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# Taxon composition and genetic variation of water frogs in the Mid-Rhône floodplain

## *Structure taxinomique et génétique des grenouilles vertes de la vallée alluviale du Rhône moyen*

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### RÉSUMÉ

Des lignées naturelles d'hybrides hémiclonaux de grenouilles vertes se reproduisent par hybridogénèse en s'accouplant avec l'espèce parentale avec laquelle elles cohabitent. De tels systèmes hôtes-hybridogènes sont localisés dans la vallée du Rhône : le système L-E au nord, le système P-G au sud. Bien que ces deux complexes hybridogénétiques constituent probablement une zone de contact, notre étude n'apporte pas d'éléments pour la situer dans la vallée du Rhône : seules *Rana ridibunda* et *R. esculenta* ont été identifiées (par électrophorèse de protéines). Il reste à tester si l'absence de *R. perezi* reflète une limite de répartition plus méridionale ou si elle occupe d'autres habitats. La comparaison des tissus somatiques et gonadiques révèle que la gamétogénèse de *R. esculenta* est de type L-E. La présence de ces hybridogènes avec *R. ridibunda* est probablement le résultat d'une immigration depuis les systèmes L-E voisins (système source-puits). Les populations de *R. ridibunda* présentent une grande variabilité génétique qui traduit l'impact d'importations récentes.

**Mots clés :** hybridogénèse, hybrides, introduction humaine, plaine alluviale du Rhône moyen, électrophorèse de protéines, *Rana ridibunda*, *Rana esculenta*

### ABSTRACT

Natural hemiclonaal hybrid lineages of water frogs reproduce by hybridogenesis, excluding one parental genome in the germ line and mating with the coexisting same parental species. Two such sexual host-hybridogen systems occur in the Rhône valley: the L-E system in the north, the P-G system in the south. Although these hybridogenetic complexes may overlap along the Rhône river, there is no evidence for a contact zone in our samples: only *Rana ridibunda* and *R. esculenta* were identified using protein electrophoresis. Whether the absence of *R. perezi* reflects a more southern distribution or its exclusive occurrence in other habitats, remains to be tested. Comparison of somatic and gonadal tissues reveals that gametogenesis of *R. esculenta* is of the L-E type: gametes carry *ridibunda* genomes. *R. ridibunda* apparently is not native, but was introduced by humans, and the *R. esculenta* in our samples is probably an immigrant from nearby L-E systems.

**Key words:** hybridogenesis, hybrids, human introduction, Mid-Rhône floodplain, protein electrophoresis, *Rana ridibunda*, *Rana esculenta*

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## VERSION ABRÉGÉE

Les grenouilles vertes sont caractérisées par des complexes hybridogénétiques. De tels complexes sont composés d'hybrides qui cohabitent avec une des deux espèces parentales (système L–E ou R–E). Les lignées hybrides se perpétuent naturellement : les hybrides excluent lors de la gamétogenèse un des deux génomes parentaux (hybridogénèse), l'accouplement avec l'espèce parentale syntopique permet de restaurer le génome éliminé (reproduction hémiclonale).

De tels systèmes hôtes–hybridogènes sont actuellement localisés dans la vallée du Rhône : le système L–E (constitué par la présence de *Rana lessonae* et de l'hybridogène *R. esculenta*) au nord, le système P–G (*R. perezi* et l'hybridogène *R. grafi*) au sud. Bien que ces deux complexes hybridogénétiques constituent probablement une zone de contact, notre étude n'apporte pas d'éléments pour situer cette zone dans la vallée du Rhône : seules *R. ridibunda* et *R. esculenta* ont été détectées (identifiées par électrophorèse de protéines). Il reste à tester si l'absence de *R. perezi* et *R. grafi* reflète une limite de répartition plus au sud ou si elle occupe d'autres habitats, les différents taxons du complexe d'hybridation ayant des affini-

tés différentielles pour les milieux faiblement oxygénés ainsi que pour l'hibernation.

La grande variabilité génétique observée parmi les populations de *R. ridibunda* ainsi que la présence d'allèles typiques de grenouilles vertes des Balkans ou du Proche-Orient sont des indices de l'impact des importations humaines de grenouilles. Une étude génétique prenant en compte un nombre de locus plus important est cependant nécessaire pour tester les hypothèses alternatives d'une dynamique de repeuplement post-glaciaire et/ou d'un peuplement issu d'importations multiples et récentes de ce taxon. Pour autant, les impacts de telles importations sur les populations naturelles sont discutés.

La composition de populations en assemblages *R. ridibunda* + *R. esculenta* ne constitue a priori pas des complexes hybridogénétiques persistants à long terme. En effet, la comparaison des tissus somatiques et gonadiques révèle que la gamétogenèse de *R. esculenta* est de type L–E, c'est-à-dire que le génome *lessonae* est exclu. Dans un tel assemblage, il n'y a plus de restauration possible des génomes *lessonae* chez l'hybride. La présence de ces hybridogènes avec *R. ridibunda* est probablement le résultat d'immigrations depuis les systèmes L–E voisins (système source–puits).

## Introduction

European water frogs (*Rana esculenta* group, Amphibia) are characterized by widespread and abundant natural interspecies hybrid lineages that persist through a hemiclonal reproduction (reviewed by Graf and Polls Pelaz [1]) termed hybridogenesis [2]: they exclude the genome of one parental species in the germ line prior to meiosis [3] and literature cited therein), endoreduplicate the genome of the other parental species, and produce haploid, unrecombined gametes with a genome of this second parental species. Hybridity is restored in each generation via sexual parasitism because the hybrids mate with the first parental species.

The first discovered [4–6], best-investigated, and most widespread system occurs in central and eastern Europe. It consists of the hybridogenetic hybrids *R. esculenta* (= *R. ridibunda* × *R. lessonae*) of both sexes, which transmit only their *ridibunda* genome to haploid gametes. Such hybrids coexist with the sexual parental host species *R. lessonae*, mating with which restores hybridity in each generation of *R. esculenta* lineages (the L–E system [7]). Similarly widespread host–hybridogen systems exist in at least two other parental species combinations, all sharing *R. ridibunda* as the source species of one genome. The second parental (host) species is *R. perezi* in southern France and eastern Spain [8–11]; *R. perezi* and its associated hybridogen *R. grafi* [12] (= *R. ridibunda* × *R. perezi*) form a P–G system analogous to the L–E system. In Peninsular Italy, in Corsica, and in Sicily, the parental host species is a taxon (possibly two) related to *R. lessonae* [13–16].

Such host–hybridogen populations are persisting reproductive systems. However, in natural populations, other

coexistences of different taxa may occur, which do not necessarily form an on-going hybridogenetic reproductive system, and for which we thus use the term 'assemblages'.

The current knowledge of the geographical distribution of the European species and host–hybridogen systems (figure 1) contains some gaps, particularly in France where the ranges of the two host species *R. lessonae* (north or east) and *R. perezi* (south or west) may overlap and therefore provide a possibility of contact zones. The northern range limit of *R. perezi* and the associated P–G system is not accurately delimited, although it has been reported to be located in the Mid-Rhône floodplain: Arano et al. [10] recorded the presence of this system in the Dombes region (near Lyon; figure 1). Direct contact between *R. lessonae* and *R. perezi*, and thus between the L–E and the P–G systems, has not been reported so far. Nevertheless, its investigation is important from an evolutionary point of view because it implies possible hybridizations between *R. lessonae* and *R. perezi*, as well as the transmission of *ridibunda* genomes of hybridogenetic hybrid lineages between two different host species [17].

In central Europe, *R. ridibunda* mainly inhabits large and deep water bodies where water is continuously renewed and refurnished with oxygen. As a consequence, *R. ridibunda* is abundant in large oxbow lakes and dead arms of large river floodplains [18–23]. In contrast, *R. lessonae* inhabits small shallow ponds [eg. 19–24]. This habitat spectrum is probably a result of lower tolerance of *R. ridibunda* to hypoxic conditions, related to different oxygen affinity of blood [25, 26], and of different modes of hibernation, *R. ridibunda* in water and *R. lessonae* on land [21]. Although dispersal of *R. ridibunda* in central Europe is limited by altitudinal barriers (eg. [23]), this spe-

cies has recently been repeatedly introduced by humans into western Europe, especially in Switzerland and France [27].

The first aim of our study was to determine the taxon composition of three water frog assemblages in the Mid-Rhône valley, using genetic markers assessed by protein electrophoresis. We investigated the presence and co-existence of *R. lessonae*, *R. perezi*, *R. ridibunda*, and their hybrids, the existence of hybridogenetic systems, and the impact of human importation of frogs, by determining sex ratios, testing for Hardy-Weinberg equilibrium of the observed genotypes, and determining the mode of gametogenesis of hybrid individuals via an electrophoretic comparison between somatic and gonadal tissues.

## Material and methods

### Sites

We investigated three water frog populations located near the active channel of the French Upper Rhône floodplain (figure 1). In the study sites, water was continuously renewed by water table flow.

Frogs were collected by hand at night during the breeding period (April–June 1995) with an electric torch.

i) Morte-de-la-Barre ( $n = 33$ ): gravel-pit pond 3 km NNW of Saint-Genix-sur-Guiers, sampled in May 1995.

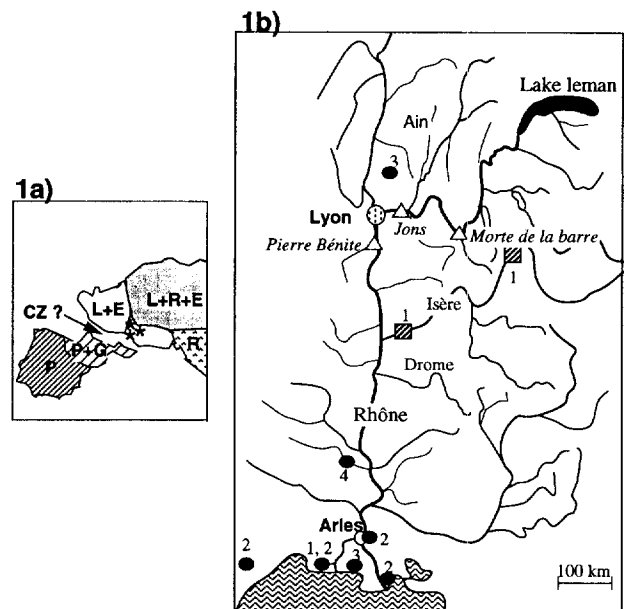
ii) Jons ( $n = 31$ ): gravel-pit pond 2 km E of the village of Jons, sampled in April 1995.

iii) Pierre-Bénite ( $n = 31$ ): regularly overflowed side-arm of the Rhône river 0.5 km E of Vernaison, sampled in May and June 1995.

### Protein electrophoresis

Skeletal muscle or gonadal tissues were used for protein electrophoresis. Tissue samples were crushed in an equal volume of a 10 mM Tris + 1 mM EDTA + 13 mM NADP solution, pH 7. Migration was performed in 12% continuous Tris citrate starch gels (gel buffer: 8 mM Tris + 3 mM citric acid, electrode buffer, 220 mM Tris + 90 mM citric acid, pH 6) for 4 h at 12 V/cm. Enzymes were revealed on gel slices using modifications of standard procedures ([28, 29] and H. Hotz, unpubl.; staining protocols available from the authors on request).

For somatic tissue, we used four enzyme loci that discriminate between at least one species pair among *R. perezi*, *R. lessonae* and *R. ridibunda* [7, 11, 14]: Creatine kinase (CK-A; E.C. 2.7.3.2), L-lactate dehydrogenase (LDH-B; E.C. 1.1.1.27), mannose-6-phosphate isomerase (MPI; E.C. 5.3.1.8), and phosphoglucosmutase (PGM-2; E.C. 5.4.2.2, formerly 2.7.5.1). For comparison, we used *R. perezi* (from Delta del Ebro, Spain), *R. grafi* (Pouzolles near Béziers, France), *R. ridibunda* (Mosina near Poznan, Poland), *R. esculenta* (Hellberg near Zürich, Switzerland), and *R. lessonae* (Poznan, Poland, and Hellberg, Switzer-



**Figure 1. a. Distribution range of two hybridogenetic complexes of the water frog group in Western Europe.**

The white area 'Contact zone?' corresponds to the hypothetical presence of the P-G as well as the L-E system. In Switzerland and France, imported *Rana ridibunda* populations are symbolized by asterisks. The others symbols represent the following taxa: P + G = *R. perezi* and *R. grafi*, L + E = *R. lessonae* and *R. esculenta*, L + R – E = *R. lessonae*, *R. ridibunda*, *R. esculenta*.

**b. Present knowledge on the ranges of the P-G and L-E systems near the hypothetical contact zone in the French Rhône basin.**

Black circles = presence of *R. perezi* and/or *R. grafi*; hatched squares = presence of *R. esculenta* and/or *R. ridibunda*; white triangles = our sample sites. The numbers refer to papers in which water frogs were identified by genetic markers. 1: Uzzell and Tunner [9], 2: Hotz et al. [11], 3: Arano et al. [10], 4: Graf et al. [8].

land). Alleles are named by lowercase letters following an established system [1, 14, 30, 31].

In the hybridogenetic hybrids, the premeiotic exclusion of one of the two parental genomes in the germ line can be detected electrophoretically in gonadal tissues: for somatically heterozygous loci, only one parental allele is expressed in normal gametocytes of the hybrids if they are hybridogenetic [eg, 8, 32–36]. We therefore compared somatic and gonadal tissues, using the loci LDH-B and MPI, of all hybrids collected, to determine whether they had a hemiclinal reproduction and, if so, which parental genome was transmitted to the gametes.

For *R. ridibunda*, goodness of fit of the observed genotype frequencies to Hardy-Weinberg expectations was tested for each population and each polymorphic locus, using  $\chi^2$  statistics.

## Results

Taxon composition of the three Rhône populations studied, determined by protein electrophoresis, is presented in table I. Water frogs of these sites belonged to *R. ridi-*

**Table I. Taxon composition and sex ratios of water frog samples from three sites in the Mid-Rhône floodplain.**

Site	<i>Rana ridibunda</i>					<i>Rana esculenta</i>				
	N	%	♀	♂	% ♀	N	%	♀	♂	% ♀
Morte-de-la-Barre	29	88	7	22	24	4	12	1	3	25
Jons	31	100	12	19	39	0	0			
Pierre-Bénite	25	81	11	12	48	6	19	2	4	33
Total	85	89	30	53	36	10	11	3	7	30

Taxa were determined by protein electrophoresis. None of the samples contained *R. lessonae*, *R. perezi* or *R. grafi*.

*bunda* and *R. esculenta*, but not to *R. lessonae*, *R. perezi* or *R. grafi*. *R. ridibunda* was the most frequent taxon (81 to 100% in samples, 89% of all individuals), whereas *R. esculenta* was recorded in low frequencies (0 to 19% in samples, 11% of all individuals).

Males predominated in all populations of both taxa (*R. ridibunda*: 52 to 76% in samples, 64% of all individuals; *R. esculenta*: 67 to 75% in samples, 70% of all individuals; table I). The male excess is statistically significant only in *R. ridibunda* from Morte-de-la-Barre ( $\chi^2$ , 1df = 7.8; 0.001 > P > 0.01).

Allele frequencies of the three variable loci studied (LDH-B, MPI, PGM-2), are given in table II, and genotype frequencies in table III. The fourth locus (CK-A) is invariant among our samples. *R. ridibunda* showed high genetic

variability: at the loci LDH-B, MPI and PGM-2, a total of two, three and three alleles, respectively, segregated. Mean observed individual heterozygosity for the four enzyme loci was 33% for Jons, 39% for Morte-de-la-Barre and 36% for Pierre-Bénite; it was 35.9% for all individuals. Mean observed heterozygosity was still much higher in the hybrid *R. esculenta*: 75% in each of the two samples (all individuals).

For each individual *R. esculenta*, we compared electrophoretic phenotypes of somatic tissue and of gonadal tissue for the enzyme loci LDH-B and MPI (table IV). Both loci were somatically heterozygous in each individual. For each hybrid and each locus, gonadal tissues, in contrast to the soma, evidenced only the allelic product characterizing the *ridibunda* parental genome. This dem-

**Table II. Electrophoretically detected allele frequencies at three variable enzyme loci in samples of *R. ridibunda* and hybridogenetic *R. esculenta* from the Mid-Rhône floodplain.**

Site	Taxon	N	LDH-B			MPI				PGM-2			
			a	c	e*	a	c	h*	j	b	c*	d	g
			100	79	64	119	100	9	135	100	94	80	47
Morte-de-la-Barre	<i>R. ridibunda</i>	29	0.69	0.31	—	0.69	0.31	—	—	0.36	—	0.57	0.07
	<i>R. esculenta</i>	4	0.50	—	0.50	0.50	—	0.50	—	—	0.50	0.50	—
Jons	<i>R. ridibunda</i>	31	0.74	0.26	—	0.27	0.73	—	—	0.52	—	0.48	—
Pierre-Bénite	<i>R. ridibunda</i>	25	0.70	0.30	—	0.44	0.54	—	0.02	0.70	—	0.30	—
	<i>R. esculenta</i>	6	0.25	0.25	0.50	0.50	—	0.50	—	—	0.50	0.50	—

The additional locus CK-A was fixed for allele *b* in all samples studied. Relative mobilities on 12% continuous Tris-citrate pH 6 starch gels (in percent, the *R. ridibunda* allele most common in our samples designated as 100) are indicated below the allele names. Alleles marked by \* characterize *R. lessonae*, the others, *R. ridibunda*.

**Table III. Electrophoretically detected genotype frequencies at three variable enzyme loci in samples of *R. ridibunda* and hybridogenetic *R. esculenta* from the Mid-Rhône floodplain.**

Site	Taxon	N	LDH-B					MPI					PGM-2					
			a/a	a/c	c/c	a/e*	c/e*	a/a	a/c	a/j	c/c	a/h*	b/b	b/d	b/g	d/d	d/g	c/d*
Morte-de-la-Barre	<i>R. ridibunda</i>	29	0.41	0.55	0.03	—	—	0.45	0.48	—	0.07	—	0.14	0.38	0.07	0.34	0.07	—
	<i>R. esculenta</i>	4	—	—	—	1.00	—	—	—	—	1.00	—	—	—	—	—	—	1.00
Jons	<i>R. ridibunda</i>	31	0.52	0.45	0.03	—	—	0.03	0.48	—	0.48	—	0.32	0.39	—	0.29	—	—
Pierre-Bénite	<i>R. ridibunda</i>	25	0.44	0.52	0.04	—	—	0.20	0.44	0.04	0.32	—	0.48	0.44	—	0.08	—	—
	<i>R. esculenta</i>	6	—	—	—	0.50	0.50	—	—	—	—	1.00	—	—	—	—	—	1.00

The additional locus CK-A was invariant (genotype *b/b*) among all samples studied. Genotypes marked by \* characterize *R. esculenta*, the others *R. ridibunda*.

**Table IV. Electrophoretic phenotypes for two enzyme loci in somatic and gonadal tissues of the hybrids *R. esculenta* from the Mid-Rhône floodplain.**

Site	Sex	N	Tissue	Electrophoretic phenotype	
				LDH-B	MPI
Morte-de-la-Barre	♀	1	Soma	*a e	*a h
			Ovaries	a	a
	♂	3	Soma	*a e	*a h
			Testes	a	a
Pierre-Bénite	♀	2	Soma	*c e	*a h
			Ovaries	c	a
	♂	1	Soma	*c e	*a h
			Testes	c	a
		3	Soma	*a e	*a h
			Testes	a	a

For somatic heterozygosities, the allelic product characterizing the *ridibunda* genome is indicated by \*.

onstrates that these hybrids were all hybridogenetic and had a gametogenesis of the L-E system type, that is, they excluded their *lessonae* genome from the germ line and transmitted their *ridibunda* genome to the gametes.

At Pierre-Bénite, within the resolution provided by the three variable loci examined, two different *R. esculenta* hemiclones (haplotypes of their *ridibunda* genome) coexisted in equal frequency. They were identified by two different *ridibunda* alleles at the locus LDH-B (a and c; table III). Only one of these two hemiclones (characterized by LDH-B a) was observed among the four hybrids at Morte-de-la-Barre (table III).

In *R. ridibunda*, genotype frequencies of each variable locus at each site (table III) were tested for goodness-of-fit to the expectations calculated from estimated allele frequencies (table II) assuming Hardy-Weinberg equilibrium. There was no significant deviation from Hardy-Weinberg expectations in any sample at any locus (Morte-de-la-Barre: LDH-B  $\chi^2$ , 1df = 2.42, MPI  $\chi^2$ , 1df = 0.47, PGM-2  $\chi^2$ , 3df = 0.51; Jons: LDH-B  $\chi^2$ , 1df = 1.00, MPI  $\chi^2$ , 1df = 1.44, PGM-2  $\chi^2$ , 1df = 1.57; Pierre-Bénite: LDH-B  $\chi^2$ , 1df = 1.42, MPI  $\chi^2$ , 3df = 1.40, PGM-2  $\chi^2$ , 1df = 0.06;  $P > 0.1$  for each comparison).

At each site, we also tested for linkage disequilibrium of two-locus genotypes in all three possible pairs among the three variable loci, using a simple goodness-of-fit  $\chi^2$  approach (data not shown), and found no evidence for deviation from Hardy-Weinberg proportions ( $P > 0.1$  for each comparison).

## Discussion

### Species composition in the Mid-Rhône valley

At the three sites investigated near the Rhône river, the water frog populations consisted mainly of *R. ridibunda* of

both sexes; at two sites we found in addition a low frequency of *R. esculenta* (12 and 19%, respectively) of both sexes. There were no *R. lessonae*, *R. perezi* or *R. grafi*.

The preponderance of *R. ridibunda* in these habitats in the vicinity of a large river correspond with this species' habitat use in central Europe: it typically inhabits (and hibernates in) large and deep lowland water bodies where water is continuously renewed and refurnished with oxygen, such as medium to large rivers, large ponds or lakes (eg, [19–23]). The absence of *R. lessonae* in the studied sites is also consistent with this species' habitat use in central Europe where it occupies small, shallow ponds in marshes and woodland and hibernates on land [21]. We assume that *R. lessonae* is present in other habitats of the Mid-Rhône valley, farther from the main channel, where it coexists with its sexual parasite *R. esculenta* (L-E systems), which probably provides the source of the small number of hybridogenetic *R. esculenta* in our samples. The presence of *R. lessonae* in this region [37] has been confirmed for Lavours' Marsh (near Morte-de-la-Barre; figure 1) in the Mid-Rhône floodplain using protein electrophoresis (A. Pagano, unpubl.).

No *perezi* genomes were detected in the studied populations. This suggests that: i) the northern range limit of *R. perezi* and its hybridogenetic associate *R. grafi* in the Rhône valley lies further south; or ii) *R. perezi* occurs in habitats that differ from those of the studied sites. The P-G system has been reported for the Dombes region (north of Lyon) [10], although data on taxon composition or electrophoretic phenotypes were not specified. Excepted the record of these authors, the northernmost localities in the Rhône drainage where *R. perezi* was identified by genetic markers were in the Camargue [11] and in the Cèze valley (north of Nîmes; J.-D. Graf, pers. comm.). Sampling transects in the Rhône valley and its surrounding hilly area from north to south, including various aquatic habitats, will provide a test of these two hypotheses, and will investigate possible contacts between the L-E and P-G systems and possible sympatry and hybridizations between *R. lessonae* and *R. perezi*.

### Population systems

At each site, both sexes of *R. ridibunda* were sampled; no locus showed a deviation from Hardy-Weinberg proportions; two-locus genotypes of all three possible pairs among the three variable loci revealed no counterevidence for panmixy at any site. We conclude that these *R. ridibunda* constituted reproducing, persisting populations, probably for several generations.

Each *R. esculenta* individual (three females, seven males) showed a hemiclinal reproductive mode of the L-E system type, as evidenced by electrophoretic comparison of somatic and gonadal tissues (table IV): the *lessonae* parental genome is excluded from the germ line and the *ridibunda* genome is clonally transmitted to gametes. Such *R. esculenta* coexisting with *R. ridibunda* cannot form a persisting host-hybrid population system, because

no source of their *lessonae* genomes is available considering their mode of gametogenesis and the absence of *R. lessonae* in our samples. The *R. ridibunda*–*R. esculenta* assemblage we studied has nothing to do with the R-E system [7]. Persisting R-E systems are only known in some regions of eastern Germany and western Poland; in these *R. ridibunda* of both sexes coexist with diploid hybridogenetic *R. esculenta* males that transmit their *lessonae* genome more often than their *ridibunda* genome to haploid sperm [38, 39]. The presence of *R. esculenta* in the *R. ridibunda*–*R. esculenta* assemblage we studied may be explained by a source–sink system [40]. We presume that at our study sites, *R. esculenta* are immigrants from nearby L-E systems occupying different habitats more distant from the main river. The two hybrid hemiclones at Pierre-Bénite probably reflect preexisting clonal diversity in surrounding L-E system populations, such as has been found in other L-E systems both within and outside *R. ridibunda*'s native range [eg, 14, 41, 42]. The *ridibunda* alleles of these two hemiclones (LDH-B *a* and *c*, MPI *a*, PGM-2 *d*) do all occur in native *R. esculenta* lineages of northern Switzerland [43; H. Hotz, unpubl.], although hemiclones containing LDH-B *a* or MPI *a* have low frequencies there. All *ridibunda* genomes of *R. grafi* lineages in southern France contain LDH-B *a*, MPI *a* and PGM-2 *d* [11]. An overview of an extended sample of loci will permit us to specify the origin of our sampled hemiclones.

### Origin of *R. ridibunda*

The occurrence of *R. ridibunda* in the Mid-Rhône floodplain may be explained by three alternative hypotheses: i) these populations are native; that is, they result from the recolonization of central Europe from southeastern refuges after the last glaciation, or ii) they result from recent human importations, or iii) they result from matings between two hemiclonal hybrids.

Although their habitats are similar to those of central European *R. ridibunda*, the occurrence of *R. ridibunda* populations in the Mid-Rhône floodplain is not trivial: this region lies well to the west and south of the species' assumed native range (eg, [22, 37]; figure 1). *R. ridibunda* has been recently imported by humans into several regions of Switzerland, including the Rhône drainage area of the Lemanic Basin [27, 44]. We hypothesize that the occurrence of *R. ridibunda* in the Mid-Rhône floodplain is a result of either expansion from the Lemanic Basin along the Rhône river, or of independent human introductions into this part of France, or of both. Genetic assessments of *R. ridibunda* populations along a transect from southwestern Switzerland to the region of Lyon (figure 1) will permit us to test these hypotheses. The presence of *R. ridibunda* in some other parts of southern France has been ascertained, again in genetically highly variable populations assumed to originate from human importation, for example in the Cèze valley north of Nîmes (J.-D. Graf, pers. comm.).

There have been multiple introductions of *R. ridibunda* into Switzerland [45], from different geographic regions probably comprising eastern and southeastern Europe and Anatolia. In France, recent importations of water frogs (mainly for culinary purposes) were (and continue to be) primarily from Balkanic countries, during the 1960s, and, from the 1980s onwards, primarily from Albany, Turkey and Egypt ([46] and unpubl. data). Of the alleles we found at the three variable loci studied in *R. ridibunda* from our study sites, LDH-B *a* and *c*, MPI *a* and *c* and PGM-2 *b* and *d* are widespread and usually common in central and eastern Europe (eg, [7, 14, 47]), and LDH-B *a* and *c*, MPI *a* and PGM-2 *d* are common in Anatolia ([31; H. Hotz, T. Uzzell and P. Beerli, unpubl.]). The rare allele MPI *j* (Pierre-Bénite) occurs in minor to moderate frequency in Anatolia, especially in the south and southeast ([31; H. Hotz, T. Uzzell and P. Beerli, unpubl.]) and is common in Cyprus (H. Hotz and T. Uzzell, unpubl.) and in Egypt (A. Pagano and H. Hotz, unpubl.). The rare allele PGM *g* (Morte-de-la-Barre) occurs in low frequency in *R. ridibunda* from the Balkans [14] and Anatolia, where it is more common in the south (H. Hotz, unpubl.; identity of the Balkan and Anatolian alleles needs confirmation). These alleles indicate the presence of imported *R. ridibunda* in natural populations. Electrophoretic examination of additional variable loci and use of other genetic markers such as mitochondrial DNA will be needed to further investigate the geographic origins of these frogs.

A potential alternative origin of *R. ridibunda* is mating between two hybridogenetic hybrids. The production of mature *R. ridibunda* from natural matings between hemiclonal hybrids *R. esculenta* from L–E systems, both sexes of which transmit the *ridibunda* genome to gametes, has been demonstrated for a population in northern Switzerland [45] and may well occur elsewhere. Nevertheless, such hybrid × hybrid matings give all-female progeny [45, 48, 49] and can therefore be ruled out as the source of the *R. ridibunda* populations we studied: our samples contained both sexes with a preponderance of males (possibly a result of sampling bias for the more active and calling males).

### Mutual impact of imported and native species

Immigrating hemiclonal *R. esculenta* can mate with *R. ridibunda* and thus have genetic impacts on populations of this species in at least two ways. i) Nuclear genetic material stemming from *ridibunda* genomes of hybrid lineages in local L-E systems can be introduced into the gene pool of reproducing *R. ridibunda* populations. Such *ridibunda* genomes of hybrid lineages may have been clonally transmitted for a relatively long time and may thus be shaped both by interclonal selection and by the expected accumulation of deleterious recessive mutations in the absence of recombination through Muller's ratchet (eg, [50, 51]). ii) *R. lessonae* mitochondrial DNA (mtDNA, maternally transmitted) can be introduced into *R. ridibunda* by matings of female *R. esculenta* with male *R. ridi-*

*bunda* (cf. [45, 52, 53]). Whether such transmission of mtDNA has in fact occurred in the Mid-Rhône *R. ridibunda* populations remains to be determined.

The imported *R. ridibunda* populations are expected to have impacts on native water frog populations. Ecological interactions include competition and predation of *R. ridibunda* on native species, which has been observed (eg, [44]). Genetic interactions are possible via matings between the genetically highly variable imported *R. ridibunda* and native species. If *R. ridibunda* occasionally colonize nearby L-E systems, or if *R. lessonae* occasionally colonize *R. ridibunda* populations, then primary hybridizations between these two parental species of *R. esculenta* are possible (*R. ridibunda* female × *R. lessonae* male rather than the reverse, for behavioral reasons [6, 49]). Such matings can found a variety of new *R. escu-*

*lenta* hemiclones with possibly different adaptive potentials, provided that the *ridibunda* genomes lead to hybridogenetic gametogenesis in hybrids with *R. lessonae*. The likelihood of this depends on the geographic origins of the imported *R. ridibunda*. Present data suggest that frogs of the *R. ridibunda* stock originating from southeastern Europe or from Anatolia will not found hemiclinal lineages when mating with *R. lessonae* but rather produce sterile non-hybridogenetic F1 hybrids (cf. [17, 36, 54]; G. Mancino, S. Bucci, M. Raghianti and F. Guerrini, unpubl. data), whereas a vast majority of *R. ridibunda* from central Poland do found hemiclinal *R. esculenta* lineages when mating with *R. lessonae* [36, 54, 55]. When arriving in native L-E systems, foreign genetic material may disrupt the hybridogenetic reproductive mode and thus have impacts on hybrid frequencies.

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