened for variation at six loci previously known to be polymorphic in waterfrogs: *sAAT*, *GPI*, *LDH-B*, *MPI*, *PGM-2*, and *PGDH* (locus designations follow Hotz et al. 1997). From the allele frequencies in the population, it was possible to assign all alleles of individual *R. esculenta* to either their *ridibunda* or their *lessonae* genome. We defined a hemiclone as a distinct haploid multilocus genotype of a *ridibunda* genome in *R. esculenta*. The hemiclone composition of the Zürich population was compared with populations still containing native L-E-systems examined by Hotz et al. (unpubl. ms) and Vorburger (2001a, b), where no potential for primary hybridizations exists.

Formation and rearing of primary hybrids

We generated primary hybrids to test whether they are indeed hybridogenetic. This is not self-evident, because *R. ridibunda* varies geographically in the ability to induce hybridogenesis, with *R. ridibunda* from the southern parts of the native range lacking it (Hotz et al. 1985). In May 1999, we used artificial fertilizations following the protocol of Berger et al. (1994) to cross ten female *R. ridibunda* from our study site near Zürich with male *R. lessonae* from the same population, producing ten families of primary hybrids. We also collected ten females from two pure *R. ridibunda* populations in the vicinity of Orbe, about 20 km north of Lausanne, western Switzerland (Figure 1). These females were crossed with the same males to produce another ten families of primary hybrids. We crossed female *R. ridibunda* with male *R. lessonae*, as opposed to female *R. lessonae* with male *R. ridibunda*, because for behavioral reasons, the first mating combination is much more likely to occur in natural populations (Tunner 1974). Frogs from western Switzerland were included in this experiment to test whether introduced *R. ridibunda* there had a higher propensity to induce hybridogenesis than those introduced near Zürich. This would help to explain why this species almost completely replaced the native waterfrogs in western Switzerland, but still occurs at low frequencies in the area of Zürich.

Thirty tadpoles of all twenty families of primary hybrids were raised in separate outdoor tanks. We marked the metamorphs by clipping one hind toe and one front toe in 20 different combinations that allowed later identification of families. To reduce the risk of losing all animals due to either unsuitable rearing conditions or the possible outbreak of diseases, we raised them in two different environments. Ten metamorphs per family were transferred to indoor terraria (one family per terrarium), and maintained on a diet of crickets and mealworms *ad libitum* at a temperature of 20–25 °C. The remaining metamorphs were released into two 10 m^2 outdoor cages containing natural vegetation and a small pond. The

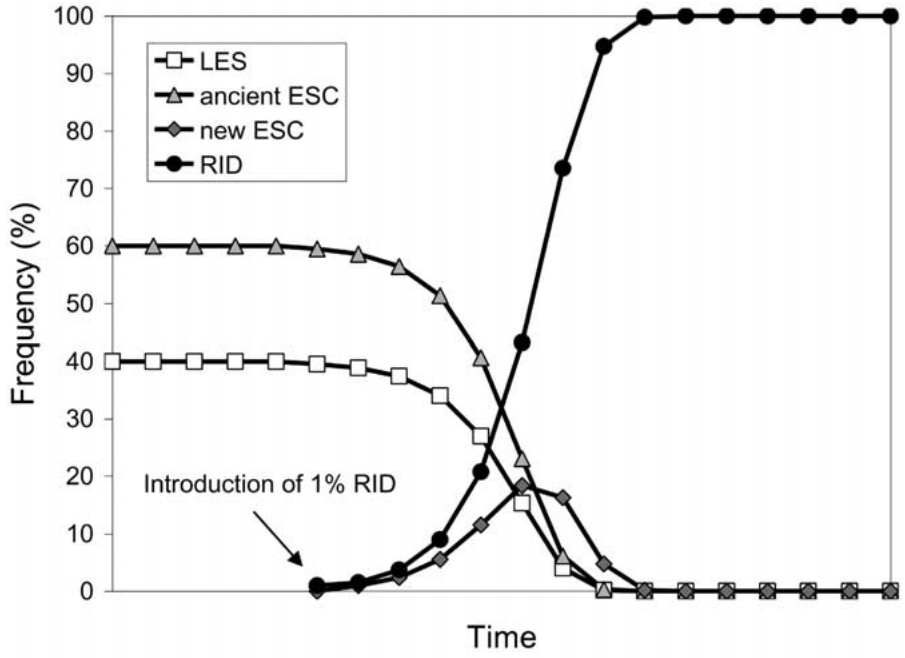

Figure 3. Results from a simple discrete-generation model, illustrating the genetically driven species replacement as described in the text. *Rana ridibunda* is introduced into an L-E-system containing 40% *R. lessonae* and 60% *R. esculenta*. For simplicity, the model makes several assumptions that are unlikely to be fulfilled in natural populations, i.e. constant population size, equal fecundity of all females and a 1:1 sex ratio in all three taxa. Survival is also assumed to be equal across taxa, only *R. ridibunda* produced by matings between two *R. esculenta* belonging to ancient hemiclones are assumed to be inviable, as it is generally observed in the field. Mating is random except for matings within the pure species *R. lessonae* and *R. ridibunda* which occur twice as frequently as under the random expectation. Without preferential mating within *R. lessonae*, stable coexistence between *R. lessonae* and *R. esculenta* before the introduction of *R. ridibunda* would not be possible. LES denotes *R. lessonae*, RID denotes *R. ridibunda*, and ESC denotes the hybrid *R. esculenta*. Hybrids can either belong to ancient hemiclones (ancient ESC) or to new hemiclones (new ESC) that were founded through primary hybridizations after the introduction of *R. ridibunda*.

frogs experienced natural temperature fluctuations and fed on naturally occurring invertebrates and a limited amount of crickets and mealworms that we added twice per week. All frogs from the indoor terraria and approximately two thirds of the frogs from the outdoor cages were overwintered in a cool room at 5 °C. The remaining frogs were left in the outdoor cages, again to split the risk of winter mortality that might occur under either condition. However, overwinter survival was almost complete under both conditions.

By the end of May 2000, the frogs from the indoor rearing had reached a mean (± SD) weight of 13.78 ± 3.27 g and a snout-vent length (SVL) of 51.10 ± 4.65 mm, and sexes could easily be distinguished by the vocal sacs and thumb pads of males. The frogs from the outdoor rearing had only achieved a mean weight of 2.33 ± 1.28 g before the first hibernation (SVL not measured) and could not be sexed by May 2000, but most of them reached sexual maturity by the beginning of the 2001 breeding season.

Reproductive mode of primary hybrids

Females. In hybridogenetic female *R. esculenta*, the exclusion of the *lessonae* genome can be detected electrophoretically in enlarged primary oocytes, because only the *ridibunda* alleles of somatically heterozygous loci are expressed in oocytes (Uzzell et al. 1980; Tunner and Heppich 1981; Hotz et al. 1985). In 2000, we sacrificed a total of 90 female primary hybrids from the indoor rearing and removed their ovaries. If possible, we prepared 20 enlarged primary oocytes of average size from each female for electrophoresis, but because ovarian development in hybrids is somewhat delayed (Wagner and Ogielska 1993), we also found some females containing fewer or no enlarged primary oocytes. We determined the electrophoretic phenotype of the somatic tissue and each individual oocyte for *LDH-B*, which exhibits fixed allelic differences between the parental species. Depending on which loci the females were additionally heterozygous for, we also determined the electrophoretic

phenotypes for one or more of the following loci: *GPI*, *MPI*, and *PGM-2*.

In 2001, we also tried to cross female primary hybrids and injected 15 individuals from the outdoor rearing with the hormone LH-RH (H-7525, Bachem, Inc.) to induce ovulation. Six females belonging to four different families ovulated, and we fertilized their eggs with sperm from field-caught *R. lessonae* (Berger et al. 1994). The resulting tadpoles were reared for two weeks after hatching and then 10 individuals per cross were sacrificed for allozyme electrophoresis. A female was considered to be hybridogenetic if all tadpoles inherited the *ridibunda* alleles of those loci for which it was somatically heterozygous.

Males. The reproductive mode of male primary hybrids was determined by crossing them with *R. lessonae* and genotyping the resulting offspring. We tested 34 males in 2000 (all reared indoors) and 36 males in 2001 (11 reared indoors, 25 reared in outdoor cages). These 70 males comprised 18 of the 20 families of primary hybrids. The basic procedure was as follows: Between 6 and 18 male primary hybrids and one field-caught male *R. lessonae* at a time were killed with 3-aminobenzoic acid ethyl ester (MS-222; A-5040, Sigma, Inc.) to remove their testes. We then prepared sperm suspensions by crushing both testes of each male in a petri dish containing 3 ml of aged tap water. One drop of the suspensions was immediately examined under a light microscope to roughly categorize sperm suspension quality into 0 (no mobile sperm visible), 1 (sporadic mobile sperm), 2 (low density of mobile sperm), or 3 (high density of mobile sperm). Then sperm suspensions were diluted to 15–20 ml and the eggs of a single *R. lessonae* were equally distributed into the sperm suspensions. Fertilization rate was estimated as the number of developing embryos divided by the total number of eggs in each petri dish. Counts were made during early cleavage when developing embryos can easily be distinguished from undeveloped eggs under a dissection microscope. Two weeks after hatching, a subsample of 10 tadpoles (or all, if there were less) per cross were sacrificed for allozyme electrophoresis. Again, a male was considered to be hybridogenetic if it transmitted the *ridibunda* alleles of heterozygous loci to all offspring. Only crosses with at least five survivors were considered in this analysis.

Viability of offspring from R. esculenta × R. esculenta *matings*

To test whether *R. esculenta* × *R. esculenta* matings can produce viable *R. ridibunda* offspring in the Zürich population, we assessed tadpole viability from as many such matings as possible. Field-caught *R. esculenta* were haphazardly paired, injected with LH-RH, and allowed to mate in outdoor tanks. Injection with LH-RH induces ovulation in females and increases sexual activity in males (Berger et al. 1994). If a pair did not produce a clutch within 5 d, we released the females, but sometimes reused the males for a second try with another female. When tadpoles were free swimming and had resorbed their yolk sacs (stage 25; Gosner 1960), we haphazardly selected 20 tadpoles from every *R. esculenta* pair and raised them in outdoor tanks. Tanks contained 80 l of tap water and were inocculated with 2.5 g of rabbit chow and 2 l of concentrated phyto- and zooplankton to start primary production. At regular intervals, *Spirulina* tablets were added as supplementary food in order to provide favourable growth conditions. Metamorphs were removed from the tanks, euthanized and stored at −80 °C for later electrophoretic analysis of the *LDH-B* locus which allowed us to examine whether the metamorphs showed a *R. ridibunda* genotype, i.e. whether their *R. esculenta* parents were hybridogenetic.

Results

Allozyme analysis

Allele frequencies are summarized for each taxon in Table 1. In *R. lessonae* and *R. ridibunda*, observed heterozygosity did not significantly deviate from that expected under Hardy-Weinberg equilibrium for any of the variable loci (χ^2-test, all $P > 0.4$). Not surprisingly, the hybrid *R. esculenta* exhibited a highly significant heterozygote excess at five loci (all $P < 0.0001$). We found no excess heterozygosity for *MPI* because more than 95% of all hybrids were homozygous for allele *c* ($P = 0.8$; Table 1). Among the 171 *R. esculenta* examined electrophoretically, we could distinguish 10 different *ridibunda* haplotypes or hemiclones (Table 2), which is more than reported for any native L-E-system examined with allozyme markers so far. Hemiclone ZH 1 was by far the most common and made up more than 90% of all *R. esculenta*. The remaining nine hemiclones were only

Table 1. Allele frequencies at six variable allozyme loci in *R. lessonae*, *R. ridibunda*, and the hybrid *R. esculenta* from our study site near Zürich. Allele designations follow the system of Hotz (1983) and Beerli (1994).

Locus	Allele	*R. lessonae* (n = 47)	*R. ridibunda* (n = 41)	*R. esculenta* (n = 171)
sAAT	*e*		0.8537	0.5000
	f		0.1463	
	g	1.0000		0.5000
GPI	*a*		0.2073	0.4649
	c			0.0175
	d	0.9894	0.7927	0.5175
	f	0.0106		
LDH-B	*a*		0.4268	0.0058
	b	0.1702		0.1345
	c		0.5732	0.4942
	e	0.8298		0.3655
MPI	*a*		0.5122	0.0205
	c	1.0000	0.4878	0.9795
PGM-2	*c*	1.0000	0.5244	0.5205
	d		0.4756	0.4795
PGDH	*c*	1.0000		0.5000
	d		0.3902	0.4854
	e		0.6098	0.0146

Table 2. Hemiclones of the study population near Zürich, defined by the allozyme haplotype of the *ridibunda* genome in the hybrid *R. esculenta*. n = number of individuals.

		Locus					
Hemiclone	n	*sAAT*	*GPI*	*LDH-B*	*MPI*	*PGM-2*	*PGDH*
ZH 1	157	*e*	*a*	*c*	*c*	*d*	*d*
ZH 2	3	*e*	*d*	*c*	*c*	*d*	*d*
ZH 3	2	*e*	*d*	*c*	*a*	*c*	*d*
ZH 4	2	*e*	*d*	*c*	*a*	*d*	*e*
ZH 5	2	*e*	*a*	*c*	*c*	*c*	*d*
ZH 6	1	*e*	*d*	*c*	*c*	*d*	*e*
ZH 7	1	*e*	*d*	*a*	*c*	*c*	*e*
ZH 8	1	*e*	*d*	*a*	*a*	*c*	*d*
ZH 9	1	*e*	*d*	*c*	*a*	*d*	*d*
ZH 10	1	*e*	*d*	*c*	*a*	*c*	*e*

represented by one to three individuals in our sample (Table 2).

The rare hemiclones appear to have originated by hybridizations between *R. ridibunda* and *R. lessonae*, because with one exception, their allozyme haplotypes represent combinations of alleles that are common among the sexual *R. ridibunda* in Zürich and therefore have a high probability of being formed by primary hybridizations within the population (Figure 4). Only hemiclone ZH 5 has a relatively low probability of being formed by primary hybridization, but this probability is still higher than that for hemiclone ZH 1 (Figure 4). The haplotype defining the most common hemiclone in our study population is only expected to show up in approximately one out of 100 *R. esculenta* produced by primary hybridzations (Figure 4), but it is identical to the most common haplotype found in all native L-E-systems that Vorburger (2001a, b) examined north of the Alps.

Reproductive mode of primary hybrids

Females. The electrophoretic analysis of enlarged primary oocytes revealed that 66 out of the 78 hybrid females that contained enlarged oocytes did not exclude the *lessonae* genome from their germline; all their oocytes expressed both alleles of somatically heterozygous loci. In 12 females we found evidence for the germline exclusion of the *lessonae* genome, i.e. oocytes that exclusively expressed the *ridibunda* alleles (Appendix 1). Surprisingly, gametogenesis was not uniform within families: all females in which genome exclusion occurred also had sisters exhibiting normal oogenesis (e.g. family 4). Moreover, we found seven females containing a mixture of oocytes, some expressing both alleles and some expressing only the *ridibunda* alleles of somatically heterozygous loci (e.g. family 4, individual 5; Appendix 1).

Among the 12 females showing evidence of genome exclusion, the incidence of hybridogenetic oogenesis was slightly higher in primary hybrids produced by *R. ridibunda* mothers from western Switzerland (9 out of 41 females) than in those produced by mothers from Zürich (3 out of 37 females). However, this difference was not statistically significant when either comparing the relative frequencies of individuals ($\chi^2 = 2.863$, df = 1, $P = 0.091$), or the relative frequencies of families in which genome exclusion occurred ($\chi^2 = 2.773$, df = 1, $P = 0.096$).

Of the six female primary hybrids that we managed to cross in 2001, none were hybridogenetic (Table 3A). All transmitted *ridibunda* and *lessonae* alleles to their offspring.

Males. Most male primary hybrids appeared to be sterile. In more than half of all suspensions, no mobile sperm could be detected under the light microscope. Accordingly, only 12 out of 70 attempted

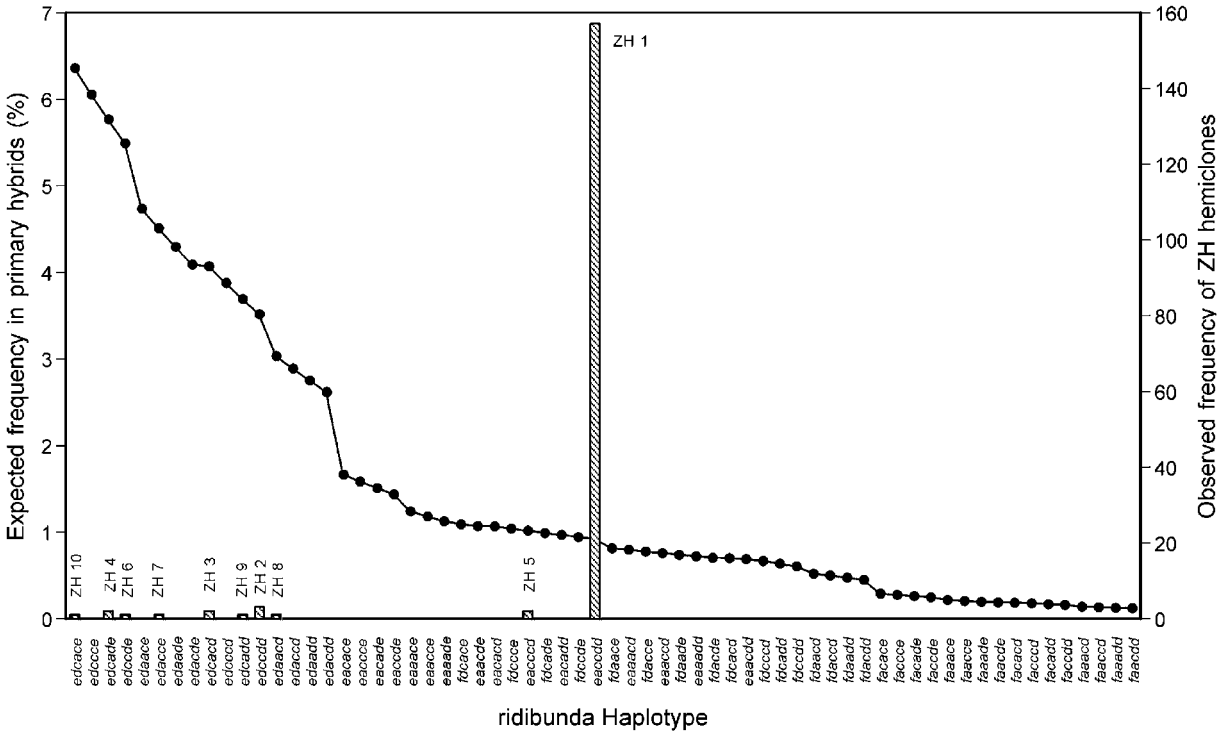

Figure 4. Observed frequencies of the ten hemiclones found in the Zürich population (bars), and expected relative frequencies of all distinguishable *ridibunda* haplotypes in primary hybrids formed within the population (line). Haplotypes are denoted by a six-letter code representing the alleles at the following loci (in this order): *sAAT*, *GPI*, *LDH-B*, *MPI*, *PGM-2*, and *PGDH*. The expected haplotype frequencies in primary hybrids were calculated from the allele frequencies in *R. ridibunda* under the reasonable assumption that the likelihood of an individual *R. ridibunda* to mate with *R. lessonae* is independent of its allozyme genotype.

crosses produced fertilized eggs, but even in those cases fertilization rate was typically low (Figure 5). Fertilization rate was positively correlated with the scores for sperm suspension quality (Spearman's ρ = 0.657, $p < 0.001$), but these scores were only poor predictors of the achieved fertilization rates (Figure 5). In contrast, sperm suspensions of *R. lessonae* used as a control contained high densities of mobile sperm (score 3, with one exception obtaining score 2), and they achieved a mean fertilization rate ($\pm$ SD) of 0.921 $\pm$ 0.151 (n = 7). This indicates that the eggs were capable of being fertilized and thus that the low fertilization success of primary hybrids indeed reflects the poor quality of their sperm.

Of the 70 male primary hybrids used for crosses, 34 had *R. ridibunda* mothers from the Zürich population and 36 from western Switzerland. All of the 12 males achieving at least some fertilization success belonged to the latter group (χ^2 = 13.678, df = 1, $P <$ 0.001), but only eight of them produced a sufficient number of tadpoles to determine their reproductive

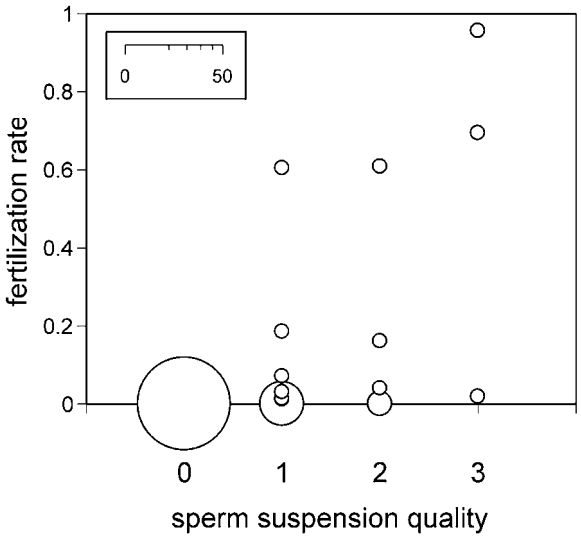

Figure 5. Bubble chart illustrating the relationship between the proportion of eggs fertilized (fertilization rate) and the sperm suspension quality scores for artificial crosses between *R. lessonae* females and primary hybrid males.

Table 3. Mode of gametogenesis for six female (A) and eight male (B) primary hybrids that produced a sufficient number of tadpoles for analysis in crosses with *R. lessonae*. Hybrids were considered to be hybridogenetic if they transmitted their *ridibunda* alleles at heterozygous loci to all offspring.

Hybrid family	Origin of *R. ridibunda* mother	Individual #	Number of tadpoles analyzed	Mode of gametogenesis
A: Female primary hybrids				
1	Zürich	5	10	Recombinant
2	Zürich	5	10	Recombinant
		6	10	Recombinant
9	Zürich	3	10	Recombinant
		4	10	Recombinant
12	Orbe	7	10	Recombinant
B: Male primary hybrids				
12	Orbe	3	10	Hybridogenetic
		4	10	Hybridogenetic
		5	10	Hybridogenetic
		6	10	Hybridogenetic
14	Orbe	7	10	Recombinant
		8	10	Recombinant
18	Orbe	2	10	Hybridogenetic
19	Orbe	4	7	Hybridogenetic

mode. Six males were hybridogenetic and two had recombination in their germlines (Table 3B). Interestingly, four of the six hybridogenetic males belonged to the same family, no. 12. The two non-hybridogenetic males also belonged to the same family, no. 14 (Table 3B).

Viability of tadpoles from R. esculenta × R. esculenta *matings*

From a total of 76 *R. esculenta* pairs that were allowed to mate in tanks between 17 May and 11 June 1999, we obtained 37 families of tadpoles for outdoor rearing; 25 females did not lay any eggs, and 14 clutches were unfertilized. The 76 pairs we started with comprised individuals from eight of the 10 hemiclones, but both parents of all the fertilized clutches belonged to the most common hemiclone ZH 1. Appendix 2 summarizes the fate of these crosses. Tadpoles from 14 fertilized clutches were fully inviable and all died at an early larval stage. For most of these families, morphological abnormalities were already evident at the hatchling stage, which is a common observation for tadpoles from *R. esculenta* × *R. esculenta* matings (Ogielska 1994). The remaining 23 crosses produced viable tadpoles. However, survival was less than 50% in eight families, and in five families development was so slow that some tadpoles, although they survived, had not reached metamorphosis until 7 October, when the experiment was taken down (Appendix 2). Moreover, a number of tadpoles that reached metamorphosis suffered from limb deformities. The majority of these tadpoles died during tail resorption, which explains the substantial mortality that occurred between developmental stages 42 and 46 in a number of crosses (Appendix 2). The limb deformities appeared to be environmentally determined, because tadpoles from eight of the surviving families were raised in the laboratory for a different purpose and did not develop any deformities (C. Vorburger, personal observation). We did not investigate what caused these deformities in the tadpoles raised outdoors, but in amphibians, parasitic trematodes are often responsible for limb malformations (e.g. Johnson et al. 1999; Johnson et al. 2001). Nevertheless, a total of 20 out of 37 *R. esculenta* × *R. esculenta* crosses produced viable froglets by the end of the experiment.

Offspring from all but one viable cross were homozygous for the *ridibunda* allele *c* of the *LDH-B* locus, indicating that their *R. esculenta* parents are hybridogenetic and only transmit the *ridibunda* genome with their gametes. However, *bc* heterozygotes showed up in a single family (cross Gr 76 × Gr 68, see Appendix 2). Since the *b* allele only occurs in *R. lessonae*, this finding suggests that recombination occurred in one of the parents. Therefore, we genotyped the offspring of this family at all the six loci we used to determine the hemiclone of their parents (Table 4). This analysis revealed that most offspring had recombinant genotypes and that it was the female (Gr 76) transmitting the *lessonae* alleles, because the male (Gr 68) possessed a different *lessonae* allele for *LDH-B*, which did not show up in the offspring (Table 4).

Discussion

We proposed a genetic mechanism that may at least in part be responsible for the rapid replacement of native waterfrogs by introduced *R. ridibunda* in Switzerland. Assuming that this mechanism indeed plays a role, we formulated three predictions that should be fulfilled in populations that are currently invaded by *R. ridibunda*. In our study population, we found some evidence

Table 4. Genotypes at six allozyme loci for parents and 13 offspring of the the *R. esculenta* × *R. esculenta* cross between female Gr 76 and male Gr 68, providing evidence for recombination in the germline of the female. Bold letters indicate the *lessonae* alleles at loci for which the parents are heterozygous.

Individual	Locus					
	sAAT	*GPI*	*LDH-B*	*MPI*	*PGM-2*	*PGDH*
Parents						
Mother (Gr 76)	*e**g***	*a**d***	***b**c*	*cc*	***c**d*	***c**d*
Father (Gr 68)	*e**g***	*a**d***	*c**e***	*cc*	***c**d*	***c**d*
Offspring						
Offspring 1	*e**g***	*a**d***	***b**c*	*cc*	***c**d*	***c**d*
Offspring 2	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 3	*e**g***	*a**d***	*cc*	*cc*	*dd*	***c**d*
Offspring 4	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 5	*e**g***	*a**d***	***b**c*	*cc*	*dd*	*dd*
Offspring 6	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 7	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 8	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 9	*e**g***	*aa*	***b**c*	*cc*	*dd*	***c**d*
Offspring 10	*ee*	*aa*	*cc*	*cc*	*dd*	*dd*
Offspring 11	*ee*	*aa*	*cc*	*cc*	*dd*	***c**d*
Offspring 12	*ee*	*a**d***	*cc*	*cc*	***c**d*	***c**d*
Offspring 13	*ee*	*a**d***	*cc*	*cc*	*dd*	*dd*

for all of these predictions: primary hybridizations can produce hybridogenetic offspring, the number of different hemiclones is large compared to native L-E-systems, and some *R. esculenta* × *R. esculenta* matings produce viable *R. ridibunda* offspring. However, the results were not always straightforward. We discuss these three findings in turn.

Reproductive mode of primary hybrids

Introduced *R. ridibunda* can found new hemiclones by producing hybridogenetic *R. esculenta* offspring upon primary hybridizations with *R. lessonae*. Primary hybrids are highly viable and have even been reported to benefit from spontaneous heterosis (Hotz et al. 1999), but based on our analysis of primary oocytes from a large number of females, hybridogenetic gametogenesis is the exception rather than the rule in primary hybrids. These results differ markedly from the other system in which the laboratory synthesis of hybridogens could be achieved: Primary hybridizations between females of *Poeciliopsis monacha* and males of *P. lucida* or *P. occidentalis* had a rather low success rate, but all surviving hybrids were spontaneously hybridogenetic (Schultz 1973; Wetherington et al. 1987).

Bucci et al. (1990) and Berger et al. (1994) have suggested that non-hybridogenetic *R. esculenta* are nearly or completely sterile. At least for males, our data support this claim. For females, the data are too scarce for a safe statement, but available evidence indicates that non-hybridogenetic *R. esculenta* may well be fertile: most female primary hybrids contained enlarged primary oocytes, and the six females spawning in artificial crosses plus one fertile female from the field were found to be non-hybridogenetic.

Based on the finding that the ability to induce the germline exclusion of *lessonae* genomes varies geographically within the native range of *R. ridibunda* (Hotz et al. 1985), we expected that either all or none of the offspring from a given primary hybridization would exhibit genome exclusion. However, the reproductive mode of primary hybrids was not consistent within families: offspring from the same hybridization could either be hybridogenetic or not. We even found individual females containing a mixture of oocytes that either did or did not express the *lessonae* alleles of somatically heterozygous loci. We do not know why the germlines of these females contain different cell lineages, some that have undergone exclusion of the *lessonae* genome, and some that possess both parental genomes. It is likely that *R. ridibunda* were introduced into Switzerland from many different areas, and therefore consisted of animals with and without the ability to induce hybridogenesis. It may be that subsequent interbreeding between these forms has broken up the putative genetic factors responsible for the inducing ability, but this is a speculative explanation as long as the molecular basis for the germline exclusion of the *lessonae* genome in hybridogenetic *R. esculenta* is unknown.

We suspected that the more rapid replacement of native waterfrogs observed in western Switzerland compared to other invaded areas like the Zürich area may be due to a higher propensity to induce hybridogenesis in *R. ridibunda* found in the west. Based on the analysis of oocytes, there was a tendency in the expected direction, but this difference was not statistically significant. Independent of the mother's origin, only a small fraction of female primary hybrids excluded the *lessonae* genome from the germline. However, male primary hybrids were significantly more likely to be at least partially fertile when their *R. ridibunda* mothers came from western

Switzerland. In fact, it was only from these males that we obtained fertilized eggs in artificial crosses. Together, these findings suggest a more important role for primary hybridizations in the invasion of *R. ridibunda* in western Switzerland than around Zürich, but it still seems that additional, probably ecological, factors need to be invoked to explain the more rapid replacement in western Switzerland.

Hemiclonal diversity in Zürich

Ten different hemiclones as detected in our study area are more than the number reported for any native L-E-system examined so far. Using the same six loci for frogs from other areas, Vorburger (2001a, b) found five populations containing 1–5 hemiclones. From a larger survey of 12 populations with comparable haplotype resolution and sample sizes, Hotz et al. (unpublished ms) report 12 populations with 1–6 hemiclones. Also, the frequency distribution of hemiclones in the invaded Zürich population is much more skewed than that of native L-E-systems. Importantly, the only common hemiclone (making up more than 90% of the hybrid population) seems to be identical to the dominant hemiclone throughout northern Switzerland, and all but one of the rare hemiclones have a high probability of being formed by primary hybridization within the population. This suggests that there was only a single hemiclone (ZH 1) present in the population before the introduction of *R. ridibunda* and that now, roughly three decades after the introduction, close to 10% of all hybrids possess *ridibunda* genomes that are derived from the introduced animals. In fact, it is unclear whether it is justified to call these rare *ridibunda* haplotypes hemiclones because unfortunately, none of the individuals belonging to a rare haplotype was involved in one of those *R. esculenta* × *R. esculenta* matings that produced fertilized eggs. Hence, we do not know whether they would reproduce by hybridogenesis, i.e. whether their *ridibunda* genomes belong to ongoing clonal lineages. Quite likely, some of these *R. esculenta* are primary hybrids and thus have a low probability of being hybridogenetic (see above). But even if only a small fraction of these *R. esculenta* are hybridogenetic, a considerable number of new clonal lineages could have been formed in this large population and produce viable *R. ridibunda* offspring in matings with other hybrids.

Viability of R. esculenta × R. esculenta *crosses*

The most puzzling result of this study comes from the *R. esculenta* × *R. esculenta* matings. We predicted that if newly formed hemiclones occur in the population, at least a fraction of these matings should produce viable *R. ridibunda* offspring. Indeed, roughly half of all fertilized clutches produced viable metamorphs. However, their parents all belonged to the single and presumably ancient hemiclone ZH 1, and such crosses are expected to be inviable (Vorburger 2001b).

Due to the limited resolution of our markers, we cannot exclude that a few of these parents actually belonged to a recently formed hemiclone with the same allozymic haplotype as ZH 1. However, this cannot be the whole explanation because this haplotype has a rather low probability of arising from primary hybridization within the Zürich population (Figure 4). An additional possibility is that what we defined as hemiclone ZH 1 comprises more than one ancient clonal lineage that happen to share the same allozymic haplotype, but do not share the same deleterious mutations. If this was the case, it is not justified to conclude that the observed viability of the *R. esculenta* × *R. esculenta* crosses in the Zürich population is a consequence of the invasion by *R. ridibunda*.

Analyzing the offspring genotypes of our crosses, we detected that one female *R. esculenta* from the field was not hybridogenetic and produced recombinant gametes. This female may either be a non-hybridogenetic primary hybrid or else belong to hemiclone ZH 1 and have undergone a breakdown of hybridogenesis. Such breakdown can arise from a variety of genetic mechanisms (Lynch 1984) and has been suggested to turn previously (hemi)clonal organisms into "hopeful monsters" which can act as a source of genetic variation that may even lead to the formation of new species (Schultz 1969; Vrijenhoek 1989). Whatever the mechanistic explanation, the occurrence of fertile, non-hybridogenetic *R. esculenta* females in the field and in experimentally produced primary hybrids suggests that "genetic swamping" by introgression (Rhymer and Simberloff 1996) should not be neglected as a possible factor in the ongoing replacement of native waterfrogs by *R. ridibunda.* Presently however, introgression does not play an important role in our study population: all individuals could clearly be classified into the three distinct taxa and no evidence for introgression was found in our samples.

Conclusions

Our study provides evidence that hybridizations between introduced *R. ridibunda* and native *R. lessonae* occur in the field, and that primary hybrids can be hybridogenetic, i.e. they can form new hemiclones. Hence, the genetic mechanism of species replacement we proposed is likely to contribute to the rapid invasion of *R. ridibunda* in Switzerland. This finding highlights the fact that species translocations into areas with closely related native species can be particularly harmful due to the potential for hybridization (Rhymer and Simberloff 1996). However, the strongly reduced fertility of male primary hybrids and the low incidence of genome exclusion in females suggest that this mechanism may not be as important as we previously suspected. Ecological advantages of *R. ridibunda* over the native waterfrogs are likely to be equally or more important and should now be thoroughly investigated. For the conservation of *R. lessonae* and *R. esculenta*, this conclusion is reassuring. While the genetically driven species replacement we proposed is essentially inevitable, something can be done about ecological factors promoting the invasion. Holenweg Peter et al. (2002) showed that *R. ridibunda* and *R. lessonae* have distinct habitat requirements. A first step would thus be to provide more of the habitat favouring *R. lessonae*, i.e. more small and highly structured breeding ponds with abundant submerged vegetation (Holenweg Peter et al. 2002). This would not only provide possible refuge areas for the native waterfrogs, it would also benefit other endangered amphibian species.

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Appendix 1. Electrophoretic phenotype at *LDH-B* for soma and individual enlarged primary oocytes of 78 *R. esculenta* females from totally 19 families of primary hybrids, produced by crossings between female *R. ridibunda* either from Zürich (A) or from Orbe in western Switzerland (B), and male *R. lessonae* from Zürich. Only *LDH-B* is reported, because results for other loci (*GPI*, *MPI*, and *PGM-2*) were consistent with *LDH-B*, i.e. genome exclusion appeared to affect the entire *lessonae* genome. From cross 7 (Kr 14 × les 3), no females large enough for analysis were availabe. SVL, snout-vent length of female hybrids; *n*, number of oocytes examined. Hyphens indicate missing data.

Family	Mother	Father	Ind. #	SVL (mm)	*n*	Electrophoretic phenotype (*LDH-B*) Soma	Oocytes
A. *Rana ridibunda* from Zürich study site							
1	Kr 13	les 5	1	65	20	*ae*	*ae*
			2	54	20	*ae*	*ae*
			3	56	20	*ae*	*ae*
			4	42	14	*ae*	*ae*
2	Kr 16	les 4	1	55	19	*ce*	*c* (18)
							ce (1)
			2	56	20	*ae*	*ae*
			3	54	19	*ae*	*ae*
			4	47	3	*ae*	*ae*
3	Kr 17	les 10	1	43	19	*ae*	*ae*
			2	37	20	*ce*	*ce*
			3	34	3	*ab*	*ab*
			4	55	9	*ab*	*ab*
			5	52	20	*ce*	*ce*
4	Kr 32	les 7	1	52	20	*ce*	*ce*
			2	58	10	*ce*	*ce*
			3	55	20	*ce*	*c*
			4	55	18	*ce*	*ce*
			5	49	14	*ce*	*c* (5)
							ce (9)
			6	49	20	*ce*	*ce*
			7	48	8	*ce*	*ce*
			8	58	20	*ce*	*ce*
5	Kr 31	les 6	1	50	10	*ae*	*ae*
			2	58	20	*ae*	*ae*
			3	58	20	*ae*	*ae*
6	Kr 34	les 9	1	51	20	*bc*	*bc*
			2	63	19	*bc*	*bc*
			3	59	20	*bc*	*bc*
8	Kr 33	les 8	1	53	20	*ce*	*ce*
			2	58	20	*ce*	*ce*
			3	53	20	*ce*	*ce*
			4	53	7	*ce*	*ce*
			5	50	19	*ce*	*ce*
9	Kr 12	les 2	1	50	20	*ce*	*ce*
			2	63	12	*ce*	*ce*
10	Kr 10	les 1	1	48	1	*ae*	*ae*
			2	–	20	*ae*	*ae*
			3	51	4	*ce*	*ce*

Appendix 1. Continued.

Family	Mother	Father	Ind. #	SVL (mm)	*n*	Electrophoretic phenotype (*LDH-B*) Soma	Oocytes
B. *Rana ridibunda* from western Switzerland							
11	Pe 14	les 8	1	50	7	*ae*	*ae*
			2	56	18	*ae*	*a* (1)
							ae (17)
			3	57	10	*ce*	*ce*
			4	50	3	*ae*	*ae*
			5	48	15	*ae*	*ae*
			6	46	20	*ae*	*ae*
			7	51	1	*ce*	*ce*
			8	46	15	*ae*	*a*
12	Pe 12	les 6	1	54	20	*ae*	*ae*
			2	50	19	*ce*	*c* (18)
							ce (1)
13	Ba 57	les 6	1	43	19	*ce*	*ce*
			2	55	20	*ce*	*ce*
			3	52	20	*ce*	*c* (5)
							ce (15)
14	Ba 37	les 4	1	54	20	*ce*	*ce*
			2	59	19	*ce*	*ce*
			3	60	4	*ce*	*c*
			4	53	2	*ce*	*ce*
			5	49	10	*ce*	*ce*
			6	53	20	*ce*	*ce*
15	Pe 11	les 7	1	51	18	*ae*	*ae*
			2	61	2	*ae*	*ae*
			3	59	3	*ae*	*ae*
			4	59	20	*ae*	*ae*
			5	46	2	*ce*	*ce*
			6	58	20	*ae*	*ae*
			7	53	10	*ae*	*ae*
			8	51	2	*ce*	*ce*
16	Pe 15	les 8	1	49	2	*ae*	*ae*
			2	53	16	*ce*	*c* (15)
							ce (1)
			3	54	12	*ae*	*a* (2)
							ae (10)
			4	49	6	*ce*	*ce*
			5	45	17	*ae*	*a*
17	Ba 30	les 1	1	57	10	*ae*	*ae*
			2	52	8	*ae*	*ae*
			3	53	3	*ae*	*ae*
18	Pe 13	les 7	1	46	20	*ce*	*c*
19	Ba 32	les 2	1	51	12	*ae*	*ae*
			2	57	20	*ae*	*ae*
			3	52	2	*ce*	*ce*
20	Ba 36	les 3	1	48	16	*ce*	*ce*
			2	57	16	*ce*	*ce*

Appendix 2. Summary of results for offspring from all *R. esculenta* × *R. esculenta* matings that produced fertilized clutches. Twenty tadpole per cross were raised in outdoor tanks, the table lists the number of tadpoles surviving to stage 42 (forelimb emergence) and stage 46 (completion of tail resorption), the number of surviving tadpoles that did not complete metamorphosis before the end of the experiment on 7 October 1999, and the *LDH-B* genotype of surviving offspring. Parents are labeled with a two-letter code for their pond of origin and a number identifying the individual. All parents belonged to hemiclone ZH 1.

Spawning date	Cross (female × male)	# Survivors to stage 42	# Survivors to stage 46	# Survivors not metamorphosed	Offspring genotype for *LDH-B*
17.05.99	Gr 6 × Gr 3	0	0	0	.
18.05.99	Gr 4 × Gr 1	0	0	0	.
19.05.99	Gr 9 × Gr 10	0	0	0	.
20.05.99	Gr 7 × Gr 12	17	17	0	cc
20.05.99	Gr 73 × Gr 69	19	19	0	cc
20.05.99	Gr 84 × Gr 85	19	12	0	cc
21.05.99	Gr 76 × Gr 68	19	13	0	cc (10), bc (3)
22.05.99	Gr 82 × Gr 83	0	0	0	.
23.05.99	Gr 81 × Gr 70	2	2	0	cc
27.05.99	Gr 89 × Gr 90	20	18	0	cc
27.05.99	Gr 95 × Gr 96	7	0	6	cc
27.05.99	Gr 101 × Gr 102	12	7	0	cc
27.05.99	Gr 87 × Gr 88	0	0	0	.
28.05.99	Gr 97 × Gr 98	0	0	0	.
28.05.99	Gr 93 × Gr 94	0	0	0	.
28.05.99	Gr 91 × Gr 92	18	16	0	cc
29.05.99	Kr 8 × Gr 105	20	18	0	cc
29.05.99	Kr 36 × Gr 104	0	0	0	.
29.05.99	Gr 109 × Gr 110	0	0	0	.
29.05.99	Ki 1 × Ki 2	3	2	3	cc
29.05.99	Ki 3 × Ki 4	15	10	1	cc
29.05.99	Ki 5 × Ki 6	0	0	0	.
29.05.99	Ki 7 × Ki 8	17	11	0	cc
01.06.99	Kr 42 × Kr 43	8	7	0	cc
01.06.99	Ki 15 × Ki 20	0	0	6	cc
03.06.99	In 28 × In 14	13	12	0	cc
03.06.99	In 26 × In 15	9	5	0	cc
03.06.99	In 25 × In 16	0	0	0	.
03.06.99	In 24 × In 17	15	0	0	.
03.06.99	In 33 × Fe 7	16	5	0	cc
03.06.99	In 30 × Fe 6	0	0	0	.
04.06.99	In 32 × Fe 8	14	13	0	cc
04.06.99	In 36 × Fe 4	4	3	0	cc
06.06.99	In 44 × Fe 1	2	2	7	cc
06.06.99	In 46 × In 19	0	0	0	.
06.06.99	In 47 × In 18	0	0	0	.
11.06.99	Fe 11 × Fe 10	8	8	0	cc