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Population composition and genetic variation of water frogs (Anura: Ranidae) from Yugoslavia

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Abstract — Species of the *Rana* kl. *esculenta* complex from twenty-one localities of the North-Western part of the Balkan Peninsula (Yugoslavia and Macedonia) were analyzed by electophoretic, morphometric and cytogenetic methods. The results of this research revealed that species that belong to the *R.* kl. *esculenta* complex from localities in Northern Yugoslavia form a *ridibunda/esculenta/lessonae* (R-E-L) population system of diploid forms of two parental species and predominantly female hybrids. Pure *R. ridibunda* populations exist south of the Sava and Danube Rivers.

The unequal sex ratio observed in water frog population systems suggests, according to Haldane's rule, that the heterogametic sex suffers the effects of hybridization. Electrophoretic analysis shows that some introgression of *ridibunda* alleles into -R. *lessonae,* as well as *lessonae* into *R. ridibunda* has taken place. The occurrence of recombinations, and cytogenetic characteristics of the chromosome complements, does not support pure hybri-dogenesis as a general mode of reproduction in the hybrid form *R.* kl. *esculenta* from the examined localities.

Key words: *Rana esculenta* complex, hybrids, cytogenetics, Haldane's rule.

INTRODUCTION

The widely distributed Western group of Palearctic water frogs comprises many different species, and a number of hybridogenetic lineages. Numerous taxa have been identified in Europe, such as: *Rana ridibunda* Pallas 1771, *Rana lessonae* Camerano 1882, *Rana perezi* Seoane 1885, *Rana esculenta* Linneaus 1758, as well as, *Rana epeirotica* (SCHNEIDER *et al.* 1984), *Rana shqiperica* (HoTZ *et al* 1987), *Rana balcanica* (SCHNEIDER *et al.* 1993), *Rana cretensis* (BEERLI *et al.* 1994) and *Rana cerigensis* (BEERLI *et al.* 1994).

Among European water frogs, the *Rana* kl. *esculenta* complex shows considerable systematic complexity. It is accepted that *R.* kl. *esculenta* is a natural hybrid between *R. ridibunda* and *R. lessonae* which is characterized by hybridogenetic reproduction. During gametogenesis in hybridogenetic forms the set of chromosomes that is derived from one parental species is completely discarded, while the set from the other parental species undergoes compensatory duplication (GRAF and POLLS-PELAZ 1989; TUNNER and HEPICH-TUNNER 1991; BERGER 1994). Thus the gametes contain an unrecom-bined genome derived from one parental species. Individuals of hybridogenetic forms live and regularly mate with one or both parental species.

In Central Europe, frogs from the *R.* kl. *esculenta* complex form seven different population systems that depend on the prevailing ecological conditions, and the genetic background of the hybrids (PLOTNER *et al.* 1994). The hemiclonal reproductive modes of *R.* kl. *esculenta* differ amongst almost all of these systems.

As we have said, in hybridogenetic reproduction one of the parental genomes is not transmitted to the next generation and is discarded before premeiotic synthesis of DNA (VINOGRADOV *et al.* 1988; VINOGRADOV *et al.* 1990). However, studies of the pattern of inheritance in diploid *Rana* kl. *esculenta* from

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the *ridibunda/esculenta* (R-E) system revealed that there is some opportunity for introgres-sion of *lessonae* alleles into *R. ridibunda*, provided that recombination between the two parental genomes occurs. Therefore, the *R. ridibunda-R.* kl. *esculenta* system has been called a "leaky hybridogenetic system" (UZZELL *et al.* 1977).

The Central European forms, *Rana lessonae* and *Rana* kl. *esculenta* extend south, to the Sava and Danube Rivers in Northern Yugoslavia, as well as Southern Romania. On the other hand, - R. *ridibunda* occurs throughout the Balkan Peninsula.

Even though Balkan water frogs were analyzed by HOTZ and *UZZELL* (1982), SINSCH and EBLENKAMP (1994), SOFIANIDOU *et al.* (1994) and SOFIANIDOU (1996), information concerning the biology and genetics of water frogs from the Northern Balkans and the Yugoslav part of the Panonian lowland is insufficient.

Cytogenetic, electrophoretic, and morphological features of the taxa that belong to the *ridibunda/esculenta/lesonae* (R-E-L) system from the Obedska Bara and other localities in Vojvodina (North Yugoslavia), as well as *R. ridibunda* from South Yugoslavia, Montenegro and Macedonia are described here. Obedska Bara is a part of Lower Posavina. It originated from a fluvial lake that was formed about 2500 BC after the traversing and separation of a meander of the Sava River from the main river flow. Since Obedska Bara is isolated from the main river flow, it has many unique ecological features (MARTINOVIC-VI-TANOVIC 1996).

MATERIALS AND METHODS

A total of 293 specimens of water frogs from 21 localities (Fig 1.) from Yugoslavia and Macedonia were analyzed electrophoretically and cytogenetically. Animals from the Obedska Bara locality were analyzed from May to October during a four-year period. Collecting data of population samples of _R. *ridibunda, R.* kl. *esculenta* and *R. lessonae* are given in Table 1. *R. shqiperica* was used as a comparable out-group species.

TABLE 1 — List of localities, species and sample sizes. Locality code numbers correspond to those in Fig. 1.

Locality code number	Region	Locality	Date	Number of specimens analyzed	*Rana ridibunda*	*R.* kl. *esculenta*	*Rana lessonae*	*Rana shqiperica*
1	Vojvodina	Petrovaradin Marsh	06.04.1995	14	0	9	5	0
2		Popovica	17.08.1995	4	4	0	0	0
3		Čortanovci	07.04.1995	5	0	5	0	0
4		Indjija	13.09.1994	7	7	0	0	0
5		Jarkovci	03.07.1994	4	4	0	0	0
6		Obedska Bara	17.09.1994	31	2	22	7	0
6			14.08.1995	14	0	2	12	0
6			03.10.1995	17	0	17	0	0
6			17.05.1996	7	0	5	2	0
6			18.06.1998	28	1	13	14	0
7		Čenta	16.10.1994	2	2	0	0	0
8		Pancevo Marsh	25.03.1995	8	0	6	2	0
9		Deliblato Sands	14.10.1994	13	13	0	0	0
10	East Serbia	Čibuklija (Danube)	14.10.1994	4	4	0	0	0
11		Zlotska Gorge	22.05.1994	3	3	0	0	0
12	Middle Serbia	Stubline	14.06.1998	8	8	0	0	0
13		Petnica (lake)	19.07.1994	16	16	0	0	0
14		Lešnica	18.08.1994	34	34	0	0	0
15	South Serbia	Niš	27.04.0995	11	11	0	0	0
16		Stara planina	28.05.1996	2	2	0	0	0
17		Trgoviste	12.05.1997	5	5	0	0	0
18	Montenegro	Virpazar	14.06.1997	21	14	0	0	7
19	Macedonia	Ohrid Lake	06.05.1997	5	5	0	0	0
20		Prespa Lake	07.05.0997	5	5	0	0	0
21		Pribacevo	09.05.1997	5	5	0	0	0
Total				293	145	79	42	7

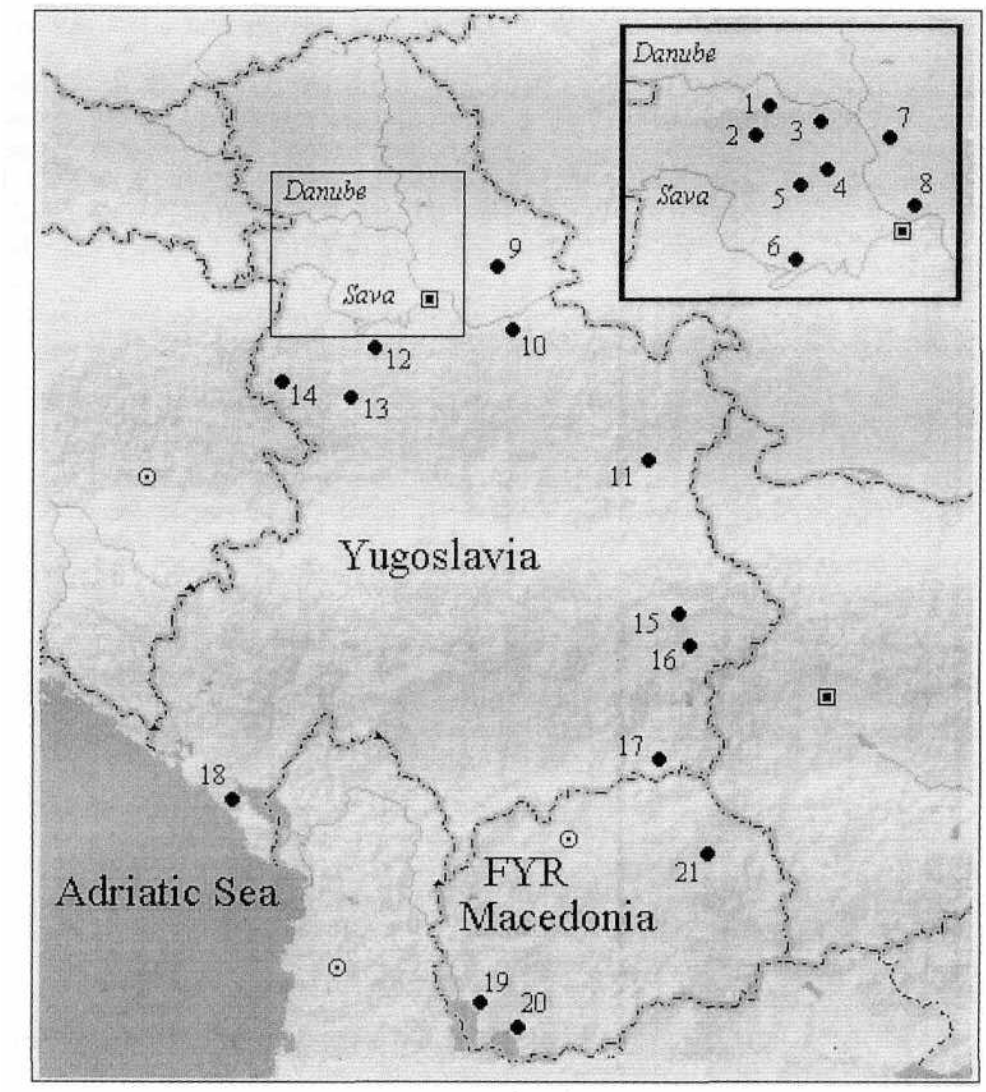

Fig. 1. — Map of collecting localities of analyzed populations of water frogs. The numbers designate the localities listed in Table 1.

The following enzymes were examined: aspartate aminotransferase *(Aat,* EC 2.6.1.1.), lactate dehydrogenase *(Ldh-1,* EC 1.1.1.27), malate dehydrogenase *(Mdh,* EC 1.1.1.37) and superoxide dismutase *(Sod,* EC 1.15.1.1.), as well as electromorphs of plasma albumin *(Alb).* Phenotypical identification of the specimens was performed by electrophoretic analysis of three diagnostic loci *(Aat, Ldh-1* and *Alb),* according to GRAF and MULLER (1979), HOTZ and UZZELL (1982), BEERLI (1994), and SANTUCCI *et al.* (1996).

We employed a vertical polyacrylamide gel system using the following buffer system: the stacking gel buffer was Tris-HCl pH 6.8, the resolving buffer was Tris-HCl pH 8.8 and the reservoir buffer was Tris-glycine pH 8.3. Skeletal muscle extracts were prepared by homogenizing small tissue samples in an equal volume of destilled water. The supernatants were applied to 10% polyacrylamide gels. Electrophoresis was performed at 200V for 4-6 hours at 4° C. The gels were stained using standard procedures (PASTEUR *et al.* 1988).

Specimens from the population in Obedska Bara were morphometrically analyzed by measuring 21 biometrical variables and by one-way ANOVA testing of 13 computed ratios.

Chromosome preparations were obtained from the bone marrow and testes according to the procedure of SCHMID (1978a). The animals received an intraperitoneal injection of 0.3% colchicine 24 h before chromosome preparation. Ag-NOR staining was performed using the "one-step" method of HOWELL and BLACK (1980). C-bands were produced using $Ba(OH)_2$ denaturation at 30° C for 15 s to 1 min, followed by incubation for 1 h in 2 x SSC at 65° C.

Measurement of chromosome lengths, the amounts of constitutive heterochromatin and estimation of the centromeric index was done from photographs of selected metaphase figures.

RESULTS

Morphometry

The procedure used for taxonomic classification of specimens examined started with morphometric analysis. The means and standard deviations for 13 calculated morphometric ratios of three water frog forms are summarized in Table 2. Two indices (DpPp/Cint and Cint/T) were found to be the most important discriminating features between the three species. One-way ANOVA analysis showed significant differences for the DpPp/Cint ($F = 30.74$, $p<0.05$), and Cint/T ($F = 15.10$, $p<0.05$) ratios. As judged by their morphological characteristics, sexual dimorphism was not found between males and females in the examined species.

TABLE 2 — Variation in measurements and ratios of species examined, standard deviations are given in paretheses.

Measurement or ratio	*R. ridibunda*	*R.* kl. *esculenta*	*R. lessonae*
L*	60.77 (5.32)	59.98 (10.35)	49.45 (6.02)
F*	27.20 (4.70)	30.48 (5.04)	23.93 (2.82)
L/T	2.09 (0.06)	2.07 (0.18)	2.26 (0.14)
L/LPp	0.59 (0.01)	0.56 (0.03)	0.59 (0.02)
F/T	0.93 (0.05)	1.03 (0.22)	1.07 (0.15)
Cint/T*	0.085 (0.002)	0.122 (0.013)	0.141 (0.015)
DpPp/Cint*	3.55 (0.39)	2.29 (0.23)	1.94 (0.26)
L/DpPp*	6.94 (0.42)	7.43 (0.57)	8.39 (0.82)
Lc/Ltc	0.89 (0.06)	0.88 (0.25)	0.89 (0.26)
Lc/Spp	6.19 (0.85)	5.53 (0.97)	5.69 (1.58)
Ltc/Spp	7.03 (1.46)	6.57 (1.52)	6.48 (1.40)
L/Ltc	3.06 (0.23)	3.00 (0.43)	3.10 (0.46)
Spcr/Spp	2.61 (0.59)	2.62 (0.49)	2.69 (0.61)
Dno/Spi	1.02 (0.02)	1.07 (0.19)	1.04 (0.36)
Spi/Dro	0.44 (0.01)	0.45 (0.06)	0.46 (0.11)

Longitudo corporalis (L), Longitudo femoris (F), Longitudo tibiae (T), Longitudo pedes posteriores (Lpp), Digitus primus pedes posteriores (DpPp), Callus internus (Cint), Longitudo capitis (Lc), Latitudo capitis (Ltc), Spatium palperbalis (Spp), Spatium canthi rostralis (Spcr), Distantia nasale oculi (Dno), Spatium internasale (Spi), Distantia rostri oculi (Dro). * = difference significant at 95% confidence level.

Protein electrophoresis

The relative electrophoretic mobility of the allelic products was used for taxonomic and diagnostic proposes. Four different allozyme phenotypes were found (Table 3., Fig. 2). The fastest alleles (a and b) for both, *Ldh-1* and *Aat* loci, were characteristic of individuals which were morphometrically identified as *Rana ridibunda*. The second allozyme phenotype was a marker for R. *lessonae* and included homozygotes for both, *Ldh-1* and *Aat* the slowest allele (c). Animals heterozygotes for both loci, were *R.* kl. *esculenta*. The fourth allozyme phenotype included individuals heterozygous for *Ldh-1* (a/c or b/c), and homozygous *ior Aat* (c/c). Between this group and specimens identified as *R. lessonae*, statistically significant differences in morphometry were not found. These two groups of *R. lessonae* had a single front band of fast mobility for *Alb*. Electromorphs of plasma albumin were displayed as slower-migrating bands (b and c) in R. *ridibunda*. *R.* kl. *esculenta* revealed a heterozygotic (a/b or a/c) phenotype for *Alb*. *R. shqiperica* differed from *R. ridibunda* in having a very slow-migrating (d) allele for *Ldh-1*. The other examined loci *(Mdh* and *Sod)* were monomorphic.

Cytogenetics

The karyotype of all the examined specimens consisted of $2n = 26$ biarmed chromosomes, with a first group of 5 large and a second group of 8 small chromosomes (Fig. 3). Chromosome pair No. 10 was characterized by a distinct secondary constriction in the long arm. The positions of the secondary constrictions corresponded with the NORs.

The quantitative karyotype characteristics of the examined species are summarized in Table 4. In contrast to the general morphological uniformity of the karyotypes of the examined species, ANOVA analysis showed significant differences in centromeric indices and relative chromosomal lengths in the group of large chromosomes. Namely, centromeric index for chromosome pair 1 is stable, but the relative chromosomal lengths showed significant differences between *lessonae* and *ridibunda* karyotype ($F = 8.87$, $p<0.01$), and *lessonae* and *shqiperica* karyotype ($F = 4.19$, $p<0.05$). The

TABLE 3 — Allele frequencies for three variable genie loci.

Species	Locality	Locus			LDH				AAT			Alb		
		Electrophoretic mobility			a	b	c	d	a	b	c	a	b	c
		Rf. velues			100	63	54	43	135	100	48	135	100	70
		N	m	f										
Rana ridibunda	Popovica	4	3	1	0.500	0.500	0.000	0.000	0.000	1.000	0.000	0.000	0.333	0.667
	Indjija	7	5	2	0.929	0.071	0.000	0.000	0.000	1.000	0.000	0.000	0.833	0.167
	Jarkovci	4	2	2	0.375	0.625	0.000	0.000	0.000	1.000	0.000	0.000	0.625	0.375
	Obedska Bara	3	1	2	1.000	0.000	0.000	0.000	0.000	0.834	0.166	0.000	1.000	0.000
	Čenta	2	–	2	0.250	0.750	0.000	0.000	0.000	1.000	0.000	0.000	0.500	0.500
	Deliblato Sands	13	4	9	0.692	0.308	0.000	0.000	0.077	0.923	0.000	0.000	0.692	0.308
	Čibuklija	4	2	2	0.375	0.625	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000
	Zlotska Gorge	3	1	2	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000
	Stubline	8	2	6	0.562	0.438	0.000	0.000	0.000	1.000	0.000	0.000	0.937	0.063
	Petnica	16	10	6	0.813	0.187	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000
	Lešnica	34	19	15	0.828	0.172	0.000	0.000	0.015	0.985	0.000	0.000	0.703	0.297
	Niš	11	3	8	1.000	0.000	0.000	0.000	0.180	0.820	0.000	0.000	0.650	0.350
	Stara planina	2	1	1	0.500	0.500	0.000	0.000	0.000	1.000	0.000	0.000	0.500	0.500
	Trgovište	5	5	–	0.900	0.100	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000
	Virpazar	14	12	2	1.000	0.000	0.000	0.000	0.036	0.964	0.000	0.000	1.000	0.000
	Ohrid Lake	5	3	2	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000
	Prespa Lake	5	4	1	1.000	0.000	0.000	0.000	0.200	0.800	0.000	0.000	1.000	0.000
	Pribačevo	5	2	3	1.000	0.000	0.000	0.000	0.100	0.900	0.000	0.000	1.000	0.000
Rana shqiperica	Virpazar	7	4	3	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000
Rana kl. *esculenta*	Obedska Bara	59	1	58	0.229	0.324	0.446	0.000	0.000	0.462	0.538	0.472	0.365	0.163
	Cortanovci	5	–	5	0.100	0.900	0.000	0.000	0.000	0.500	0.500	0.500	0.125	0.375
	Petrovaradin Marsh	9	1	8	0.111	0.667	0.222	0.000	0.000	0.500	0.500	0.500	0.375	0.125
	Pančevo Marsh	6	1	5	0.333	0.500	0.167	0.000	0.000	0.500	0.500	0.500	0.333	0.166
Rana lessonae	Obedska Bara	35	13	22	0.178	0.000	0.822	0.000	0.000	0.071	0.929	1.000	0.000	0.000
	Petrovaradin Marsh	5	4	1	0.000	0.200	0.800	0.000	0.000	0.000	1.000	1.000	0.000	0.000
	Pančevo Marsh	2	2	–	0.000	0.000	1.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000

difference between the centromeric index of chromosomal pair No. 2 ($F = 4.54$, $p<0.01$), and of the relative chromosomal lengths of chromosomal pairs No. 3 ($F = 3.49$, $p<0.05$), and No. 4 ($F = 7.22$, $p<0.01$) were significant between *R. lessonae* and *R. shqiperica.*

In the group of small chromosomes, variation in the quantitative chromosome characteristics were observed only in the 10th chromosome pair, due to the position of the secondary constrictions and NORs.

Comparative analysis of the distribution of heterochromatin provided information for the cytogenetic determination of individuals which were characterized by electrophoretic and morphometric phenotype as *Rana ridibunda.* The karyotype of R. *ridibunda* from all the localities, had a very clear C-band positive region in the short arm of the 3rd chromosome pair (Fig. 4). This marker was absent from karyotypes of *R.* kl. *esculenta* and *R. lessonae* but was just noticable in the karyotype of R. *shqiperica.*

The karyotype of R. kl. *esculenta* had regions of constitutive heterochromatin mostly in the centromeric and telomeric regions of chromosomes. In the karyotype of the hybrid form, R. kl. *esculenta,* we did not observe any differences in the distribution of C-bands between homologous chromosomes, however, homologous chromosomes No. 1 very often displayed differences in relative chromosome length (Fig. 3c). Statistical analysis by ANOVA revealed that the differences in the relative chromosomal lengths were significant ($F = 8.87$, $p<0.01$) between

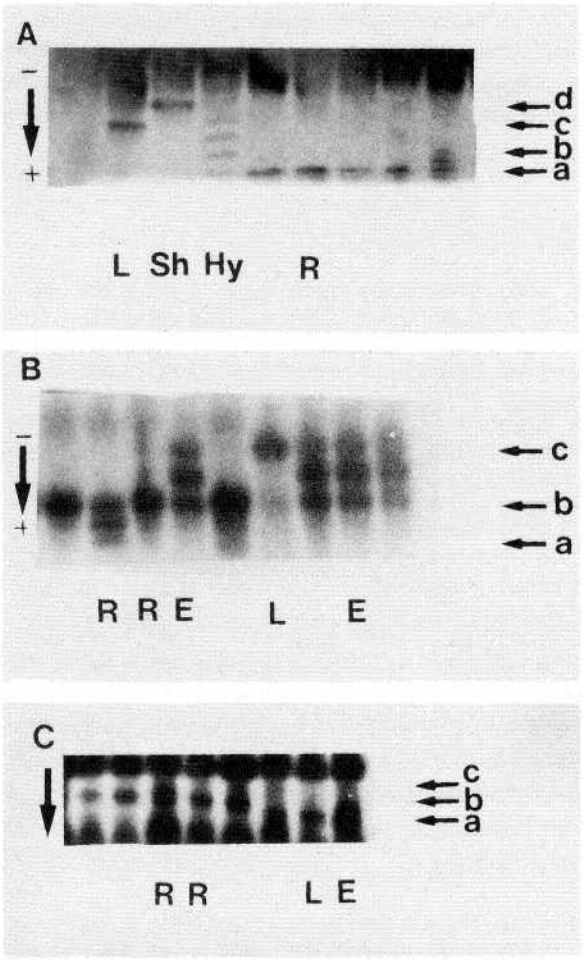

Fig. 2. — Electrophoretic patterns of two enzymes a) *Ldh-1,* b) *Aat,* c) albumin in populations of water frogs. R- *R. ridibunda;* L- *R. lessonae;* E- R. kl. *esculenta;* Sh- *R. shqiperica;* Hy- hybrid between *R. ridibunda* and R. *shqiperica.*

chromosomes No. 1 in karyotypes of *R. ridibunda* and R. *lessonae,* as well as between homologous chromosomes of 1st pair in *R.* kl. *esculenta.*

DISCUSSION

Allozyme identification of species of the R. kl. *esculenta complex*

Among the 293 frogs from different areas of Yugoslavia and Macedonia that were examined electrophoretically, four basic allozyme phenotypes were identified. The most important discriminating loci between the examined species were *Alb* and *Aat.*

Pure *R. ridibunda* populations were found south of the Sava and Danube Rivers. Fifty-nine specimens from the Obedska Bara locality, and 20 from other localities in Vojvodina (Northern Yugoslavia) were heterozygotes for all three of the examined polymorphous loci. According to their heterozygotic allozyme phenotype and morphology, those specimens represented *R.* kl. *esculenta.* In the whole R. kl. *esculenta* sample only three males (3.8%) were found.

In the examined sample of R. *lessonae* from the Obedska Bara locality 3 males and 10 females (35.7%) were heterozygous (a/c) for the *Ldh-1* locus, which means that a degree of introgression of the *ridibunda* allele (a) into the *lessonae* genome had occurred. Also, introgresion of the *ridibunda Ldh-1* allele (b) into R. *lessonae* was observed in the Petrovaradin Marsh locality. Moreover, the slow *lessonae Aat* allele was found in the R. *ridibunda* sample from the Obedska Bara locality. According to allozyme profiles suggested by BEERLI (1994), no natural polymorphism for *Ldh-1* or *Aat* was found in *lessonae* or *ridibunda* populations.

The results of enzyme studies have previously identified individuals with recombined genomes in several population systems (UZZELL and BERGER 1975; UZZELL *et al.* 1977; TUNNER 1979; PLOTNER 1990). Moreover, the results of morphometric investigations provided evidence that the number of recombined loci in R. kl. *esculenta* was probably higher then expected solely on the basis of enzymological studies (PLOTNER 1990). As a result of morphometric and DNA investigations of European water frogs, PLOTNER *et al.* (1994) suggested that R. kl. *esculenta* has a high inter-individual and population-specific variability.

Authors preparing taxonomic or systematic reviews of water frogs of the genus *Rana* that are based on the results of protein electrophoresis should take into consideration the occurrence of recombinations in R. kl. *esculenta* despite the proposed hybridogenetic mode of reproduction. It seems that the suggested allozyme profiles of species of the *Rana esculenta* complex are not generally applicable to natural populations. Therefore, the increase in the number of the "currently recognized" *Rana (Pelophilax)* species is probably due to the relatively small size of the analyzed samples. Mod-

TABLE 4 — Quantitative characteristics of chromosomes in karyotypes of the examined species: centromeric index/100 (length of short arm/length of whole chromosome), and percent length of chromosome pairs in relation to total genome length. Standard de viations are in parentheses.

Chromo-some	*Rana ridibunda*	*Rana* kl. *esculenta*	*Rana lessonae*	*Rana shqiperica*	Type of chromo-some	*Rana ridibunda*	*Rana* kl. *esculenta*	*Rana lessonae*	*Rana shqiperica*
	Centromeric index/100					Relative length of chromosomes (%)			
1	0.45 (0.01)	0.45 (0.01)	0.45 (0.01)	0.46 (0.01)	m	16.12 (0.32)	14.56 (0.82) 16.74 (0.79)	14.86 (0.93)	16.5 (0.7)
2	0.37 (0.03)	0.38 (0.02)	0.38 (0.02)	0.34 (0.03)	sm	12.95 (0.59)	13.29 (0.65)	12.82 (0.70)	13.3 (0.3)
3	0.30 (0.02)	0.31 (0.03)	0.31 (0.02)	0.32 (0.02)	sm	12.32 (0.40)	11.86 (0.55)	12.10 (0.50)	12.4 (0.6)
4	0.38 (0.03)	0.40 (0.03)	0.43 (0.03)	0.40 (0.02)	sm	11.26 (0.66)	11.21 (0.72)	10.99 (0.55)	11.6 (0.5)
5	0.42 (0.02)	0.44 (0.03)	0.43 (0.02)	0.42 (0.02)	m	9.59 (0.55)	9.67 (0.48)	9.70 (0.47)	9.6 (0.7)
6	0.43 (0.01)	0.44 (0.02)	0.43 (0.02)	0.41 (0.03)	m	5.64 (0.34)	6.03 (0.37)	5.68 (0.34)	5.9 (0.4)
7	0.24 (0.03)	0.25 (0.03)	0.25 (0.03)	0.24 (0.02)	st	5.17 (0.29)	5.42 (0.46)	5.51 (0.33)	4.8 (0.3)
8	0.39 (0.02)	0.39 (0.02)	0.38 (0.02)	0.40 (0.03)	sm	5.14 (0.40)	5.19 (0.30)	5.25 (0.38)	5.4 (0.4)
9	0.42 (0.03)	0.41 (0.03)	0.42 (0.03)	0.41 (0.02)	m	4.49 (0.35)	4.48 (0.40)	4.51 (1.48)	4.1 (0.3)
10	0.32 (0.02)	0.33 (0.02)	0.33 (0.01)	0.32 (0.03)	sm	4.85 (0.44)	4.91 (0.33)	5.14 (0.39)	5.0 (0.4)
11	0.29 (0.03)	0.29 (0.03)	0.28 (0.02)	0.26 (0.03)	st	4.51 (0.39)	4.56 (0.29)	4.83 (0.46)	4.3 (0.2)
12	0.35 (0.03)	0.34 (0.03)	0.34 (0.04)	0.33 (0.03)	sm	3.98 (0.34)	3.82 (0.34)	4.14 (0.38)	3.8 (0.3)
13	0.34 (0.03)	0.36 (0.04)	0.36 (0.04)	0.32 (0.02)	sm	3.41 (0.38)	3.48 (0.30)	3.75 (0.37)	3.0 (0.3)

m - metacentric
sm - submetacentric
st - subtelocentric

ern methods, such as RAPD identification (ZEISSET and BEEBEE 1998), will no doubt prove to be useful in resolving the presently confused state of water frog taxonomy that are due to frequent revisions of taxonomic concepts (DUBOIS and GUNTHER 1982; DUBOIS 1998).

Cytogenetic analysis

The cytogenetic evidence presented here cannot be used to support hybridogenesis *sensu stricto* as a mode of reproduction in the examined population systems. There is no difference in C-band patterns between homologous chromosomes in the hybridogenetic form *R.* kl. *esculenta*. The difference in the relative chromosomal lengths between homologous chromosomes No. 1 in the *R. esculenta* karyotype can be accepted as significant in view of the fact that this phenomenon is often found in the chromosome complement of *R.* kl. *esculenta* but not in the other three species. VINOGRADOV *et al.* (1990) based their studies on the finding that the -R. *ridibunda* genome contained 16 % more DNA than the *R. lessonae* genome. Perhaps this excess DNA is located mostly in the first and the largest chromosomal pair.

In contrast to many populations from Central Europe, where triploids were found (BERGER 1983; TUNNER and HEPPICH-TUNNER 1992; PLOTNER *et al.* 1994), it appears that only diploid forms exist in the examined part of the Balkan Peninsula.

The distribution of C-bands does not provide enough evidence for a cytogenetic identification of species from the *R.* kl. *esculenta* complex. No differences between the three taxa were detected in chromosome No. 11. This chromosome was karyologically characterized first by HEPPICH (1978). MIURA (1995) did not establish the existence of this difference in karyotypes of either *R. lessonae* or *R. ridibunda.* TUNNER and HEPPICH (1982) found C-bands on chromosome No. 2 in an intercalary position of the short arm in the karyotype of *R. ridibunda* from Greece. This is probably the same band as we observed (3p pc) in *R. ridibunda* from other localities. KOREF-SANTIBANEZ and GUNTHER (1980) showed in most metaphase plates that *R.* kl. *esculenta* had one metacentric *("lessonae"),* and one submetacentric *("ridibunda")* chromosome No. 12. In our examined samples we did not establish any differences in chromosome morphology, except for chromosome pair No. 1.

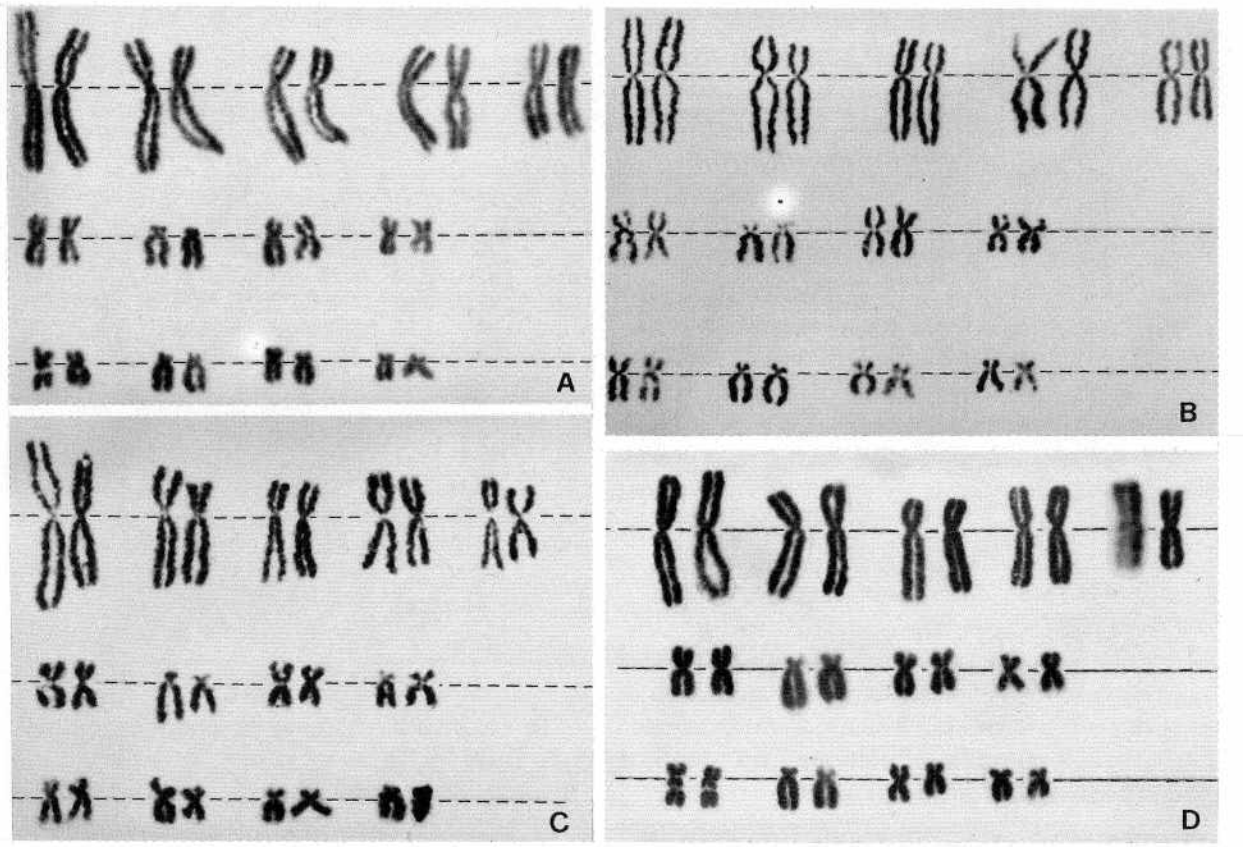

Fig. 3. — Giemsa-stained karyotypes of a) *Rana ridibunda,* b) *R. shqiperica,* c) R. kl. *esculenta* and d) R. *lessonae.*

Sex chromosomes in R. kl. *esculenta and Haldane's rule*

The analysis of BrdU-replication patterns by SCHEMPP and SCHMID (1981) demonstrated the presence of sex-specific chromosomes of the XX/XY type in R. kl. *esculenta.* They observed an extremely late replicating region in the Y chromosome; such a late replicating region is not found in the X chromosome. In thekaryotype *of Rana* kl. *esculenta* homologous sex chromosomes do not show any heteromorphisms in either Q-, C- or R-banding patterns.

According to the work of TUNNER and HEP-PICH (1982) and the data presented here, we propose that the chromosome pair No. 3 in *R. ridibunda* karyotype is similar to the sex chromosome pair No. 4 described by SCHEMPP and SCHMID (1981). The chromosomal pair No. 3 belongs to the large chromosomal group with the smallest centromeric index and a very prominent C-positive heterochromatin at 3p pc. Moreover, the 3p pc band is characteristic of the R. *ridibunda* karyotype (SCHMID 1978b; TUNNER and HEPPICH 1982; data presented here) and is sometimes observed in R. *shqiperica.* In view of the karyotype characteristics of R. kl. *esculenta* described by SCHEMPP and SCHMID (1981) and the fact that animals from Macedonia were used, it is likely that the authors actually studied frogs that belonged to R. *ridibunda* rather than to R. kl. *esculenta.* Our electrophoretically identified specimens from Macedonia were all R. *ridibunda,* except proportion of alleged R. *ridibunda* x R. *balcanica* hybrids (unpublished observations).

Regardless of whether the sex chromosomes belonged to the 3rd or 4th chromosomal pairs or whether they were identified in R. kl. *esculenta* or R. *ridibunda,* in the majority of species

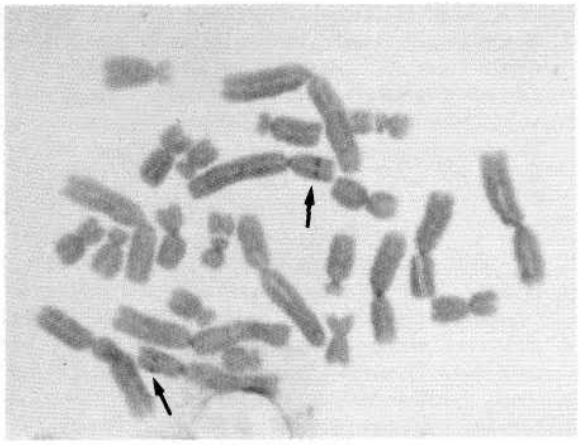

Fig. 4. — C-banded chromosomes of *R. ridibunda;* arrows show 3p pc band.

of the genus *Rana* the males are the heterogametic sex (HiLLis and GREEN, 1990; MIURA, 1994). A general observation pointed out by HALDANE (1922) is that if one sex is absent, rare or infertile in an interspecific cross, it is always the heterogametic sex (Haldane's rule). Despite READ and NEE (1991) who have expressed doubts about the significance of Haldane's rule, many examples of sex-specific effects in interspecies crosses have been reported. They have been noted in: *Lepidoptera* (SPERLING 1994), *Drosophila* (ORR 1987; CABOT *et al.* 1994; FORD and AQUADRO 1996), birds and mammals (ORR 1997) and in plants (GERSTEL 1954; CHRISTIE and MACNAIR 1984). In hetrepetofauna, Haldane's rule was described in salamanders (male heterogamety) of the genus *Triturus* (SPURWAY 1953), and in interspecific crosses between lizards of the genus *Lacerta,* where hybrid sterility occurs in heterogametic females (RYKENA 1991).

Since among the 79 electrophoterically, morphometrically and cytogenetically characterized specimens of -R. kl. *esculenta* from Vojvodina (Northern Yugoslavia or the region north of the Sava and Danube Rivers) only 3.8% males were found, we can assume that Haldane's rule operated in this particular hybrid form.

Population composition of population systems of water frogs from Yugoslavia

UZZELL and BERGER (1975) made the first classification of the water frog population systems according to genotypes. They recognized the *lessonae/esculenta* (L-E), *ridibunda/esculenta* (R-E) and pure *esculenta* (E) systems. A more detailed classification of the recently identified population systems according to geno-typic structures and sex ratios was provided by PLOTNER and GRUNWALD (1991).

The most common population system is the L-E system (UZZELL and BERGER 1975). Less frequent are populations where *R.* kl. *esculenta* is associated with *R. ridibunda* in the R-E system. Pure hybrid populations exist in Germany, Poland, Sweden and Denmark (EBENDAL 1979; BERGER 1988; GUNTHER 1991; PLOTNER and GRUNNALD 1991). In Hungary, TUNNER and HEPICH-TUNNER (1992) found a new population system of water frogs which consists of *R. ridibunda* and 3n *R.* kl. *esculenta* males. GUBANY and KOR-sos (1992) analyzed Hungarian water frogs from L-E populations.

According to TUNNER and HEPPICH-TUNNER (1992), there is variety of differently structured populations of water frogs. It includes *R. lessonae,* and *R.* kl *esculenta 2n* or 3n males and females in different sex ratios.

The population system from the Obedska Bara locality consists of *2n R. lessonae* males and females, *2n R.* kl. *esculenta* females and rare males and very rare *2n R. ridibunda* males and females. Generally, population systems of water frogs in Vojvodina consists of only pure *R. ridibunda* populations, or L-E-R systems in which *R. ridibunda,* as well as, *R.* kl. *esculenta* males are rare.

Reproductive mode in R. kl. *esculenta*

If *R.* kl. *esculenta* clonally inherited its genome, it would be more uniform in its characteristics than the parental species. Increasing genetic diversity in *R.* kl. *esculenta* was demonstrated by the results of morphometric studies, DNA fingerprint data, allozyme analyses and their ecological plasticity (UZZELL and BERGER

1975; PLOTNER and GRUNWALD 1991; PLOTNER *et al.* 1994; data presented here).

The following characteristics of the reproductive and inheritance modes in *R.* kl. *esculenta* can be summarized as follows:

1. Genetic diversity in clonally reproducing "species" can be caused by accumulation of mutations in the clonally inherited genome (HoTZ 1983), or as a result of variation in the genome of the syntopic parental species.

2. Interspecific crosses lead to the production of many recombinant gametes and zygotes, but can be eliminated by gametic selection or by selective mortality. In some populations gametocytes in which recombinations occur do not form functional gametes (UZZELL *et al.* 1977). The rare true hybrids male examined here and in Central Europe (TUNNER 1974; BERGER and BERGER 1992) can also be included in gametic and/or zygotic selection, as well as sexual disbalance in hybrid forms whose fitness depends on the ecological and population-specific genetic factors.

UZZELL *et al.* (1980) concluded that the *R. ridibunda* genome contains factors which are responsible for the induction of hybridogen-esis. Moreover, *R. ridibunda* varies geographically in its capacity for excluding one genome in hybrids (Hoxz *et al.* 1985; GUERRINI *et al.* 1997). Further investigation will provide more information and an estimate of the levels of recombinations between the two parental genomes. However, questions dealing with hybridogenesis as a mode of reproduction, and hemiclonal inheritance in the *R.* kl. *esculenta* complex as a mechanism which prevents and neutralizes recombinations remain unanswered.

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