



Population structure, mate choice, and genome transmission in naturally formed pairs in a *Pelophylax lessonae*–*Pelophylax esculentus* hybridogenetic system

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Abstract

Central European water frog *Pelophylax esculentus* is a hybrid that lives sympatrically and forms genetic systems with one of its parental species, in this case L-E (*P. lessonae* - *P. esculentus*). Hybrids are restored in each generation due to hybridogenesis, a unique mode of hemiclinal reproduction. We investigated changes in the species composition, mate choice, and genome transmission in naturally formed pairs in the L-E hybridogenetic system from the Raków fish pond complex (SW Poland). Our observations showed that the genetic system remained the same for over a decade but the share of the taxa and the sex ratios within the species changed. The frequency of *P. lessonae* decreased by about 20% in favor of *P. esculentus*. The proportion of males to females of *P. lessonae* increased twofold and of *P. esculentus* fourfold. We also found that the stability of the L-E population was ensured by a specific female mate choice pattern with the dominance of homotypic *P. lessonae* and heterotypic ♀*P. esculentus* × ♂*P. lessonae* pairs (almost 90% of the pairs collected from nature). The youngest females and males that formed a successful amplexus were 3 years old. We noted a male-male competition manifested by the exclusion of young males (before the third hibernation) from the participation in the mating. An analysis of genome transmission to gametes revealed that females *P. lessonae* transmitted Lx, while *P. esculentus* transmitted the Rx and/or Lx genomes. Males of *P. lessonae* transmitted the Lx or Ly genomes, while *P. esculentus* transmitted the Lx, Ly, and/or Rx genomes. The high proportion of the L genomes transferred to gametes enables both the restoration of the parental species *P. lessonae* and the regular renewal of a new generation of hybrids.

Significance statement

Central European water frog complex includes two species: *Pelophylax ridibundus* (genotype RR) and *P. lessonae* (LL), and their natural hybrid, *P. esculentus* (RL). Hybrid individuals of both sexes reproduce due to a specific mode of reproduction, i.e., hybridogenesis, in which hybrids eliminate one of the parental genomes (R or L) from the germ line prior to meiosis and transmit the other one (not recombined, i.e., clonal) to the gametes. The hybrids live in mixed populations with one of the parental species and are restored in each generation. This, in turn, allows a kind of balance to be maintained in a population due to specific mate choice and genome transmission. Our research showed that the stability of the studied population was ensured by female preference to *P. lessonae* and male-male competition manifested by the exclusion of males under 3 years of age from the participation in the mating.

Keywords Hybridogenesis · Water frogs · Mate choice · Skeletochronology

Introduction

Central European water frog complex includes two species and their hybrid, which occur sympatrically but differ ecologically (Lada et al. 1995; Pagano et al. 2001). The pool frog *Pelophylax lessonae* (LL) prefers small ponds,

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the marsh frog *P. ridibundus* (RR) inhabits lakes, large fish ponds, and rivers, while their natural hybrid, the edible frog *P. esculentus* (RL; E), is a generalist usually co-occurring in mixed populations with one of the parental species. Such mixed populations form two main genetic systems: L-E and R-E, each living in an environment suitable for the parental species (Uzzell and Berger 1975; Berger 1983; Pagano et al. 2001; Rybacki and Berger 2001; Hoffmann et al. 2015a). The L-E systems are known to persist over time and are the most widespread in Central Europe (Rybacki and Berger 2001). *Pelophylax esculentus* is a bisexual hybrid that can also create the E-E system, but only when it is composed of diploids and triploids (Mikulíček and Kotlík 2001; Rybacki and Berger 2001; Christiansen 2009).

Maintenance of the hybrids over generations is possible due to hybridogenesis, a unique mode of hemiclinal reproduction (Schultz 1969). Hybrid individuals eliminate one of the parental genomes (the one belonging to the parental species present in the population) from the germ line prior to meiosis and transmit the other one (not recombined, i.e., clonal) to the gametes (Berger 1973a; Graf and Polls Pelaz 1989; Chmielewska et al. 2018, 2022). In the L-E system, which is the focus of this study, most hybrids eliminate the L genome and transmit the R genome to the gametes. When a hybrid that produces the R gametes mates with *P. lessonae* that produces the L gametes, a new generation of the RL progeny appears. However, the progeny usually suffers from abnormal embryogenesis and development and eventually dies (for review, see Ogielska 2009; Doležalková-Kašánková et al. 2022) mostly as a result of deleterious mutations carried by the R gametes (Vrijenhoek 1989; Bove et al. 2014) and the aneuploid R gametes (Chmielewska et al. 2022) that lead to aneuploid embryos and tadpoles (Christiansen et al. 2005; Christiansen 2009). The RL survivors will recapitulate the same way of genome elimination and clonal gamete formation which results in the constant supply of new generations of hybrids to the population. On the other hand, *P. lessonae* seems to suffer as a species because some part of its genome disappears from the pool with the high rate of death of the hybrid offspring (Joly 2001).

Besides predictable and repeatable composition of the genomes transmitted by the gametes, the breeding behavior and mate choice are crucial for the stability of the genetic systems. In water frogs, competition between the males and the mate choice in females seem to be key mechanisms in sexual selection, as was shown in a series of experiments (Bergen et al. 1997; Engeler and Reyer 2001) and in mathematical models and computer simulations (Abt and Reyer 1993; Reyer et al. 1999; Hellriegel and Reyer 2000; Som et al. 2000). As a result of these studies, the authors concluded that females from the L-E systems mate in an assortative way and prefer male *P. lessonae* to *P. esculentus*. The weakness of these models is the scarcity of empirical

data taken from nature. Female choice also manifests in eliminating the undesirable male by allowing other males to scramble and compete (Lengagne and Joly 2010). Reyer et al. (1999) reported that females release fewer eggs and reduce clutch sizes when grasped by an undesired *P. esculentus* male.

Mating behavior of the males is based on competition for the female and males from the L-E systems differ in mating strategies. The field observations revealed that the male *P. lessonae* are very active and move across the breeding site grasping the approaching females while *P. esculentus* are territorial and use to stay at their positions (Blankenhorn 1977; Lada et al. 1995; Lengagne et al. 2006, 2008). The males differ also in mating calls: *P. lessonae* calls longer and with a higher frequency in comparison to *P. esculentus* (Hoffmann and Reyer 2013) and females preferred *P. lessonae* over *P. esculentus* (Roesli and Reyer 2000).

Taken together, the relations between the genome transmission by the gametes of the hybrids, mating behavior, and stability of the L-E systems are fairly complex. Our knowledge of the pairing of frogs from the L-E systems mostly comes from observations under experimental conditions, with the exception of Lengagne et al. (2006, 2008), Hoffmann and Reyer (2013), and Hoffmann et al. (2015b). However, still little is known about the mate choice among water frogs in nature and about the possible change in the share of the taxa in the population over time, and our study provides such data. We especially focused on three issues: (1) the stability of the population structure, (2) the age of males and females in naturally formed pairs *in amplexus*; to the best of our knowledge, this life-history trait has never been studied in water frog complexes for the mate choice aspect and (3) the genome composition of the gametes transmitted by the hybrid parents from naturally formed pairs, including sex inheritance.

Material and methods

Study area and material collection

The study was carried out in two neighboring ponds (pond area 12 ha and 0.5 ha, pond maximum depth 1.7 m and 2.2 m, respectively) that are a part of the fish pond complex in Raków village, 10 km north-east of Wrocław city (southwestern Poland, 51.175 N, 17.278 E). The study area is surrounded by a forest, meadows, and cereal fields. The common carp *Cyprinus carpio*, the grass carp *Ctenopharyngodon idella*, the silver carp *Hypophthalmichthys molitrix*, and the wels catfish *Silurus glanis* are farmed in these ponds.

The main part of the study was conducted in years 2006–2008. Sampling of the adults was attempted during and immediately after the breeding season (in May and the

beginning of July), whereas the juveniles were collected between May and September. The frogs were caught by hand or net at night after being blinded by a flashlight. Afterwards, they were transported to the laboratory in wet fabric bags. In total, 721 frogs (excluding recaptures) were caught: 560 adults (including 63 pairs in amplexus) and 161 juveniles. All of them were sexed and measured (see below). The sex of each individual was determined by the presence of nuptial pads and vocal sacs in males. A decade later, during the breeding season in May 2017, water frogs were caught from the same ponds and by the same methods and a total of 125 adults were collected. They were also sexed and buccal swabs were taken from each of these frogs for further species identification (details below). The frogs were released back into their habitat except for 63 females and 63 males caught in amplexus and used for crossing experiments and cytogenetics in 2006–2008. It was not possible to record data blind because our study involved focal animals in the field.

Taxonomic identification and ploidy assessment

For species determination of the frogs collected in 2006–2008 ($N=721$), the following measurements were made using a digital calliper to the nearest 0.05 mm: snout-vent length (SVL), tibia length (T), digitus primus of the hind leg length (DP), callus internus length (CI), and 2 indices best distinguishing the taxa: DP/CI and T/CI (Berger 1988, 2008); the frogs were also weighed using Pesola (± 0.5 g). In addition to morphometry, body colouration (according to Kierzkowski et al. 2013), and shape of CI (Kierzkowski et al. 2011) were taken into account. To confirm the correct morphology-based taxa identification, a cytogenetic analysis with AMD-DAPI (actinomycin D – 4',6-diamidino-2-phenylindole) was performed for all individuals caught in amplexus ($N=126$) and their progeny; this method allowed to distinguish the R and L chromosomes (Heppich et al. 1982; Ogielska et al. 2004).

To obtain a sufficient number of metaphase plates, adult frogs were injected peritoneally with 1 mL of 0.3% colchicine (Sigma) 24 h before preparation; in a few cases (when the females were assessed gravid and in the case of a rare mating between a *P. lessonae* female and a *P. esculentus* male in cross no. 43) after failed initiation of ovulation, the procedure was repeated the next day. Tadpoles and metamorphosing individuals were kept in 0.01% colchicine overnight. Immediately before the tissue preparation, the frogs were anesthetized with 0.5%, and the tadpoles with 0.25% solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical Co.) in water. The intestine was dissected, hypotonized in distilled water for 20 min, and fixed in a freshly made Carnoy's fixative. The tissues were stored in the fixative at -20 °C. Genome composition was determined on 10–20 metaphase plates that were examined using a Carl

Zeiss microscope equipped with a fluorescence lamp with appropriate filters.

The cytogenetic analysis of the frogs collected in 2006–2008 was already performed before 2017. Because this method is much more laborious, we decided to determine the genotype of all frogs collected in 2017 ($N=125$) on the basis of length polymorphism of the serum albumin intron-1 (SAI-1; Hauswaldt et al. 2012). DNA was extracted using the GeneMATRIX TISSUE DNA Purification Kit (EURx Ltd., Poland) and the amplification was done with the primers PeI-SA-F1 (5'-TCCATACAAATGTGCTAA GTAGGTT-3') and PeI-SA-R2 (5'-GACGGTAAGGGG ACATAATTCA-3') designed by Hauswaldt et al. (2012). PCR cycling conditions followed the protocol according to Kolenda et al. (2017a): initial denaturation for 3 min at 95 °C followed by 35 cycles of (i) 30 s denaturation at 94 °C, (ii) 30 s annealing at 53 °C, and (iii) 60 s elongation at 72 °C and (iv) the final extension for 7 min at 72 °C. PCR products were separated electrophoretically on a 1.5% agarose gel; then, the bands were compared with a 100-bp size marker and previously sequenced amplicons (Kolenda et al. 2017a).

Ploidy of all hybrid *P. esculentus* collected in 2006–2008 ($N=313$) was assessed by screening erythrocytes on blood smears under the Axiostar Plus microscope (Zeiss) using $\times 20$ lens and KS400 software (Zeiss) according to the following rule: if the long axes of erythrocytes did not exceed 24 μm , the individuals were classified as diploids, and if the long axes were equal or exceeded 29 μm , the individuals were classified as triploids (Ogielska et al. 2004; Kierzkowski et al. 2011). Blood for the smears was taken directly to a microscope slide from the cut fingertips. The smears were air-dried for ca. 1 h and stored in darkness.

Skeletochronological analysis

The individual's age was estimated for 325 randomly chosen frogs collected in 2006–2008. The analysis was performed using the skeletochronological method which is based on counting the lines of arrested growth (LAGs) formed during hibernations, when the physiological processes slow down (Smirina 1994; Sinch 2015). The protocol developed by Rozenblut and Ogielska (2005) was used. Toe clips were fixed in 4% formaldehyde and stored in -20 °C. Soft tissue was removed from the phalanges manually and bones were decalcified in 1:1 mixture of 10% formic acid and 4% formalin for a maximum of 5 h. Then the phalanges were washed in distilled water (4 times, 15 min. each). Decalcified bones were sectioned into 10- μm thick sections by freezing microtome Leica CM 1850 UV in -20 °C, and stained with 0.05% cresyl violet. At least 20 cross-sections of the middle portion of diaphysis of phalangeal bone per individual were examined under a Carl Zeiss Axioscope 20 light microscope at magnifications ranging

from $\times 10$ to $\times 40$. In order to avoid an underestimation caused by the endosteal resorption of year-1 growth zone and LAG-1 that might have occurred, “back calculation” approach was applied (Rozenblut and Ogielska 2005). This analysis is based on the comparison of LAG diameters in adult frogs with the bone diameter of juveniles before or right after the first hibernation (which is equal to LAG-1).

Progeny of natural pairs and genomic composition of gametes

The 63 natural pairs in amplexus were transported to the laboratory (each pair in a separate bag) to obtain progeny. Females which were collected before ovulation were artificially fertilized in the laboratory according to Berger et al. (1994) by their mates from the natural amplexus. The day before the procedure, the females were injected intraperitoneally with salmon luteinizing hormone-releasing hormone (LHRH, H-7525.0001, Bachem) in the amount of 6.25mg/kg of body weight. When ovulation started, males were anesthetized; their testes were dissected and homogenized in 2–5 mL of standing tap water. Then, the female’s belly was gently squeezed and an emerging stripe of ovulated oocytes was placed on the bottom of a plastic dish and the testes homogenate was poured over the layer of eggs; after ca. 20 min, more water was added to cover the eggs. After about 1 h, the eggs were checked to see if they were successfully fertilized: the fertilized eggs rotated with the darker animal pole upwards. From the moment of fertilization, the embryos were controlled and unfertilized eggs and dead embryos were removed. The stages of development were determined according to Gosner (1960). After hatching (stage 20), the tadpoles were transferred to larger containers. The breeding conditions were close to the natural ones: tadpoles were kept in containers filled with standing water at the density of 4–5 individuals per 1L enriched with vegetation from the pond (Berger 1973b). The containers were placed outdoors and were covered from above with a sheet of agrotexile. Every 2 or 3 days, the containers were inspected and dead individuals were removed.

The genomic composition of the gametes that were successfully transferred by the *P. esculentus* parents was deduced from the genotypes of their progeny after AMD-DAPI staining and control crosses (Berger 1970, 1973b). In the case of RL \times RL pairs, we could not predict which gametes were provided by which parent. To answer this question, a control cross for one of the hybrid parents from each cross was made with a partner of the parental species (*P. lessonae*) with a predictable genome composition of the gametes (only L). The sex of the tadpoles was determined according to the morphology of the gonads.

Statistical analysis

The empirical data were verified to the normal distribution by the Shapiro-Wilk’s test. Non-parametric tests were used in a statistical analysis due to the non-normal distribution of variables. The Chi-square test was used for the comparison of qualitative variables. *U* Mann-Whitney test was used to compare one feature between two groups. The Kruskal-Wallis ANOVA by ranks test with post hoc Dunn’s multiple comparisons test of mean ranks were applied to compare one feature among several groups.

When testing of the mate choice in frogs, three variables were considered as potentially significant: SVL, body weight, and individual age. Spearman’s correlation coefficient was used to check the relationships between these variables. The relationship between SVL and weight was significant in the case of *P. esculentus* (in males and females) and *P. lessonae* (in males and females); thus, we rejected weight from further analysis (see Supplementary Table S1 for details). The verification of the obtained results was carried out at a significance level of 0.05 according to the statistical methods and principles provided by Zar (2010) and Sokal and Rohlf (2012).

All calculations were carried out using STATISTICA 14 software (TIBCO Software Inc. 2020).

Results

All ($N=846$) collected frogs were identified by morphology (see Tables 1, S2 for summarized data). Individuals caught in amplexus ($N=126$) and all frogs collected in 2017 ($N=125$) were additionally checked by a cytogenetic analysis of intestine epithelium cells by AMD/DAPI ($2n = 26$) or on the basis of length polymorphism of the serum albumin intron-1, respectively, and the obtained results were consistent. According to the erythrocyte size, all frogs were diploid.

Taxonomical composition and population structure

Years 2006–2008 Among the adults, *P. lessonae* ($N=379$; 67.7%) outnumbered *P. esculentus* ($N=181$; 32.3%), with the species ratio 1:0.48 (*P. lessonae*:*P. esculentus*) (Fig. 1A, Table 1). The opposite trend was found for the juveniles (frogs after the first and second hibernation), where *P. esculentus* ($N=132$; 82.0%) was the most common, followed by *P. lessonae* ($N=27$; 16.8%) and *P. ridibundus* ($N=2$; 1.2%) (see Table S2 for details). The sex ratio of adult males to females reached 1:4.0 for *P. esculentus* and 1:0.2 for *P. lessonae* (Fig. 1B–C). The differences in the sex ratio between both taxa was significant (chi-square test $\chi^2 = 207$, $df = 1$, test, $p < 0.0001$). The females were significantly larger (*U* Mann-Whitney test: *P. lessonae* $Z=6.11$, $p < 0.0001$, *P.*

Table 1 Morphometry and taxonomical indices of adult frogs collected between 2006 and 2008

	Genotype	Sex	N	Min	Max	Mean	SD
SVL	<i>P. esculentus</i>	Male	36	32.0	77.0	57.7	10.7
		Female	145	41.1	91.9	67.5	11.9
	<i>P. lessonae</i>	Male	314	30.0	61.5	46.5	4.6
		Female	65	36.4	64.5	51.1	6.5
Mass	<i>P. esculentus</i>	Male	36	3.5	49.0	22.6	10.2
		Female	145	5.0	92.0	33.2	16.3
	<i>P. lessonae</i>	Male	314	2.0	23.0	9.7	4.4
		Female	65	2.5	32.0	15.5	5.6
Age	<i>P. esculentus</i>	Male	19	3	5	4.0	0.7
		Female	59	3	7	4.3	1.0
	<i>P. lessonae</i>	Male	170	3	8	4.3	1.1
		Female	40	3	6	4.0	0.9
Dp/Ci	<i>P. esculentus</i>	-	181	1.48	2.80	2.12	0.24
	<i>P. lessonae</i>	-	379	1.1	2.26	1.57	0.20
T/Ci	<i>P. esculentus</i>	-	181	5.93	9.31	7.63	0.66
	<i>P. lessonae</i>	-	379	4.56	8.02	6.11	0.64

esculentus $Z=3.69$, $p=0.0002$) and heavier than the males (U Mann-Whitney test *P. lessonae* $Z=7.49$, $p<0.0001$, *P. esculentus* $Z=3.68$, $p=0.002$) (Table 1).

Individual age was successfully estimated in all examined individuals ($N=325$). Age distribution of the frogs collected during the whole season in each study year ranged from one and two (juvenile individuals after the first and second hibernation) to eight (after the eighth hibernation) years (Table 1, S2, Fig. 2A–B). Adult females reached ages from 3 to 7 years, while males from 3 to 8 years. Four- and five-year old individuals dominated in both sexes. One- and two-year old juveniles were absent in the breeding arena and evidently did not take part in the reproduction; this was confirmed by females and males found in amplexus where the youngest individuals were 3 years old.

Year 2017 A decade later, *P. esculentus* ($N=65$; 52%) slightly dominated over *P. lessonae* ($N=59$; 47.2%) and the species ratio was 1:0.9 (*P. esculentus*:*P. lessonae*) (Fig. 1A). Additionally, the presence of one (0.8%) adult female *P. ridibundus* was noted. The sex ratio (males:females) reached 1:1.1 for *P. esculentus* and 1:0.1 for *P. lessonae* (Fig. 1B–C).

Mate choice

Four different types of pairs in amplexus were found in the years 2006–2008 (Table S3). The most frequent was ♀ *P. lessonae* × ♂ *P. lessonae* ($N=29$; 46%), followed by ♀ *P. esculentus* × ♂ *P. lessonae* ($N=26$; 41.3%), ♀ *P. esculentus* × ♂ *P. esculentus* ($N=7$; 11.1%), and ♀ *P. lessonae* × ♂ *P. esculentus* ($N=1$; 1.6%).

The length of the females found in amplexus ranged from 46.0 to 90.9 mm, while the length of the males ranged from 40.4 to

67.4 mm. In most cases ($N=60$; 95%), the females were significantly longer than the males (U Mann-Whitney test, $U=467.5$, $p<0.001$). In the remaining three pairs (5%), the males were slightly longer than the females, but not more than 3.3 mm.

Among the frogs in pairs, 3- and 4-year-old individuals of both sexes dominated. Three groups of pairs showing different patterns, but with no significant differences in frequency (chi-square test $\chi^2=3.81$, $df=2$, $p=0.15$), were found: (1) pairs of frogs of the same age ($N=25$; 40%) were the most common; (2) females older than the males were found in 33% of pairs ($N=21$) and the difference in age between the sexes in this group was significant (U Mann-Whitney test, $U=35.0$, $p<0.001$); and (3) in 27% ($N=17$) the pairs demonstrated the opposite pattern; i.e., males significantly older than the females were found (U Mann-Whitney test, $U=35.0$, $p<0.001$). There were also significant age differences in both sexes between different groups (ANOVA Kruskal-Wallis test; females $H=22.78$, $p<0.001$, males $H=27.99$, $p<0.01$, see Fig. 3 for details).

Genomes transmitted to the progeny by parents from natural pairs

Out of 63 pairs collected in amplexus, 41 yielded viable offspring. In the remaining 16 pairs (including the only ♀ *P. lessonae* × ♂ *P. esculentus* pair), the females were most probably caught just after ovulation judging by the swollen cloaca, palpation of the abdomen, and slim appearance, and had not laid eggs any more, and in 6 pairs, the progeny was not obtained due to the failure of fertilization or death at the early embryonic stages (Table S3). The type of gametes was determined by two parameters: the transmitted genome (L or R) and type of the sex determinant (x or y). The pairs that

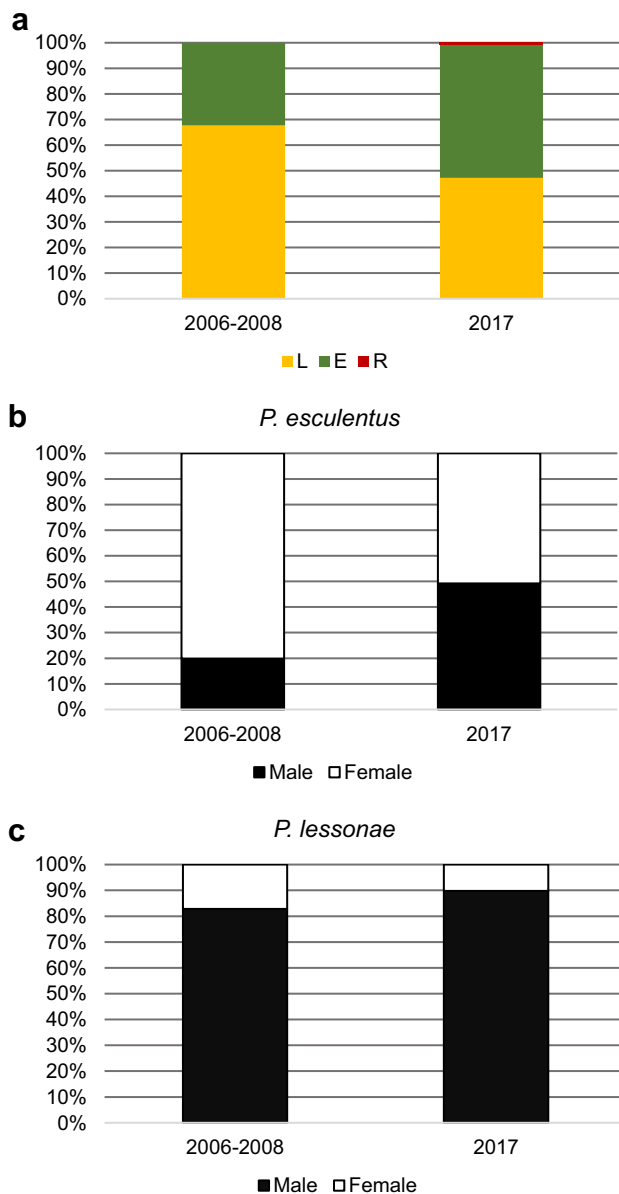


Fig. 1 a Different ratios of the taxonomic composition of water frogs in the Raków fish ponds complex between 2006–2008 and 2017. **b** Sex ratio differences in *P. esculentus* and **c** *P. lessonae* in the Raków fish ponds complex between 2006–2008 and 2017. L *P. lessonae*, E *P. esculentus*, R *P. ridibundus*

the yielded progeny were sorted into three groups depending on the taxa involved:

- (1) 22 ♀*P. lessonae* × ♂*P. lessonae* pairs. Out of 22 crosses, 16 produced the *P. lessonae* offspring of both female (LxLx) and male (LxLy) sexes. One cross (no. 6), fathered by male no. 6 which transmitted only Ly, resulted in an all-male offspring (LxLy). The reverse situation was found in five crosses (no. 21, 23–25, 37) fathered by males no. 22a, 24a, 25a/7, 26a, and 78a

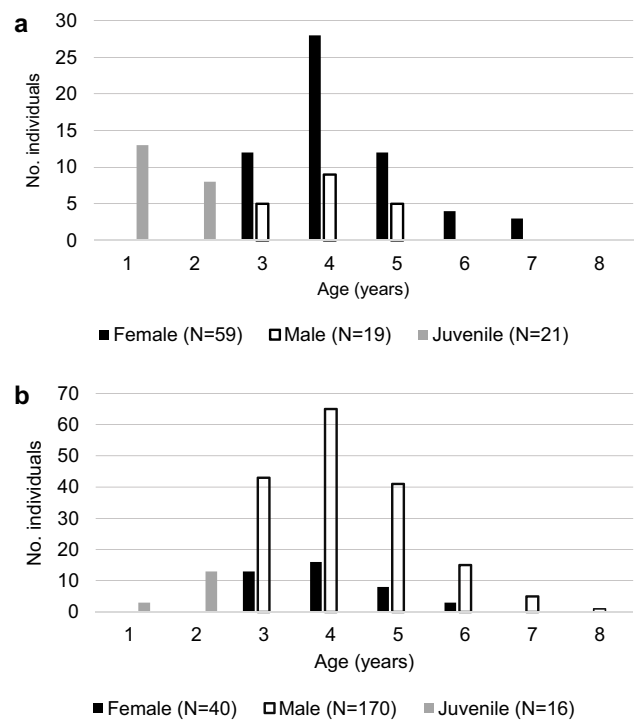


Fig. 2 Age distribution in *P. esculentus* (a) and *P. lessonae* (b) from the Raków fish ponds complex in 2006–2008

which transmitted only Lx, resulting in an all-female (LxLx) offspring (Tab. S3).

- (2) 13 ♀*P. esculentus* × ♂*P. lessonae* pairs. All *P. esculentus* females transmitted the Rx gametes and 5 of them (no. 21, 80b, 11, 12, and 14) transmitted simultaneously also the Lx gametes. The *P. lessonae* males transmitted the Lx and Ly gametes, but in four cases (no. 20, 27a/07, 5, and 11), they passed only the Lx gametes. The resulting progeny represented all types of genetic compositions of frogs inhabiting the population, i.e., *P. esculentus* females (RxLx) and males (RxLy) and *P. lessonae* females (LxLx) and males (LxLy). The result that deserved attention was the restoration of both sexes of the parental species *P. lessonae* from the five hybrid mothers that produced the Lx gametes.
- (3) 6 ♀*P. esculentus* × ♂*P. esculentus* pairs. Five females (no. 31b, 16, 69b, 88b, 1b) transmitted the Rx gametes and one (no. 26b) transmitted the Lx gametes. One male (no. 26a) transmitted Rx, another (no. 88a) transmitted Ly, yet another (no. 1a) Lx and Ly and the other three males (no. 31a, 17, and 69a) transmitted the Lx gametes. Four pairs (crosses no. 57, 62, 18, 29) yielded a progeny of the RxLx genotype, i.e., *P. esculentus* females, one (no. 47) gave RxLy, i.e., *P. esculentus* males, and another one (no. 54) yielded RxLx and RxLy genotypes, i.e., *P. esculentus* females and males; in that way, both sexes of the hybrids were restored.

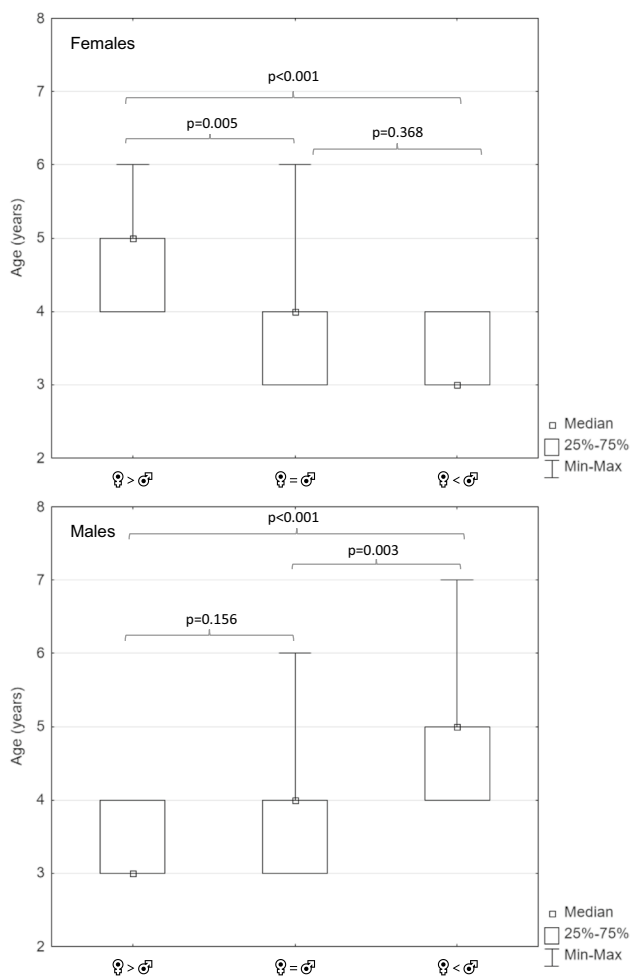


Fig. 3 Age differences in males and females between three groups of natural pairs in amplexus (females older than males, females and males of equal age, females younger than males). ♀ females, ♂ males

Control crosses We have successfully examined one *P. esculentus* male (no. 26a) and four *P. esculentus* females (31b, 16, 69b, 1b) from group (3). The genotypes of the progeny from the control crosses confirmed the results obtained for the hybrid parents from natural crosses.

When we pooled the successful gametes produced by females and males at the population level, we found that females contributed the Rx and Lx genomes, namely *P. lessonae* ($N = 22$) transmitted only Lx, whereas *P. esculentus* ($N = 19$) transmitted the Rx genomes (94.7% of females) and/or Lx genomes (31.6%). Males of *P. lessonae* ($N=35$) transmitted the Lx (97.1% of males) and/or Ly (74.3%) genomes, while of *P. esculentus* ($N=6$) transmitted the Lx (66.7%), Ly (33.3%), and Rx (16.7%) genomes (Fig. 4).

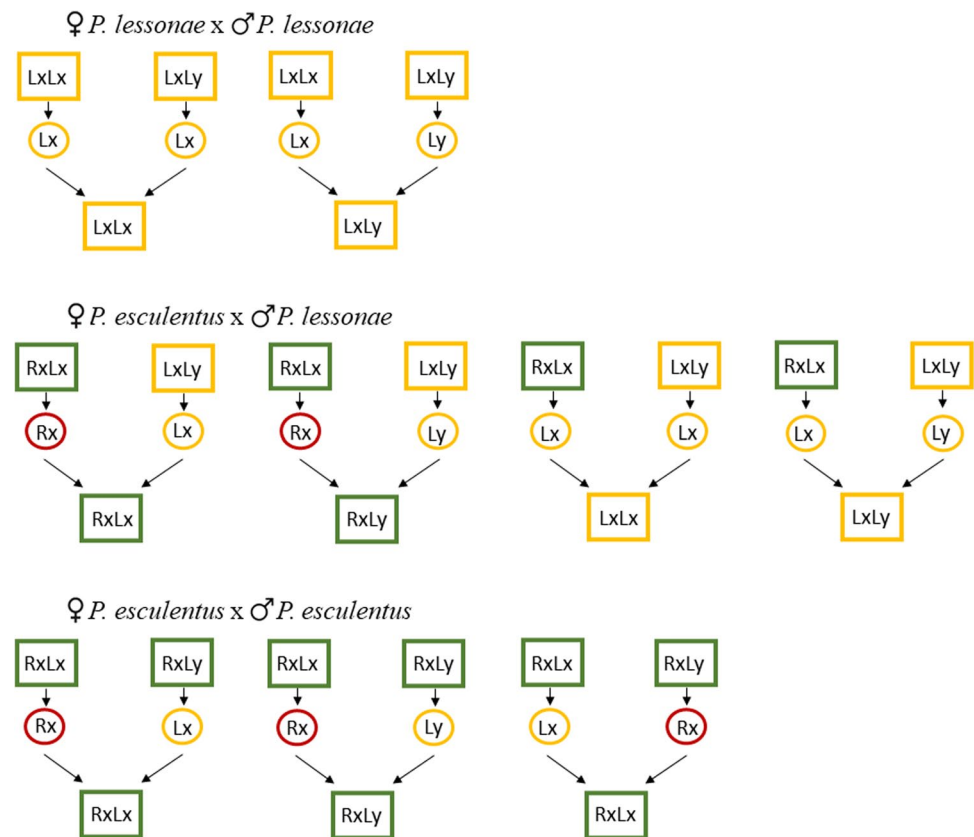
Discussion

Taxonomical composition and population structure

The studied population forms the L-E genetic system which is considered to be the most frequent in Central Europe (Rybacki and Berger 2001; Hermaniuk et al. 2020). The forested area surrounding the ponds (the wintering site for *P. lessonae*) and a complex of aquatic habitats suitable for frogs and tadpoles, such as flat and sunny pond shores, high density of reeds, and submerged plants (e.g., hornwort *Ceratophyllum demersum*), offer protection against fish and provide suitable conditions for the maintenance of the frog population. The ponds that have been selected for the study are very similar in terms of use, and their management has not changed over the decades (established before the World War II and renovated in 1978). This eliminates the possible factor of environmental influence and provides a good model for research on the structure of populations and the mode of reproduction of taxa that compose them. The large number of the collected frogs and sampling over three consecutive years constitutes a representative sample of the studied population. The comparison between years 2006–2008 and 2017 led to the conclusion that the population type (L-E) after 10 years remained the same but the share of taxa and the sex ratios within the species changed. The frequency of *P. lessonae* decreased by about 20% in favor of *P. esculentus*. The proportion of males to females of *P. lessonae* increased twofold and of *P. esculentus* fourfold. A single adult female *P. ridibundus* found in 2017 came probably from another nearby fish pond complex. Her origin from the RLxRL cross is less possible, because we did not find *P. ridibundus* progeny in such natural pairs (Fig. 4) and the presence of a single *P. ridibundus* has probably no significant effect on the stability of the population.

We found significant differences in the taxa shares between the juveniles (1- and 2-year-old frogs) and the adults collected in 2006–2008. In the juvenile group, *P. esculentus* outnumbered *P. lessonae* (82.0%:16.8%) while in the adults, the ratio was in favor of *P. lessonae* (32.3%:67.7%). Both *P. lessonae* and *P. esculentus* males preferred *P. esculentus* females, which are much larger than *P. lessonae* females and their clutches contain even three times more eggs (Berger 1977). For this reason, Berger and Berger (1992) and Abt Tietje and Reyer (2004) concluded that their progeny dominated during tadpole stages. However, the matter may not be quite so simple because a very large proportion, even up to 100%, of hybrid tadpoles suffer from various abnormalities

Fig. 4 Transmission of genomes and sex chromosomes by natural pairs of water frogs from the L-E system. ♀ females, ♂ males



(arrest at mid-blastula transition, incomplete blastopore closure-exogastrulation, oedema, curvature of the body axis, microcephaly, and eye underdevelopment) and die before metamorphosis (Berger 1967; Ogielska-Nowak 1985; Ogielska 1994). Moreover, Abt Tietje and Reyer (2004) suggested higher mortality rate of hybrids during the metamorphosis due to worse handling of stress at this crucial moment of their lives and then a similar survival rate at the juvenile stage. On the other hand, the dispersal rate after metamorphosis is higher in *P. lessonae* than in *P. esculentus* (Holenweg Peter 2001). This means that the stability of the L-E genetic systems is influenced by various factors, which can influence the taxa ratio. As we presented in this study, the shift in the taxa share changed even under relatively stable condition of fish ponds. Generally, changes over time in populations are poorly studied and further long-term observations are needed to find a presumed rule explaining fluctuations in the taxonomic composition of water frog populations (see also Abt Tietje and Reyer 2004).

Apart from natural fluctuations, also anthropogenic factors can affect the species ratio in mixed populations of water frogs. In the population under study, Kolenda et al. (2017b) found in 2015 a high prevalence of *Batrachochytrium dendrobatidis* (*Bd*) infection, the fungus associated with the global decline in amphibians. We suggest that *P. esculentus* may have

increased in frequency because it appears to be more resistant to *Bd* as it secretes greater antimicrobial peptide diversity that is more effective against *Bd* when compared to *P. lessonae* (Daum et al. 2012). Infection of *Bd* can also be a cause of a slightly smaller body size of *P. lessonae* adults when compared to other populations in Poland (Berger 2008; Table 1). However, the effect of the *Bd* infection on the stability of the water frog systems requires further studies.

Mate choice

In the hybridogenetic reproductive mode (Schultz 1969; Graf and Polls Pelaz 1989; Lamatsch and Stöck 2009), hybrids are typically restored by backcrosses with a parental species. In the L-E system, ♀ *P. esculentus* × ♂ *P. lessonae* crosses are the most relevant for the maintenance of hybrids (Uzzell and Berger 1975). However, this type of crossing is not the only one that can be formed in the L-E systems (see the “Results” section). Lada et al. (1995) studied the breeding behavior and pair formation in various populations of water frogs in Eastern Europe in the region of the Tsna River (Volga drainage system). Among a variety of pure hybrid and mixed populations, they caught 32 pairs in amplexus and reported that in all pairs, the females were bigger than the males with the exception of one pair where both partners were of the same length. According to this rule, the naturally formed ♀ *P. lessonae* × ♂ *P. esculentus* pairs were very rare. Lada et al.

(1995) found 3 such pairs with big *P. lessonae* females. In our sample, we found only one such amplexus (cross no. 128), where the female (55.6 mm) was only slightly bigger than the male (54.9 mm). Unfortunately, this female did not lay eggs, even after two hormone injections. We cannot rule out that the male grasped the female when she was escaping from the breeding arena already after oviposition. The study of Günther and Plötner (1990) on 55 naturally formed ♀ *P. esculentus* × ♂ *P. esculentus* pairs captured in amplexus in a pure E-E system revealed that 7.3% of males were bigger than the females and there was no clear evidence for significant size-related preferences.

A series of experiments conducted under controlled laboratory conditions supported the observations from nature and clearly indicated that female *P. esculentus* and *P. lessonae* preferred to mate with *P. lessonae* males and this preference led to assortative mating, whereas males did not discriminate between the female taxa (Abt and Reyer 1993; Reyer et al. 1999). This breeding behavior was aptly defined by Engeler and Reyer (2001) as “choosy females and indiscriminate males”. The importance of the assortative mating for the persistence and stability of the L-E systems over time was supported by theoretical models (Hellriegel and Reyer 2000; Som et al. 2000). Only Bergen et al. (1997) reported that results of other experiments were not always so clear and showed that mating was not assortative but rather depended on the male availability.

Age of pairs in amplexus

The age of adults was not a barrier in the mate choice. The youngest females and males that took part in the breeding and formed successful amplexus were 3 years old. The age of the females is consistent with time needed for the completion of vitellogenesis in diplotene oocytes (Wagner and Ogielska 1990). However, Berger (1970) reported that females from his rearing yielded ripe oocytes even when they were 1 year old (*P. lessonae*) or 2 years old (*P. esculentus*), most probably due to very good feeding conditions. The results were somewhat surprising for males because sexual maturity is achieved by *P. lessonae* usually after the second hibernation, i.e., when they are 2 years old (Haczkiwicz et al. 2017). In semi-natural conditions and with good food supply, *P. lessonae* males can produce some mature spermatozoa earlier and successfully fertilize eggs even after the first hibernation (Berger 1970, 1973c). Most probably, younger males are likely not to be admitted into the breeding arena by competing older males. This seems to be a behavioral barrier not mentioned in the literature so far.

Genomes transmitted to progeny by parents from natural pairs

Water frogs have the XX female and XY male sex determination system. The gametes produced by females always contain the X chromosomes, while the gametes produced by

males may contain the X or Y chromosomes (Schempp and Schmid 1981; Stöck et al. 2021).

The most common pairs found in our study were homotypic *P. lessonae*. It might seem that they will produce offspring with the predictable sex ratio of 1:1. However, one male transmitted only the Ly gametes resulting in sons only, whereas other five males transmitted only the Lx gametes resulting in daughters only. A similar situation was found in *P. lessonae* males in heterotypic pairs with *P. esculentus* females, where four fathers transmitted only the Lx gametes. These results indicate that the skewed sex ratio in some populations may be the result of a disturbed transmission of the sex chromosomes not only by hybrids but also by the parental species, in this case *P. lessonae*.

We also found a significant deviation from the known mechanism of hybridogenesis. A classic scenario assumes that the hybrid restores itself in backcrosses by transmitting the genome of the parental species that does not occur in the population (Berger 1973a). In our study, five (38%) *P. esculentus* females transmitted simultaneously two kinds of genomes: Rx and Lx and these, when fertilized by *P. lessonae*, enabled the restoration of the parental species (hybridolysis according to Plötner and Schmeller 2001). These results suggest a much greater potential for diverse combinations of the gametes and genome introgression in the L-E system than previously suggested (Berger 1973a). The observed variety of genomes transferred by hybrids and *P. lessonae* (Rx, Lx, and Ly) ensures a successful restoration of hybrids and species of both sexes. However, the limitation of our study in predicting the population composition is the examination of offspring at the stages before reaching sexual maturity. Therefore, we do not know whether the species restored in this way will be a permanent component of the population.

Conclusions

Our study sheds new light on the maintenance of the L-E systems of water frogs. We confirmed the stability of the taxa composition that persisted for over 10 years under a constant and suitable habitat of two adjoining fish ponds. The stability is ensured by a specific female mate choice pattern with the dominance of homotypic *P. lessonae* and heterotypic ♀ *P. esculentus* × ♂ *P. lessonae* pairs (almost 90% of all pairs collected during the breeding period). For the first time, we observed a male-male competition manifested by the exclusion of young sexually mature males (before the third hibernation) from participating in the mating. Thus, the individual age should be included as an important variable when studying the mate choice in amphibians.

Another important feature affecting the population structure is the production of differentiated (simultaneously R and L) gametes by hybrids of both sexes. A high share of the L genomes transmitted to the gametes enables both the

restoration of the parental species *P. lessonae* and the regular renewal of a new generation of hybrids. Sex inheritance followed the XX/XY system. New generations of males of both taxa were formed only by the Ly gametes produced by both *P. lessonae* and *P. esculentus* males because the Ry gametes were not transferred by male hybrids in the studied population. Furthermore, long-term monitoring of the L-E (and other) genetic systems of water frogs including species composition, sex ratio, age, mate choice, and gamete production is necessary to fully understand the features that shape the population persistence.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethical approval All procedures were approved by the Ministry of Environment (DOPg-4201-02-74/05/kl), Regional Director of Environmental Protection in Wrocław (DPZ-WG.6401.02.2.2017.dł), and Local Committee of Ethics (103/2007). All applicable national and institutional guidelines for the use of animals were followed.

Competing interests The authors declare no competing interests.

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