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Interspecific hybrids of *Rana ridibunda* without germ line exclusion of a parental genome[1]

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Summary. Hybrids of *Rana ridibunda* × 2 unnamed Balkan taxa show no evidence, in electrophoretic comparison of soma and oocytes I for 4 enzyme loci, of germ line exclusion of their non-*ridibunda* genome. In this they differ from hybrids *R. ridibunda* × *R. lessonae,* × an unnamed Italian taxon, and × *R. perezi,* which in most cases clonally pass only their *ridibunda* genome to gametes.

The western Palearctic water frog *Rana ridibunda* Pallas 1771 (Amphibia) has been reported as a parental species of 3 different groups of natural interspecific hybrid lineages, the other parental species being *R. lessonae* Camerano 1882 in central Europe[2,3], *R. perezi* Seoane 1885 in southern France[4], and an unnamed taxon related to *R. lessonae* in peninsular Italy[5]. In all 3 cases, diploid hybrids of both sexes reproduce by 'hybridogenesis[6]': only their *ridibunda* genome is usually transmitted to progeny, the entire non-*ridibunda* genome being lost in the germ line before completion of gametogenesis (*R. ridibunda* × *R. lessonae*[7,8]; × *R. perezi*[4]; × Italian taxon[5]). Such hemiclonal[9] reproduction also occurs in fishes (interspecific *Poeciliopsis* hybrids[10]) and possibly in newts (interspecific *Triturus* hybrids[11]). The hybridogenetic genome exclusion can be detected electrophoretically in gonadal samples[4,12-17] and in individual oocytes I[14-16]: for somatically heterozygous loci, only *ridibunda* alleles are expressed in normal gametocytes of the hybrids.

Recently, 2 related additional taxa, each specifically distinct from and sympatric with *R. ridibunda,* have been discovered in the Adriatic parts of the Balkan peninsula; one in SW Yugoslavia[18], and the other in NW Greece[18,19]. Both are electrophoretically, immunologically and morphologically distinct from all other European water frog species; they are possibly most nearly related to *R. lessonae* and the Italian non-hybrid taxon[18]. Whether the 2 new taxa are conspecific is not known. Interspecific hybrids of both sexes in low frequencies have been detected electrophoretically in 4 of 7 syntopic populations of *R. ridibunda* and either new Balkan taxon[18]. To investigate whether these hybrids also show hybridogenetic gametogenesis, we compared electrophoretic phenotypes of their soma and their individual oocytes or testis extracts.

Material and methods. The 7 hybrids *R. ridibunda* × either new Balkan taxon discussed here were collected in August and September 1980 and 1981 in Yugoslavia (5 ♀ at Virpazar, Skadarsko Jezero, Crna Gora) and in Greece (1 ♀ + 1 ♂ at 10 km NW Igoumenitsa, Epeiros). For comparison we used electrophoretically identified hybrids, *R. ridibunda* × *R. lessonae* (May and June 1980: surroundings of Zürich, Switzerland; May 1981: surroundings of Poznań, Poland; all were syntopic with *R. lessonae:* the *'lessonae-esculenta* system'[20]), × Italian taxon (September 1980: Tarsia, Calabria, Italy), and × *R. perezi* (May 1981: SW Arles, Bouches-du-Rhône, France). Small pieces of frozen skeletal or skeletal and heart muscle and of testes were crushed in equal volumes of water; individual enlarged oocytes I of average size were squashed, adding a drop of water. Samples were applied on filter paper tabs to 12–15% starch gels pH 5.7/8.0 (electrode buffer 0.22/0.69 M tris + 0.09/0.14 M citric acid, gel buffer 0.008/0.023 M tris + 0.003/0.005 M citric acid). Proteins were separated horizontally at 13 V/cm for 3–4 h (pH 5.7 gels) or at 9 V/cm for 4–6 h (pH 8.0 gels) in a refrigerator. We examined 4 enzymes that partly discriminate the 2 parental species[18] and that were scorable in gonads; they were localized on gel slices in 0.5–1% agar overlays with modified standard chromogenic methods[21,22]: glucosephosphate isomerase (GPI, EC 5.3.1.9), lactate dehydrogenase (LHD, EC 1.1.1.27), NAD-dependent malate dehydrogenase (MDH, EC 1.1.1.37) and a peptidase cleaving leucyl-tyrosine and leucy-valine (Pep, EC 3.4).

Results and discussion. The 6 ♀ and 1 ♂ hybrids *R. ridibunda* × either new Balkan taxon, all of which appear to be diploid according to erythrocyte size[18], were somatically heterozygous 11 times for GPI and LDH-1, the 2 enzyme loci examined in gonads for all of them. All individual oocytes of each ♀, with 1 exception, and testis extracts of the ♂ gave the same pattern as the soma, whether the soma was homozygous or heterozygous (table, fig. D). This is supported by the ♀ hybrid from Greece being heterozygous for MDH-1 in the soma[18] and in 18 oocytes, and by 1 ♀ hybrid from Yugoslavia being heterozygous for Pep-2 in the soma[18] and in 17 oocytes. The exception, a ♀ somatically heterozygous for GPI and LDH-1, had a heterozygous pattern of LDH-1 in all oocytes examined, but only the translation product of the *a* GPI allele, characteristic of

R. ridibunda (table). It is possible that this reflects an early germ line exclusion of the new Yugoslavian taxon chromosome that bears the GPI locus. Other GPI data alternatively suggest a possibly tissue specific preferential expression of the *a* allele: 1 *R. ridibunda* ♂ and 1 probable B_1 backcross ♂ to *R. ridibunda*[18] from Greece, both somatically heterozygous for GPI, in testis extracts preponderantly exhibited the *a* allelic product in a 2-banded pattern without the middle heterodimer band. Moreover, a few hybrids *R. ridibunda* × *R. lessonae* and × Italian taxon, despite apparent lack of the *a* allele in their syntopic non-*ridibunda* parent, showed only the *a* allelic product in the soma[23].

In the diploid hybrids *R. ridibunda* × *R. lessonae,* × Italian taxon, and × *R. perezi,* examined with the same methods, testis extracts of somatically heterozygous ♂ for LDH-1 showed the *ridibunda* allelic product predominating in a faintly heterozygous pattern; for GPI, heterozygous patterns were seen, the middle band often being least intense or absent. These indications of heterozygosity are probably due to somatic tissues of the testes. On the other hand, each ♀ of these hybrids that was somatically heterozygous for GPI or LDH-1, in all oocytes showed only a single allelic product, characteristic for *R. ridibunda* (table, fig. A–C), as has been reported earlier[4,12–16]. The Pep-2 locus again gave analogous results for 1 hybrid ♀ each from Switzerland and from Poland and for 2 hybrid ♀ from Italy. The general pattern found in the *R. ridibunda* × either new Balkan taxon hybrids is discordant with these observations; these Balkan hybrids show no evidence of hybridogenetic gametogenesis. A premeiotic germ line exclusion of the non-*ridibunda* genome in the other hybrids has been proposed to explain hybridogenetic gametogenesis[4,15–17,24]. It seems to be induced by the *ridibunda* genome[14]. Possibly it is a consequence of a germ line specific inactivation of the non-*ridibunda* genome, interfering with centromere-spindle fiber coupling[14], or is directly connected to centromere differences between the 2 genomes[16,17], that only in the germ line lead to exclusion of 1 genome. This exclusion seems to be followed by a regulatory endoreduplication of the *ridibunda* genome[14,16,17,24], after which an orderly meiosis occurs, with synapsis of sister chromatid derived chromosomes. Whether the genomes of both new Balkan taxa differ from those of *R. lessonae,* the Italian taxon, and *R. perezi* by being resistant to germ line exclusion induced in hybrids by the *ridibunda* genome, or whether genomes of western Balkan *R. ridibunda* do not contain the factors inducing hybridogenesis in hybrids, or both, cannot presently be decided.

R. ridibunda × *R. lessonae* hybrids produce, in low frequencies, large 2 n gametes containing both parental genomes that give 3 n progeny[25–27]; they obviously correspond to very large oocytes I in which both parental allele products are active[15,23] and that we believe to be tetraploid[25]. We found no triploids, and examined no unusually large oocytes in hybrids *R. ridibunda* × either new Balkan taxon. While it is clear that these hybrids are not hybridogenetic, our electrophoretic data would be consistent with their exclusively producing diploid gametes that either do not lead to viable offspring or that develop gynogenetically. However, 3 apparently diploid backcross individuals have been detected electrophoretically[18], and the low hybrid frequency strongly suggests that they do not reproduce by gynogenesis. Moreover, preliminary data on lampbrush chromosomes suggest that a haploid number of bivalents is not exceeded in oocytes of *R. ridibunda* × new Yugoslavian taxon hybrids[28].

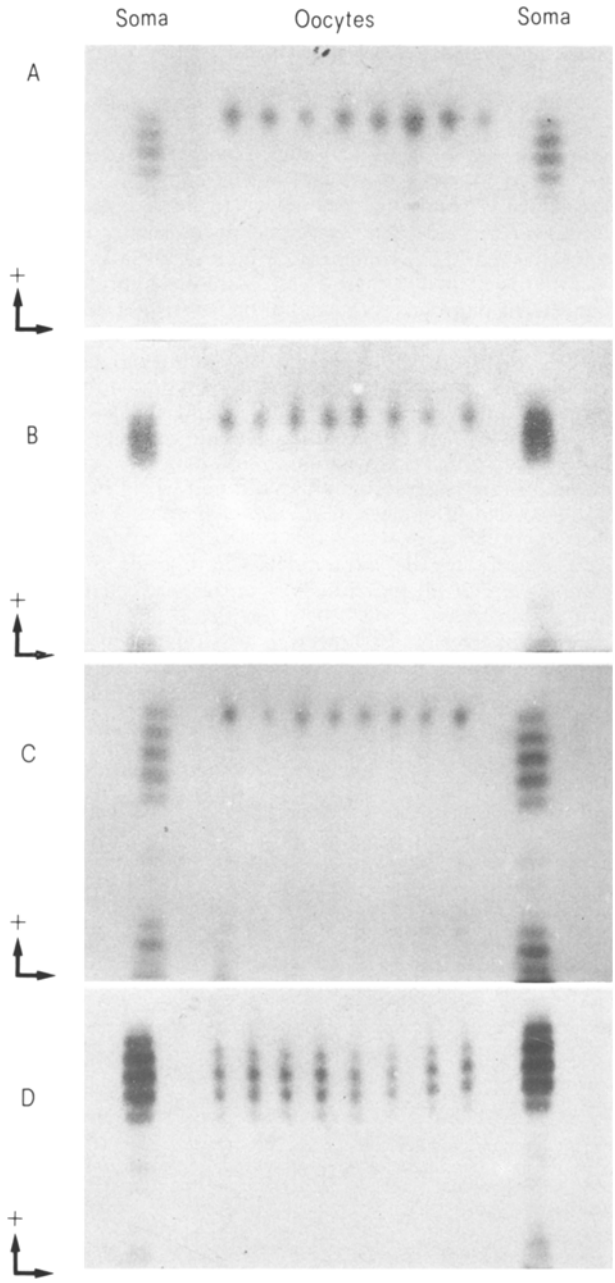
The scarcity of the western Balkan hybrids (0–21% in samples, 7% of all individuals[18]) suggests effective antihybridization mechanisms[29] between the 2 largely syntopic parental species. It is, however, also related to the non-hybridogenetic gametogenesis of these hybrids: they do not represent hemiclonal lineages, but rather F_1 individuals from single hybridizations. The very scant evidence of successful backcrosses to either parental species[18] suggests that reproduction of hybrids is limited, possibly due to

Electrophoretic phenotypes for 2 structural loci of somatic tissues and of individual primary oocytes of ♀ interspecific hybrids involving *Rana ridibunda* as a parental species

Parental species	Origin	N	Tissue	GPI				LDH-1					
				n	*a* −48	*d* −100	*e* −110	*n*	*a* 100	*b* 88	*c* 79	*d* 68	*e* 64
R. ridibunda x new Balkan taxa	Yugoslavia	3	Soma		(X)	X			(X)			X	
			Oocytes	25, 24, 28	X	X		34, 36, 28	X			X	
		1	Soma		(X)	X			(X)			X	
			Oocytes	33	X			42	X			X	
		1	Soma			X			(X)			X	
			Oocytes	28		X		28	X			X	
	Greece	1	Soma			X			(X)			X	
			Oocytes	24		X		26	X			X	
R. ridibunda x *R. lessonae*	Switzer-land	1	Soma		(X)	X					(X)		X
			Oocytes	22	X			26			X		
	Poland	1	Soma		(X)	X			(X)	X			
			Oocytes	15	X			16	X				
		1	Soma		(X)	X			(X)				X
			Oocytes	14	X			16	X				
R. ridibunda x Italian taxon	Italy	3	Soma		(X)	X					(X)		X
			Oocytes	15, 14, 12	X			26, 16, 16			X		
R. ridibunda x *R. perezi*	France	3	Soma		(X)		X		(X)			X	
			Oocytes	10, 8, 8	X			10, 16, 16	X				

N, number of females examined; *n*, number of individual oocytes examined per female; GPI, glucosephosphate isomerase; LDH-1, lactate dehydrogenase, B locus; *a–e*, allelic variants at a locus[18]. Approximate relative mobilities of allelic products (in percent) refer to 12% continuous tris-citrate starch gels pH 5.7 at 13 V/cm for 3 h. Electrophoretic phenotypes are indicated by X; for somatic heterozygosities the allelic product characterizing *R. ridibunda* is indicated by ◯.

pairing difficulties of the 2 parental genomes during meiosis I. The regularly observed high frequencies of the hybridogenetic hybrids *R. ridibunda* × *R. lessonae*[30–32], × Italian taxon[5,23], and × *R. perezi*[23] do not indicate more frequent primary hybridizations between their parental species: Autochthonous *R. ridibunda* populations are not known in the areas of the Italian taxon and of *R. perezi*; and in those parts of the range of *R. ridibunda* × *R. lessonae* lineages where both parental species occur, they usually are not syntopic because of habitat differences[33–35]. Rather, the hybrid abundance is a consequence of their inheriting the *ridibunda* genome hemiclonally. They perpetuate their advantage of a highly heterozygous 'F_1 state' via sexual parasitism on gametes of their syntopic non-*ridibunda* parent; and because in the germ line they exclude the non-*ridibunda* genome and endoreduplicate the *ridibunda* genome, they avoid meiotic difficulties due to having 2 different genomes.



Electrophoretic phenotypes of lactate dehydrogenase: Comparison of somatic tissues (skeletal and heart muscle) and of 8 individual primary oocytes of interspecific hybrids involving *Rana ridibunda* as a parental species. In case of heterozygous tissues, the anodally situated homotetramers of the LDH-1 locus show a 5-banded pattern. *A* ♀ hybrid *Rana ridibunda* × *Rana lessonae* from Poland; soma heterozygous *a/e*, oocytes only *a* (table). *B* ♀ hybrid *Rana ridibunda* × Italian taxon from Italy, soma heterozygous *c/e*, oocytes only *c* (table). *C* ♀ hybrid *Rana ridibunda* × *Rana perezi* from France; soma heterozygous *a/d*, oocytes only *a* (table). *D* ♀ hybrid *Rana ridibunda* × Yugoslavian taxon from Yugoslavia; soma heterozygous *a/d*, oocytes also (table). Arrows indicate origin and anodal direction. 12% continuous tris-citrate starch gels pH 5.7, 13 V/cm for 3 h.

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