SALAMANDRA **54**(1) | 11–20 | 15 February 2018 | ISSN 0036–3375

Composition and age structure of the *Pelophylax esculentus* complex (Anura; Ranidae) population in inland Croatia

Kristina Čavlović[1], Ivana Buj[2], Dean Karaica[3], Dušan Jelić[4] & Lukáš Choleva[5,6]

[1)] Division for human reproduction, Department of Obstetrics and Gynecology, Clinical hospital Center Rijeka, Cambierieva 17/5, 51000 Rijeka, Croatia

[2)] Department of Zoology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia

[3)] Molecular Toxicology Unit, Institute for Medical Research and Occupational Health, 10000 Zagreb, Croatia

[4)] Croatian Institute for Biodiversity, Croatian Herpetological Society HYLA, Lipovac I., no 7, 10000 Zagreb, Croatia

[5)] Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics of the CAS, v. v. i., Academy of Sciences of the Czech Republic, Rumburská 89, 27721 Liběchov, Czech Republic

[6)] Department of Biology and Ecology, Faculty of Science, University of Ostrava, Chittussiho 10, 710 00 Ostrava, Czech Republic

Corresponding author: Kristina Čavlović, e-mail: kristina.cavlovic2@gmail.com

Manuscript received: 12 July 2016
Accepted: 8 June 2017 by Stefan Lötters

Abstract. *Pelophylax esculentus* is a vertebrate animal with hemiclonal heredity, attracting the interest of many reproductive and evolutionary biologists. It is a hybrid between *P. ridibundus* and *P. lessonae*. These three taxa form the so-called *Pelophylax esculentus* complex with a population structure usually comprising a hybrid taxa and one parental species. Data on population types at the southernmost distribution area of their sympatry are rare. Here we sampled five sites in inland Croatia in order to analyse the population structure, sex ratio and age structure. The individual genotypes of 93 randomly collected water frogs were verified with allozyme markers for three species-specific polymorphic loci. In order to estimate population age structure, the annual growth rate (skeletochronology) and growth index profiles were also investigated. The growth index profiles were analysed by an estimation of number of lines of arrested growth visible in the cross-section of femur bones. Our results revealed the presence of the R-E-L population with a dominance of *P. esculentus*. *Pelophylax ridibundus* was the least abundant taxon but with a relatively high age estimate of eight years on average. Its annual growth rate did not differ from the remaining two species. Gene introgression of mostly *ridibundus* alleles was also observed in hybrids. Most profiles of gonads in hybrids showed presence of both parental genomes with dominance of *ridibundus* alleles. The study area represents one of the southernmost distributions of the hybrid taxon in Europe, making it attractive to study gene flow and impact of *P. esculentus* on *P. ridibundus*, a typical water frog representative of the Balkan Peninsula.

Key words. Hybridogenesis, population composition, allozyme markers, hemiclonal reproduction, gene introgression, skeletochronology.

Introduction

The Edible Frog, *Pelophylax esculentus* (Linnaeus, 1758), is of particular interest to evolutionary biologists and population ecologists because of its hybrid origin and unusual reproduction mode. It represents a natural hybrid taxon between the Pool Frog, *P. lessonae* (Camerano, 1882), and the Marsh Frog, *P. ridibundus* (Pallas, 1771). The genomic constitutions of the parental species are denoted as LL and RR, respectively. *Pelophylax esculentus* reproduces via hybridogenesis. In this hemiclonal reproduction, a clonal copy of either *lessonae* or *ridibundus* genome is transmitted to gametes (Graf & Polls-Pelaz 1989, Bergen et al. 1997, Holenweg Peter et al. 2002, Pruvost et al. 2013). The other genome is excluded during the first division of gametogenesis (Tunner & Heppich-Tunner 1991). Therefore, hybrids have to backcross with a parental species to receive an excluded genome and produce a new generation of hybrids (Graf 1986, Semlitsch et al. 1996, Vorburger & Reyer 2003, Christiansen & Reyer 2011). As a result, *P. esculentus* maintains a permanent F1 genotypic constitution.

The compositions of population systems in water frogs vary throughout Europe (Plötner et al. 1994, Holsbeek & Jooris 2009, Christiansen & Reyer 2011, Mayer et al. 2013, Pruvost et al. 2013, Hoffmann et al. 2015). The type of population system depends on the environmental variables and genetic background of the hybrids (Sas 2010). *Pelophylax esculentus* usually has a diploid constitution and persists in mixed populations with *P. lessonae* (here called

the L-E system) or *P. ridibundus* (the R-E system) (Graf & Polls-Pelaz 1989). Alternative population systems such as those that involve the presence of triploid individuals (LLR and LRR) forming all-hybrid populations (E-E system) are also known, mostly from northwestern Europe (Christiansen & Reyer 2011, Pruvost et al. 2013, Pruvost et al. 2015, Hoffmann et al. 2015). The L-E system represents the most widespread population type (Graf & Polls-Pelaz 1989). Relatively rare are populations where all three taxa coexist within the so-called R-E-L system found in Latvia (Borkin et al. 1986), Switzerland (Hotz et al. 1992), Hungary (Gubányi 1992), central and northwestern Ukraine (Mezhzherin & Morozov-Leonov 1993), Czech Republic (Kotlík & Šůlová 1994), European Russia (Lada et al. 1995, Borkin et al. 1986, 2002), western Germany (Schröer & Greven 1998), Romania (Sas 2010), Slovakia (Pruvost et al. 2013, Mikulíček et al. 2014), northern Serbia (Spašić-Bošković et al. 1999, Krizmanić & Ivanović 2010) and Croatia (Berger et al. 1988). *Pelophylax ridibundus* is assumed to be ethologically isolated in the R-E-L system (Hotz et al. 1992). It is known that in some cases *P. ridibundus* migrates toward ponds with *P. lessonae* and *P. esculentus* during the breeding period converting the L-E system into the R-E-L system (Sas 2010).

After the initial recognition that *P. esculentus* is of a hybrid origin (Berger 1966, 1967), a number of studies were performed on morphological investigations, experimental hybridizations (e.g. Berger 1968, Günther 1973, Ebendal 1979), cytological analyses (e.g. Heppich & Tuner 1979, Heppich et al. 1982) and molecular genetic analyses (e.g. Engelman 1972, Graf & Müller 1979, Graf & Polls-Pelaz 1989, Hauswaldt et al. 2012) mostly on populations from central, western and northwestern Europe (Plötner 2005, Pruvost et al. 2015 and citations therein).

Considering *P. esculentus* hybrid´s ability to serve as a vehicle for the transfer of *P. lessonae* genes into *P. ridibundus* in the area of sympatry (Plötner et al. 2008), suggesting the impact of hybrids on the parental taxon especially north from the Balkan Peninsula, our knowledge on *P. esculentus* distribution is important. Previous studies suggested that water frogs are indigenous in Croatia (Jelić et al. 2012, Jelić 2014) and morphological analyses point out a presence of all three taxa in the region (Gasc et al. 1997, Jelić et al. 2012, Karaica et al. 2016). In this investigation we aimed to describe the population structure of the *P. esculentus* complex in the inland part of Croatia from different aspects. Its taxonomic composition was investigated by means of allozyme analyses, whereas skeletochronology was employed in order to investigate the age structure of the complex population in question. Furthermore, we have analysed the sex structure of this population. All investigation aspects: genetic, age and sex structure, are important in describing a status of a certain population, in particular its ecological relationships and stability. Since population structure and demographic parameters have not been analysed for populations in Croatia, the unexplored southern limits of this complex' range, we believe that obtained data could contribute significantly not only in widening knowledge on this unusual biodiversity component, but also for making scientific bases for its conservation.

Materials and methods

Sampling

The sample sites have been described in detail by Karaica et al. (2016). In brief, the sampling took place near the Ilova River (Danube River system) in the northwestern part of Croatia (Bjelovar-Bilogora County; 100–125 m a.s.l.) at five localities described as follows: the pond near the fish hatchery (Grubišno polje: 45°42'31" N 17°08'52"E), two sites located near the Ilova River channel (Ulovčev mlin: 45°39'54"N, 17°13'36"E; Mali Zdenci: 45°40'10"N, 17°08'26"E), and the wet meadows near the brook (Velika Barna: 45°44'09"N, 17°06'53"E; Velika Jasenovača: 45°43'41"N, 17°06'01"E). Distances between the localities ranged from 4.5 to 8 km. The collection of samples was approved and carried out with the permission of the Directorate for Nature Protection, Ministry of Culture of the Republic of Croatia (permit number given in the acknowledgements). Frogs were collected by a hand net during day or night. Sex was determined by the presence or absence of vocal sac openings, thumb pads and/or by direct inspection of gonads. Those frogs that had not yet developed the external sex characters and, simultaneously, their inspection of gonads were not done, we further call as subadults. Taxon identification was first based on external morphological characters (Günther 1990, Plötner 2005). The results of our morphology investigation were in details described in Karaica et al. (2016). The determination of the species was later verified by isozyme (allozyme) electrophoresis. Samples of somatic and gonadal tissues were isolated and frozen at -20°C for further genetic observation. A Chi-square test was used in order to detect possible differences between the observed population sex ratio and the expected 1:1 sex ratio. Software Packages Microsoft© Excel 2010 and Statsoft© Statistica Version 7 were employed for data analyses. The frogs were marked and preserved in 75% ethanol at the Department of Zoology, Faculty of Science, University of Zagreb.

Allozyme analysis

Approximately 1cm^3 of skeletal muscles and gonads were crushed separately and homogenised in an equal volume of pH 8.5 Tris NaCl extraction buffer (Valenta et al. 1971, Appendix II). Following cold centrifugation at 4°C, equal volumes of the supernatant were mixed. The mixture was frozen overnight in a deep freezer at -70°C. The enzymes were separated horizontally using gel electrophoresis method in a refrigerator at 4°C. The enzymes were separated on 11% starch gel using a pH 8.6 Tris-citrate buffer system (Valenta et al. 1971, Appendix II) and pH 6.0 Tris-citrate acid electrode buffer (Uzzell & Berger 1975, Appen-

dix II). The supernatants mixtures were applied to the gels on filter paper tabs (Whatman no 3.). The investigated loci were already described as polymorphic and species-specific (Uzzell & Berger 1975, Beerli 1994) for the following enzymes: aspartate aminotransferase (Aat; EC 2.6.1.1), lactate dehydrogenase (Ldh-1; EC 1.1.1.27) and phosphoglucomutase (Pgm-2; EC 5.4.2.2). The enzymes were stained using procedures similar to those described by Harris & Hopkinson (1976), Buth & Murphy (1980) and Pasteur et al. (1987, Appendix II). We used a previously determined water frog as a biological standard for allele mobility at every allozyme run. The allele homology was identified after Kotlík & Šůlová (1994) for Aat, Uzzell et al. (1980) for Pgm-2, and Vorburger (2001) and Vorburger & Ulrich-Reyer (2003) for Ldh-1. In our analysis, we assumed that bands with equivalent mobility are controlled by the same allele. Stained gels were photographed. The fastest visualised allele products were designated as a. Samples that revealed unclear patterns were reprocessed again. The relative intensity of bands has also been studied to estimate how many copies of the gene coding the product were expressed in the tissue of each individual. Here, the asymmetrical staining intensity would potentially correspond to triploids (Tunner 2000). Allele frequency was measured as the proportion of a particular allele among all of the allele copies being considered.

Skeletochronology

In order to estimate the age of the 93 genotyped water frogs from our population sample, we used a skeletochronology method that has already been applied on a variety of amphibian species from temperate and tropical regions (Kumbar & Pancharatna 2001, Lin & Hou 2002, Yilmaz et al. 2005, Guarino et al. 2008). During dissection, we successfully isolated 81 femoral bones without any damage, while the remaining 12 bones had damaged diaphysis and were excluded from the analysis. The bones were stored in 70% ethanol for further processing. Each bone was then transferred into distilled water for 24 h, fixed with 10% formalin for 24–48 h and decalcified with Rapid Decalcifier solution (Apex Engineering Product Corporation) for 5–8 h. After decalcification, the residual decalcifying solution was removed under running tap water. The processed bone tissue was cross-sectioned at a thickness of 20–25 μm at -25°C using a Leica Cryocut 1800 cryostat. These cross-sections were stained with Ehrlich's hematoxylin in the dark for 5–10 min, rinsed in water two times for another 5–10 min, then mounted in a drop of glycerine gelatine on glass microscope slides and covered with cover slides. Sections were examined with a Zeiss AxioVert 200 microscope (Carl Zeiss Microimaging) and photographed with a mounted Zeiss Axiocam MRC camera using Carl Zeiss Axiovision Rel.4.6 software (Carl Zeiss Microimaging GmbH).

Estimations of individuals' age were done on at least four mid-diaphysis sections which have the thickest cortical bone. The assumption was that the number of bright zones followed by so-called dark lines of arrested growth (LAGs; developed during hibernation period) seen in the periosteal bone represents growth during a single year (Castanet & Smirina 1990, Erismis 2011). Lines of arrested growths on bone sections were difficult to read in two individuals (one from *P. ridibundus* and one from *P. esculentus*), thus they were not included in the statistical analysis. While examining the photographs of the bone cross-sections, two authors counted the LAGs independently. When discrepancies in the estimated number of LAGs occurred, the authors rechecked photographs of that particular cross-section and came to a consensus regarding the final number. Nonparametric statistical Kruskal-Wallis test ($\alpha = 0.05$) was used to detect possible differences in estimated age (age structure) and annual growth rate data (total body length/estimated age ratio) between all three water frog species in the studied *Pelophylax* complex population.

Results

Population composition

All three taxa, *P. lessonae*, *P. ridibundus* and *P. esculentus* of both sexes, were found in the Ilova River drainage, Croatia. Out of the 109 specimens caught, 93 (85.3%) in total were electrophoretically determined with species-specific allozyme markers. The remaining 16 specimens (14.7%) were not analysed due to a lack of tissue for analysis or inactivity of somatic tissue enzymes. Using the allozyme markers, the majority of specimens (66.7%) were identified as *P. esculentus*, while 25.8% belonged to *P. lessonae* and 7.5% to *P. ridibundus* (Fig. 1).

Out of the 93 frogs, 61 were adult frogs from which 26 were females (27.9% of totally collected frogs) and 35 were males (37.6% of totally collected frogs). There were 32 subadults where sex determination was impossible (34.4%). Female to male sex ratios did not differ significantly from the expected 1:1 ratio (Chi-square, df = 1, p = 0.25). Among the collected *P. esculentus* (n = 62) individuals, 27.4% were females (n = 17), 37.1% were males (n = 23) and 35.5% were subadults (n = 22). Out of the 24 *P. lessonae* individuals we

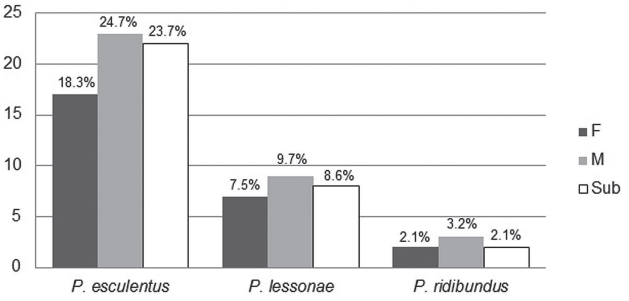

Figure 1. Sex composition and proportion of subadult individuals of the studied *Pelophylax* individuals in Croatia. Abbreviations: F – females, M – males, Sub – subadults.

Table 1. The allele frequencies investigated at the three loci for *Pelophylax ridibundus, P. lessonae* and *P. esculentus*. Abbreviations: Aat – aspartate aminotransferase, Ldh-1 – lactate dehydrogenase, Pgm-2 – phosphoglucomutase.

Locus	Aat		Pgm-2		Ldh-1			
Allele	a	b	b	c	a	b	c	d
P. ridibundus	1.000	0.000	0.000	1.000	0.250	0.000	0.750	0.000
P. lessonae	0.000	1.000	1.000	0.000	0.000	0.429	0.000	0.571
P. esculentus	0.510	0.490	0.578	0.422	0.096	0.061	0.588	0.254

identified, 29.2% were females (n = 7), 37.5% males (n = 9), and 33.3% subadults (n = 8). *Pelophylax ridibundus* (n = 7) consisted of 28.6% females (n = 2), 42.8% males (n = 3), and 28.6% subadult individuals (n = 2) (Fig. 1).

Based on the pattern of the species-specific allozyme loci, three *P. ridibundus* males, six *P. lessonae* (four males, two females) and 29 *P. esculentus* (14 males, 9 females and six subadults) were sampled in Grubišno polje, 16 *P. lessonae* (five males, three females, and eight subadults) and 25 *P. esculentus* (three males, seven females and 15 subadults) were sampled at Ulovčev mlin. Two subadult *P. ridibundus*, two *P. lessonae* females and seven *P. esculentus* (six males and one female) were found at the locality of Mali Zdenci. A single subadult *P. esculentus* was found at Velika Barna and two *P. ridibundus* females were found at Velika Jasenovača.

Allozyme genotype composition

Aat. Two alleles typical for both species were found (a and b). All of the *P. ridibundus* were homozygous for the allele a, while all of the *P. lessonae* were homozygous for the allele b. Most of the *P. esculentus* individuals showed a heterozygous combination (a/b), while one individual had a homozygous combination typical for *P. ridibundus* (a/a). The staining intensity of bands did not appear to differ between alleles.

Pgm-2. Two alleles (b and c) were found. Parental species showed homozygous allele combinations for this locus. *Pelophylax lessonae* individuals expressed the faster moving allele b, while *P. ridibundus* individuals showed the expression of the slower allele c. Hybrids showed heterozygosity in most cases, but there were 9 hybrids expressing alleles typical for *P. lessonae* (b/b) individuals, while only two contained a homozygous combination typical for *P. ridibundus* (c/c). The staining intensity of bands was symmetrical.

Ldh-1. At this locus, four alleles were found (a, b, c and d). The investigated *P. ridibundus* individuals expressed c/c and a/c genotypes, while *P. lessonae* had b/b, d/d or b/d allele combinations. Hybrids showed all kinds of variations of heterozygosity between the two parental species (a/d, b/c and c/d), except in two individuals that had an expression of a/c alleles and 14 individuals that had an expression of c/c alleles typical for *P. ridibundus*. The staining intensity of bands was asymmetrical in 14 hybrid individuals, of which one frog showed b/**c**, one a/**c**, four **a**/d and eight individuals expressed the **c**/d allele combination pattern (more intensely stained bands on the gel electrophoresis are in bold). This phenomenon was observed in 34.8% males and 11.8% females.

Table 2. Summary of detected allele gene introgressions in *Pelophylax esculentus*. The underlined letters represent alleles specific for *P. ridibundus*; non-underlined alleles typical for *P. lessonae*. Abbreviations: N – number of individuals; Aat – aspartate aminotransferase, Pgm-2 – phosphoglucomutase, Ldh-1 – lactate dehydrogenase.

N	Aat	Pgm-2	Ldh-1
1	a/a		
9		b/b	
2		c/c	
14			c/c
2			a/c
1			a/**c**

The allele frequencies for each taxon are summarized in Table 1.

The gene introgression (gene flow from one taxon to another) was found in 24 hybrid individuals among the three loci examined (38.7% of all hybrids). The mostly introgressed was the Ldh-1 locus with 17 individuals expressing *P. ridibundus*-like alleles only. There were 14 hybrids homozygous for the allele c (Grubišno polje, Ulovčev mlin), whereas two individuals (Mali Zdenci) were heterozygous with the a/c allele combination, and one with the a/**c** allele combination. At the Pgm-2 locus, 9 hybrid individuals (Grubišno polje, Ulovčev mlin) expressed b/b alleles typical for *P. lessonae*, while two individuals (Grubišno polje, Ulovčev mlin) expressed the *P. ridibundus*-like variant (c/c). A single introgression was recorded in the Aat locus at *P. esculentus* (Mali Zdenci) with the a/a pattern typical for *P. ridibundus* (Table 2). No gene introgression from *P. ridibundus* into *P. lessonae* and vice versa was found among the loci.

Hemiclonal reproduction

Pelophylax esculentus specimens were examined for allelic activity in testes and ovaries for three loci to estimate a basic type of a gamete production in hybridogenetic hybrids.

Table 3. The expression of alleles for investigated loci in gonads of *Pelophylax esculentus*. Abbreviations: F – females, M – males, Aat – aspartate aminotransferase, Pgm-2 – phosphoglucomutase, Ldh-1 – lactate dehydrogenase; underlined alleles typical for *P. ridibundus*; non-underlined alleles typical for *P. lessonae*. More intensely expressed alleles are in bold.

Locality	Ulovčev mlin		Mali Zdenci		Grubišno polje	
Allele	F (n=7)	M (n=3)	F (n=1)	M (n=6)	F (n=9)	M (n=14)
Aat	5 aa 1 ab	2 ab		4 ab	5 aa 2 ab	10 ab
Pgm-2	3 bc 2 bb 1 cc	2 bc	1 bc	4 bc	4 bc 3 cc	7 bc 3 bb
Ldh-1	7 cc	2 **c**d	1 ac	1 **c**d 1 cd 2 a**d**	6 cc 1 **a**d	3 **c**d 2 cd 2 b**c** 2 cc 2 **a**d

Aat. Female hybrids showed a heterozygous somatic allele combination a/b except a single individual with the a/a allele combination from Mali Zdenci. Females from Grubišno polje and Ulovčev mlin translated the a/a allele combination in ovaries typical for *P. ridibundus* except for three of them that had the a/b allele combination. The individual with the a/a allele somatic combination showed inactivity in oocytes for this locus. Males had somatic a/b allele combination with the same pattern in the testes.

Pgm-2. Most hybrid females showed a heterozygous b/c pattern both in somatic tissues and ovaries, except for three females (Grubišno polje) expressing c/c profiles typical for *P. ridibundus*. The females with b/b or c/c allele combination (Ulovčev mlin) in the somatic tissue displayed the same pattern in the ovaries. All hybrid males expressing b/c alleles in the somatic cells showed the same expression in their testes (Grubišno polje, Ulovčev mlin, Mali Zdenci). Likewise, the males from Grubišno polje showing a somatic b/b combination displayed the same allelic pattern in the gonads.

Ldh-1. Females with somatic c/c allele combination expressed the c/c pattern in gonads, as typical for *P. ridibundus* genome (Grubišno polje, Ulovčev mlin). A single individual shared a heterozygous a/c allele combination both in somatic tissues and gonads (Mali Zdenci). One hybrid female from Grubišno polje with the **a**/d allele somatic combination showed the same expression in ovaries. There was also one female from the same locality showing a b/**c** allele somatic expression, while the only c allele was visible in the gonads.

The hybrid males with the c/d allele somatic combination showed c/d (Grubišno polje, Mali Zdenci) or c/c (Grubišno polje) allele combinations in testes. Two males from Mali Zdenci with the somatic combination a/c and a/d showed the a/**d** pattern in gonads. Two males from Grubišno polje showing the b/c somatic allele combination expressed the b/**c** profile in their gonads. All males expressing **c**/d (Ulovčev mlin, Mali Zdenci, Grubišno polje) or **a**/d (Grubišno polje) somatic allele combinations had the same variant in testes.

Most *P. esculentus* testes (89.8%) had both parental alleles in their profiles, although *P. ridibundus*-specific allele was usually more intense; in total 6.1% had the *P. lessonae*-like pattern and 4.1% showed the *P. ridibundus*-like pattern. The most common pattern in the ovaries of hybrid females was that of the *P. ridibundus*-like type (66.6%). In 4.8%, the *P. lessonae*-like genome was expressed, while other gonads showed the pattern typical for hybrids (28.6%). Still, those alleles coming from *P. ridibundus* were more intense. Data are summarized in Table 3.

Age structure and annual growth rate

The age structure of the studied water frogs is summarized in Figure 2, whereas Figures 3 and 4 show a distribution of the estimated age and annual growth rate, respectively. The majority of *P. ridibundus* individuals (66.6%) were at least seven years old. In contrast, 90% of the *P. lessonae* and *P. esculentus* were placed into the age group between one

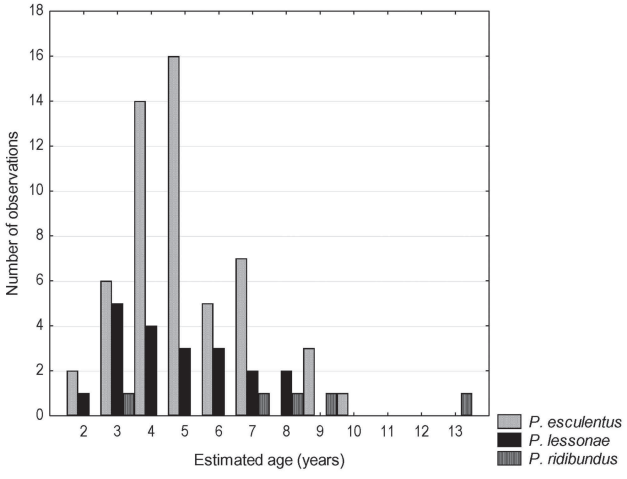

Figure 2. The age structure of the investigated *Pelophylax* individuals in Croatia.

Table 4. Summary of age estimates and number of lines of arrested growth (LAGs) of *Pelophylax* individuals. Abbreviation: n – number of individuals.

Estimated age / Number of LAGs	*P. esculentus* / n	*P. ridibundus* / n	*P. lessonae* / n
2	2	0	1
3	6	1	5
4	14	0	4
5	16	0	3
6	5	0	3
7	7	1	2
8	0	1	2
9	3	1	0
10	1	0	0
13	0	1	0
Average age (mean ± standard error)	5.1 ± 0.24	8.0 ± 1.61	4.8 ± 0.40
Standard deviation	1.75	3.60	1.79
Total number	54	5	20

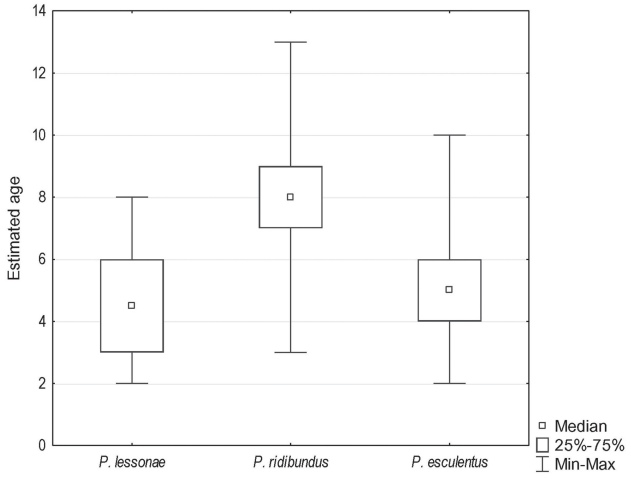

Figure 3. Box plots with the distribution of estimated age of the studied *Pelophylax* individuals in Croatia.

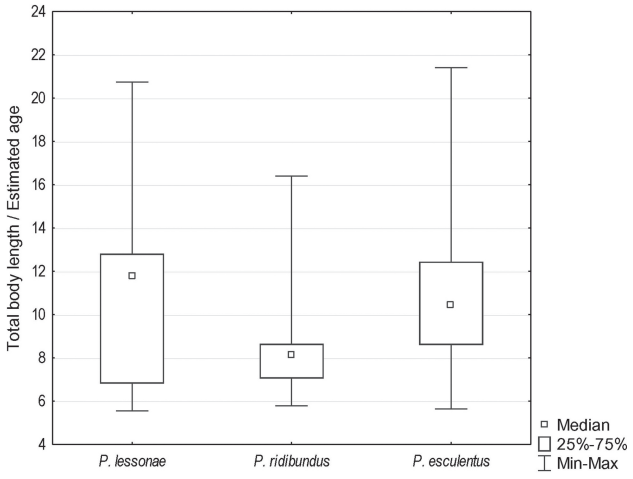

Figure 4. Box plots with the annual growth rate (total body length/estimated age ratio) distribution for the studied *Pelophylax* populations in Croatia.

and seven years. The youngest individuals were identified as being two years old (*P. lessonae* and *P. esculentus*), while the oldest frog was a *P. ridibundus* individual that had 13 years. The oldest *P. esculentus* and *P. lessonae* individuals were estimated to have 10 years and eight years, respectively. The average age was 8.0 ± 1.61 years for *P. ridibundus* (n = 5; Table 4), 4.8 ± 0.40 years for *P. lessonae* (n = 20), and 5.1 ± 0.24 years for *P. esculentus* (n = 54). We did not find a statistically significant difference between taxa in their age (n = 79, p = 0.096; Kruskal-Wallis test), nor in their annual growth rate (n = 79, p = 0.47).

Discussion

Pelophylax water frogs can easily move from one pond to another at least within 2.5 km distance (Juszczyk 1953). Distances between the studied localities are generally small and, therefore, it is likely that we studied a single population rather than several isolated populations. Genetic analyses supported the presence of all three taxa, *P. lessonae, P. esculentus* and *P. ridibundus*, so we classify the population as the R-E-L system.

As in our case, hybrids usually dominate in most of the investigated R-E-L systems, likely due to their better vitality in the F1 hybrid state linked to a wider ecological tolerance when compared to the parental species (Kotlík & Šůlová 1994, Spašić-Bošković et al. 1999, Borkin et al. 2002, Mayer et al. 2013). The representation of the investigated population is described as follows: *P. esculentus*: *P. lessonae*: *P. ridibundus* = 9:3:1.

The staining intensity of bands was asymmetrical in hybrid specimens for the Ldh-1 locus only. As we do not have other data measuring ploidy level, we cannot conclude yet

that the gene dose pattern observed is an effect of polyploidy.

Although distinguishing between incomplete lineage sorting and genomic introgressions is a difficult task (Choleva et al. 2014), the episodic appearance of hybrid individuals with the recombinant genotype associated with observations of *lessonae* genes in the *ridibundus* genome (Uzzell & Berger 1975, Uzzell et al. 1977, Tunner 1979, Mezhzherin & Morozov-Leonov 1997) or rarely vice versa (Günther & Hähnel 1976, Kotlik 1996), is attributed to the role of introgressions (Uzzell et al. 1977). Our observations of some introgressed loci in *P. esculentus* (Table 2), together with the presence of both parental genomes still present in the gonads of some hybrids (Table 3) suggest that hybridogenetic genome exclusion can be incomplete, take place in meiosis itself or be absent at all (Doležálková et al. 2016). As a result, the presence of both parental genomes in meiosis can be associated with a recombination followed by the production of at least partly recombinant gametes (Uzzell et al. 1977, Tunner & Heppich 1981). Parallel explanation for the presence of both parental genomes in gonads at some loci may be a background expression of somatic tissues forming analysed reproductive organs. At this point, crossing experiments or a detailed study of gamete production will need to be performed in order to understand the mechanism and type of gamete production in the Croatian population.

Water frog populations from the L-E system normally contain both sexes of *P. esculentus* and *P. lessonae*. Populations belonging to the R-E system mostly include hybrids of a male sex only (Graf & Polls-Pelaz 1989, Ragghianti et. al. 2007). In natural populations throughout most of central Europe and northern Italy in general, *P. esculentus* females somewhat outnumber the males (Berger 1988). An explanation for this phenomenon could be the higher reproductive success of *P. esculentus* females. In fact, matings between *P. lessonae* males and *P. esculentus* females result in offspring with an even sex ratio. Conversely, crosses between *P. lessonae* females and *P. esculentus* males produce an all-female progeny as a result of complex mechanisms of sex determination, hybridogenesis and mating behaviour (Som & Reyer 2006). Contrary to our expectations to find more hybrid females, we found a larger number of males (23:17), which is a rather rare phenomenon (Günther et al. 1979, Tunner 1979, Wijnands 1979, Berger 1988, Spašić-Bošković et. al 1999, Krizmanić & Ivanović 2010). Our results also differ from populations found in the Pannonian Basin in Croatia, where a high number of females was observed by Berger (1988). However, it should be noted that we collected our specimens during the breeding season, when water frogs usually show a strongly skewed operational sex ratio (OSR) with males outnumbering females (Wells 1977). Alternative explanations for our observation hypothesize that the genetic basis of sex determination in water frogs could be more complex than a simple XX-XY mechanism (Berger 1988).

Despite only seven *P. ridibundus* were found, we consider the species as a self-perpetuating taxon because both males and females were found in the R-E-L population. The pattern of *P. esculentus* gamete production remains yet to be studied in detail; however a dominant allozyme pattern in gonads of hybrids was that of *ridibundus*-like. This suggests that *P. esculentus* produce *ridibundus* gametes and use *lessonae* gametes either from *P. lessonae* or from other hybrid for self-reproduction. Therefore, from a reproductive perspective the R-E-L system is functionally a typical L-E system. Although *P. ridibundus* can be produced by homotypic matings between two *P. esculentus* individuals, the results would be female sex only (Hotz et al. 1992), which is not the case for this population.

Data related to the age structure of water frogs are generally rare. By comparing all three taxa within the R-E-L system our study results showed that the estimated average age of *P. ridibundus* was relatively high (eight years), whereas *P. lessonae* and *P. esculentus* were between two and five years old. While the comparative data are not known for *P. lessonae*, *P. ridibundus* age estimates from Greece and Turkey ranged between 2.96 and 5.58 years (Yilmaz et al. 2005, Kyriakopoulou-Sklavonounou et al. 2008, Gül et al. 2011), and *P. ridibundus* with *P. esculentus* from Poland were between 3.7 and 4.4 years of age (Socha & Ogielska 2010).

These data are close to our age estimates for *P. lessonae* and *P. esculentus* but not for *P. ridibundus* species (Fig. 2 and 3). Currently, we have no clear explanation for the high age of studied *P. ridibundus* individuals. Despite that, our statistical analysis did not reveal differences in the estimated age or in the annual growth rates between these three species. Also, we must note that our sample size of *P. ridibundus* was rather small and could have affected our statistical analyses. Thus, to get better idea about the relationship between the taxa and their age structure in the R-E-L system, more studies are needed that focus on species microhabitat preferences, analysis of food competition and mating success rates.

The studied area represents one of the southernmost distributions of the hybrid taxon *P. esculentus* not only in the Balkan Peninsula but considering its European distribution in general. The closest sites of the southernmost distribution are known from Italy, Slovenia and Romania (Plötner et al. 2008). Data from Serbia (Krizmanić & Ivanović 2010) also help to define a southern line of *P. esculentus´* occurrence. Considering the active role of *P. esculentus* for interspecies transfer and the presence of a contact zone between the southern limits of *P. esculentus* and the northern limits of *P. ridibundus* populations that are considered as still non-introgressed at least in mitochondrial DNA (Plötner et al. 2008), the region certainly deserves further attention in the context of studying *P. ridibundus* genetic resistance against *P. esculentus*-mediated introgressions.

Acknowledgements

The authors thank Mateja Bulaić and Jelka Škoton for their support during field work and technical assistance. We also thank

Věra Šlechtová (Laboratory of Fish Genetics, IAPG CAS, v.v.i., Liběchov) for her kind help during genetic analyses. Lukáš Choleva received support No. RVO 67985904 from the Academy of Sciences of the Czech Republic and No. 15-19947Y from the Czech Science Foundation (GA CR). Sampling permit was provided by the Directorate for Nature Protection, Ministry of Culture of the Republic of Croatia (No. 532-0801-01/3-07-02).

References

Beerli, P. (1994): Genetic isolation and calibration of an average protein clock in western Palearctic frogs of the Aegean region. – Dissertation University of Zurich, Zurich.

Bergen, K., R. D. Semlitsch & H.-U. Reyer (1997): Hybrid female matings are directly related to the availability of *Rana lessonae* and *Rana esculenta* males in experimental populations. – Copeia, **1997**: 275–283.

Berger, L. (1966): Biometrical studies on the population of water frogs from the environs of Poznan. – Annales Zoologici Warszawa, **23**: 303–324.

Berger, L. (1967): Embryonal and larval development of F1 generation of green frogs of different combinations. – Acta Zoologica Cracoviensia, **12**: 123–160.

Berger, L. (1968): Morphology of the F1 generation of various crosses within *Rana esculenta* complex. – Acta Zoologica Cracoviensia, **13**: 301–324.

Berger, L. (1988): On the origin of the genetic systems in European water frog hybrids. – Zoologica Poloniae, **35**: 5–32.

Berger, L., T. Uzzell & H. Hotz (1988): Sex determination and sex ratios in western Palearctic water frogs: XX and XY female hybrids in the Pannonian basin? – Proceedings of the Academy of Natural Sciences of Philadelphia, **140**: 220–239.

Borkin, L. J., I. A. Caune, M. M. Pikulik & T. M. Sokolova (1986): Distribution and structure of the green frog complex in the USSR. – pp. 675–678 in: Roček, Z. (ed.): Studies in Herpetology. – Charles University Press, Prague.

Borkin, L. J., S. N. Litvinchuk, E. I. Mannapova, M. V. Pestov & J. M. Rosanov (2002): The distribution of green frogs (*Rana esculenta* complex) in Nizhny Novgorod Province, Central European Russia. – Russian Journal of Herpetology, **9**: 195–208.

Buth, D. G. & R. W. Murphy (1980): Use of nicotine amine adenine dinucleotide (NAD) – dependent glucose-6-phosphate dehydrogenase in enzyme staining procedures. – Stain Technology, **55**: 173–176.

Castanet, J. & E. Smirina (1990): Introduction to the skeletochronological method in amphibians and reptiles. – Annales des Sciences Naturelles, Zoologie, **11**: 191–196.

Choleva, L., Z. Musilova, A. Kohoutova-Sediva, J. Paces, P. Rab & K. Janko (2014): Distinguishing between incomplete lineage sorting and genomic introgressions: complete fixation of allospecific mitochondrial DNA in a sexually reproducing fish (*Cobitis*; Teleostei), despite clonal reproduction of hybrids. – PLoS ONE: e80641. doi: 10.1371/journal.pone.0080641.

Christiansen, D. G. & H.-U. Reyer (2011): Effects of geographic distance, sea barriers and habitat on the genetic structure and diversity of all-hybrid water frog populations. – Heredity, **106**: 25–36.

Doležálková, M., A. Sember, F. Marec, P. Ráb, J. Plötner & L. Choleva (2016). Is premeiotic genome elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus *Pelophylax*?. – BMC Genetics, **17**: 100.

Ebendal, T. (1979): Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). – Mitteilungen aus dem Zoologischen Museum in Berlin, **55**: 143–152.

Engelman, W. E. (1972): Disk-electrophorese der Serumproteine von Wasserfröschen. Ein Beitrag zur Diskussion über den Hybridcharakter von *Rana esculenta* L. – Acta Biologica et Medica Germanica, **29**: 431–435.

Erismis, U. C. (2011): Abundance, demography and population structure of *Pelophylax ridibundus* (Anura: Ranidae) in 26-August National Park (Turkey). – North-Western Journal of Zoology, **7**: 5–16.

Gasc, J. P., A. Cabela, J. Crnobrnja-Isailovic, D. Dolmen, K. Grossenbacher, P. Haffner, J. Lescure, H. Martens, J. P. Martinez Rica, H. Maurin, M. E. Oliveira, T. S. Sofianidou, M. Veith & A. Zuiderwijk (eds) (1997): Atlas of amphibians and reptiles in Europe. – Collection Patrimoines Naturels, 29, Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle & Service du Patrimoine Naturel, Paris.

Graf, J.-D. (1986): Population genetics of the *Rana esculenta* complex: A model. – pp. 175–180 in: Roček Z. (ed.): Studies in Herpetology, Prague.

Graf, J.-D. & W. P. Müller (1979): Experimental gynogenesis provides evidence of hybridogenetic reproduction in the *Rana esculenta* complex. – Experientia, **35**: 1574–1576.

Graf, J.-D. & M. Polls-Pelaz (1989): Evolutionary genetics of the *Rana esculenta* complex. – pp. 289–301 in: Dawley R. M. & J. P. Bogart (eds): Evolution and ecology of unisexual vertebrates. – New York State Museum, Albany/NY.

Guarino, F. M., I. Di Gia & R. Sindaco (2008): Age structure in a declining population of *Rana temporaria* from northern Italy. – Acta Zoologica Academiae Scientarum Hungaricae, **54**: 99–112.

Gubányi, A. (1992): Distribution of green frogs (*Rana esculenta* complex, Anura: Ranidae) in Hungary. – pp. 205–210 in: Korsós, Z. & I. Kiss (eds): Proceedings of the 6th Ordinary General Meeting of the Societas Europaea Herpetologica, Budapest. – Hungarian Natural History Museum, Budapest.

Gül, S., N. Özdemir, N. Üzum, K. Olgun & B. Kutrup (2011): Body size and age structure of *Pelophylax ridibundus* populations from two different altitudes in Turkey. – Amphibia-Reptilia, **32**: 287–292.

Günther, R. (1973): Über die verwandtschaftlichen Beziehungen zwischen den europäischen Grünfröschen und den Bastardcharakter von *Rana esculenta* L. (Anura, Amphibia). – Zoolischer Anzeiger, **190**: 250–285.

Günther, R. (1990): Die Wasserfrösche Europas (Anura-Froschlurche). Neue Brehm-Bücherei 600. – A. Ziemsen Verlag, Wittenberg-Lutherstadt.

Günther, R. & S. Hähnel (1976): Untersuchungen den Genflus zwischen *Rana ridibunda* und *Rana lessonae* sowie die Rekombinationsrate bei der Bastardform *Rana "esculenta"* (Anura, Ranidae). – Zoologischer Anzeiger, **197**: 23–38.

Günther, R., T. Uzzell & L. Berger (1979): Inheritance patterns in triploid *Rana "esculenta"*. (Amphibia, Salientia). – Mitteilungen aus dem Zoologischen Museum in Berlin, **55**: 35–57.

Harris, H. & D. A. Hopkinson (1976): Handbook of enzyme electrophoresis in human genetics. – North-Holland, Amsterdam.

Hauswaldt, J. S., M. Höer, M. Ogielska, D. G. Christiansen, D. Dziewulska-Szwajkowska, E. Czernicka & M. Vences (2012): A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). – Molecular Ecology Resources, **12**: 797–805.

Heppich, S. & H. G. Tunner (1979): Chromosomal constitution and C-banding in homotypic *R. esculenta* crosses. – Mitteilungen aus dem Zoologischen Museum in Berlin, **55**: 111–114.

Heppich, S., H. G. Tunner & J. Greilhuber (1982): Premeiotic chromosome doubling after genome elimination during spermatogenesis of the species hybrid *Rana esculenta*. – Theoretical and Applied Genetics, **61**: 101–104.

Hoffmann, A., J. Plötner, N. B. Pruvost, D. G. Christiansen, S. Röthlisberger, L. Choleva, P. Mikulíček, D. Cogălniceanu, I. Sas-Kovács, D. Shabanov, S. Morozov-Leonov & H. U. Reyer (2015): Genetic diversity and distribution patterns of diploid and polyploid hybrid water frog populations (*Pelophylax esculentus* complex) across Europe. – Molecular Ecology, **24**: 4371–4391.

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

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

Hotz, H., P. Beerli & C. Spolsky (1992): Mitochondrial DNA reveals formation of nonhybrid frogs by natural matings between hemiclonal hybrids. – Molecular Biology and Evolution, **9**: 610–620.

Jelić, D., M. Kuljerić, T. Koren, D. Treer, D. Šalamon, M. Lončar, M. Podnar-Lešić, B. Janev-Hutinec, T. Bogdanović & S. Mekinić (2012): Red book of amphibians and reptiles of Croatia. – Ministry of Environmental and Nature Protection & State Institute for Nature Protection, Zagreb.

Jelić, D. (2014): Checklist of Croatian amphibians and reptiles with bibliography of 250 years of research. – Natura Sloveniae, **16**: 17–72.

Juszczyk, W. (1953): The migrations of the aquatic frog *Rana esculenta* L. – Bulletin international de l'Académie polonaise des sciences et des lettres. Classe des sciences mathématiques et naturelles. Série B: Sciences naturelles 1951, **2**: 341–369.

Karaica, D., I. Buj, K. Čavlović & V. Mičetić Stanković (2016): Comparative morphology and ecology of the *Pelophylax esculentus* complex in Croatia. – Salamandra, **52**: 161–170.

Kotlík, P. & K. Šůlová (1994): Syntopic occurrence of three taxa of water frogs in Czech Republic. – Zoologica Poloniae, **39**: 417–424.

Kotlík, P. (1996): Hybridogenesis in water frogs: Genetic analysis of populations of *Rana esculenta* complex in the Czech Republic (Amphibia, Ranidae). – Dissertation Charles University, Prague.

Krizmanić, I. I. & A. Ivanović (2010): Population systems of the *Pelophylax complex* in the southern part of its range. – Folia Zoologica, **59**: 215–222.

Kumbar, S. M. & K. Pancharatna (2001): Determination of age, longevity and age at reproduction of frog *Microhyla ornata* by skeletochronology. – Journal of Biosciences, **26**: 265–270.

Kyriakopoulou-Sklavonounou, P., P. Staylianou & A. Tsiora (2008): A skeletochronological study of age, growth and longevity in a population of the frog *Rana ridibunda* from southern Europe. – Zoology, **111**: 30–36.

Lada, G. A., L. J. Borkin & A. E. Vinogradov (1995): Distribution, population systems and reproductive behaviour of green frogs (hybridogenetic *Rana esculenta* complex) in the central Chernozem territory of Russia. – Russian Journal of Herpetology, **2**: 46–57.

Lin, Y. L. & P. C. L. Hou (2002): Applicability of skeletochronology to the anurans from a subtropical rainforest of southern Taiwan. – Acta Zoologica Taiwanica, **13**: 21–30.

Mayer, M., O. Hawlitschek, A. Zahn & F. Glaw (2013): Composition of twenty green frog populations (*Pelophylax*) across Bavaria, Germany. – Salamandra, **49**: 31–44.

Mezhzherin, S. V. & S. J. Morozov-Leonov (1993): Population genetic analysis of the structure of hybrid populations of the *Rana esculenta* L. complex (Amphibia, Ranidae). – Tsitologiia i Genetika, **27**: 63–68.

Mezhzherin, S. V. & S. Morozov-Leonov (1997): Gene diffusion in hybrid populations of green frogs *Rana esculenta* L., 1758 complex (Amphibia, Ranidae) from the Dnepr Basin. – Genetika, **33**: 358–364.

Mikulíček, P, M. Kautman, J. Kautman & N. B. M. Pruvost (2014): Mode of hybridogenesis and habitat preferences influence population composition of water frogs (*Pelophylax esculentus* complex, Anura; Ranidae) in a region of sympatric occurrence (western Slovakia). – Journal of Zoological Systematics and Evolutionary Research, **53**: 124–132. doi: 10.1111/jzs.12083.

Pasteur, N., G. Pasteur, F. Bonhomme, J. Catalan & J. Britton-Davidian (1987): Manual technique de génétique par electrophorese des proteins. Techniques et Documentation. – Lavoasier, Paris.

Plötner, J. (2005): Die westpaläarktischen Wasserfrösche. Von Märtyrern der Wissenschaft zur biologischen Sensation. – Laurenti-Verlag, Bielefeld.

Plötner, J., J. C. Becker & K. Plötner (1994): Morphometric and DNA investigations into European water frogs *Rana* kl. *esculenta* synklepton (Anura, Ranidae) from different population systems. – Journal of Zoological Systematics and Evolutionary Research, **32**: 193–210.

Plötner, J., T. Uzzell, P. Beerli, C. Spolsky, T. Ohst, S. N. Litvinchuk, G.-D. Guex, H. U. Reyer & H. Hotz (2008): Widespread unidirectional transfer of mitochondrial DNA: a case in western Palearctic water frogs. – Journal of Evolutionary Biology, **21**: 668–681.

Pruvost, N. B. M., A. Hoffmann & H.-U. Reyer (2013): Gamete production patterns, ploidy, and population genetics reveal evolutionary significant units in hybrid water frogs (*Pelophylax esculentus*). – Ecology and Evolution, **3**: 2933–2946.

Pruvost, N. B., P. Mikuliček, L. Choleva & H. U. Reyer (2015): Contrasting reproductive strategies of triploid hybrid males in vertebrate mating systems. – Journal of Evolutionary Biology, **28**: 189–204.

Ragghianti, M., S. Bucci, S. Marracci, C. Casola, G. Mancino, H. Hotz, G.-D. Guex, J. Plötner & T. Uzzell (2007):

Gametogenesis of intergroup hybrids of hemiclonal frogs. – Genetics Research, **89**: 39–45.

Sas, I. (2010): The *Pelophylax esculentus* complex in North-Western Romania: distribution of the population systems. – North-Western Journal of Zoology, **6**: 294–308.

Schröer, T. & H. Greven (1998): Verbreitung, Populationsstrukturen und Ploidiegrade von Wasserfröschen in Westfalen. – Zeitschrift Feldherpetologie, **5**: 1–14.

Semlitsch, R. D., S. Schmiedehausen, H. Hotz & P. Beerli (1996): Genetic compatibility between sexual and clonal genomes in local populations of the hybridogenetic *Rana esculenta* complex. – Evolutionary Ecology, **10**: 531–543.

Socha, M. & M. Ogielska (2010): Age structure, size and growth rate of water frogs from central European natural *Pelophylax ridibundus – Pelophylax esculentus* mixed populations estimated by skeletochronology. – Amphibia-Reptilia, **31**: 239–250.

Som C. & H.-U. Reyer (2006): Variation in sex ratio and evolutionary rate of hemiclonal *Rana esculenta* populations. – Evolutionary Ecology, **20**: 159–172.

Spašić-Bošković, O., I. Krizmanić & M. Vujošević (1999): Population composition and genetic variation of water frogs (Anura: Ranidae) from Yugoslavia. – Caryologia, **52**: 9–20.

Tunner, H. G. (1979): The inheritance of morphology and electrophoretic markers from homotypic crosses of the hybridogenetic *Rana esculenta*. – Mitteilungen aus dem Zoologischen Museum in Berlin, **55**: 89–109.

Tunner, H. G. (2000): Evidence for genomic imprinting in unisexual triploid hybrid frogs. – Amphibia-Reptilia, **21**: 135–141.

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

Tunner, H. G. & S. Heppich-Tunner (1991): Genome exclusion and two strategies of chromosome duplication in oogenesis of a hybrid frog. – Naturwissenschaften, **78**: 32–34.

Uzzell, T. & L. Berger (1975): Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae*, and their hybridogenetic associate, *Rana esculenta*. – Proceedings of the Academy of Natural Sciences of Philadelphia, **127**: 13–24.

Uzzell, T., R. Günther & L. Berger (1977): *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia Salientia). – Proceedings of the Academy of Natural Sciences of Philadelphia, **128**: 147–171.

Uzzell, T., H. Hotz & L. Berger (1980): Genome exclusion in gametogenesis by an interspecific *Rana* hybrid: evidence from electrophoresis of individual oocytes. – Journal of Experimental Zoology, **214**: 251–259.

Valenta, M., J. Hylgaaed-Jensen & E. S. Jensen (1971): Interaction of veronal, pyrophosphate, citrate and protein with lactate dehydrogenase isozyme determination and kinetics. – Acta Veterinaria Scandinavica, **12**: 15–35.

Vorburger, C. (2001): Heterozygous fitness effects of clonally transmitted genomes in waterfrogs. – Journal of Evolutionary Biology, **14**: 602–610. doi: 10.1046/j.1420-9101.2001.00307.x.

Vorburger, C. & H. Ulrich-Reyer (2003): A genetic mechanism of species replacement in European waterfrogs? – Conservation Genetics, **4**: 141–145.

Wells, K. D. (1977): The social behaviour of anuran amphibians. – Animal Behaviour, **25**: 666–693.

Wijnands, H. E. J. (1979): Partial ecological isolation of *Rana lessonae* and *Rana esculenta* as a mechanism for maintenance of the hybrid form, *Rana esculenta* (Anura, Ranidae). – Mitteilungen aus dem Zoologischen Museum in Berlin, **55**: 131–142.

Yilmaz, U., B. Kutrup, Ü. Çobanoglu & Y. Özoran (2005): Age determination and some growth parameters of a *Rana ridibunda* population in Turkey. – Acta Zoologica Academicae Scientiarum Hungaricae, **51**: 67–74.