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Filling a gap in central Europe: morphological and genetic variation in the hybridogenetic complex of water frogs (genus *Pelophylax*) in Slovakia

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Abstract. The central European complex of water frogs (*Pelophylax esculentus* complex) is one of the best studied hybridogenetic complexes in vertebrates, but also one of the most complicated due to different modes of hybrid reproduction. Although genetic and ecological research on water frogs has been going on for more than fifty years, many areas of Europe are still underexplored, which is also the case of eastern Slovakia. In this study, we fill this gap and investigate local populations from morphological and genetic perspectives. During field surveillance and using genetic markers, we found 146 individuals of *P. ridibundus*, 17 of *P. lessonae* and 116 diploid hybrids of *P. esculentus* in 21 localities. In addition to pure populations of *P. ridibundus*, we recorded the syntopic occurrence of hybrids with parental species. The sex ratio in parental species was equal, but females strongly dominated in hybrids. Based on several clonality tests, it can be assumed that hybrids primarily eliminate the *lessonae* genome during gametogenesis and form clonal gametes with the *ridibundus* genome. The high clonal diversity observed in the *ridibundus* genome of hybrids is most likely the result of multiple primary hybridization events in sympatric populations. All individuals of *P. lessonae*, 86.2% of *P. esculentus* and 8.3% of *P. ridibundus* had *lessonae* mtDNA. Introgressed individuals of *P. ridibundus* shared the same mitochondrial haplotypes with the two other taxa, suggesting relatively recent unidirectional introgression.

Key words. Amphibia, Anura, Ranidae, clonal reproduction, habitat preferences, haplotype diversity, hybridogenesis, mtDNA introgression, ploidy level.

Introduction

The vast majority of vertebrates reproduce sexually, with males and females producing gametes through meiosis. Asexual reproduction is rare and typically results from disrupted meiosis, leading to clonal or partially clonal offspring (Bengtsson 2009). Most of the obligate asexual vertebrates originate from interspecific hybridization and often include polyploid forms (Neaves & Baumann 2011, Choleva et al. 2012, Neiman et al. 2014, Stöck et al. 2021). Three main asexual reproductive modes exist: parthenogenesis, where females produce clonal offspring without males; gynogenesis, where sperm activates but does not fertilize eggs; and hybridogenesis, where hybrids discard one parental genome and reproduce with males of a sexual species (Avise 2008, Lehtonen et al. 2013, Janko et al. 2023).

The dependence of gynogenetic and hybridogenetic forms on their sexual relatives has led to complex reproductive, ecological and evolutionary interactions between the individual players in sexual-asexual complexes. One of the best-studied, but also most complicated, is the central European complex of water frogs (*Pelophylax esculentus* complex). The complex consists of two parental species, *Pelophylax ridibundus* (RR) and *P. lessonae* (LL), which interbreed to produce the hybrid *P. esculentus* (LR). Hybrids reproduce hybridogenetically, that is, they eliminate the parental genome of one species (usually *P. lessonae*) during gametogenesis and produce clonal gametes with the genome of the other parental species (usually *P. ridibundus*), backcrossing with individuals of the species whose genome they have eliminated. Such crosses result in hybrid offspring that have half of the genome clonal and the other

sexual (Uzzell & Berger 1975, Graf & Polls Pelaz 1989, Plötner 2005).

Pelophylax esculentus often co-occurs with one of the parental species. In syntopic occurrence with *P. lessonae*, hybrids produce clonal gametes with the *P. ridibundus* genome (R gametes). This population system has been named the L-E system (Günther 1983, 1990). A kind of mirror system is R-E, in which hybrid individuals, usually males, form gametes predominately with the *P. lessonae* genome (L gametes) and interbreed with syntopic individuals of *P. ridibundus* (Uzzell et al. 1977, Doležálková-Kaštánková et al. 2018). Some hybrid males in R-E populations form both L and R gametes simultaneously (Doležálková-Kaštánková et al. 2021). Numerous triploid individuals of two types, LLR and LRR, occur in the northern (N Germany, Poland, Denmark, S Sweden) and eastern (E Ukraine) parts of the *P. esculentus* range (Hoffmann et al. 2015). These may produce partially recombined gametes carrying the genome of one or the other parental species. Together with diploid individuals that produce both haploid and diploid gametes, they can form pure hybrid populations independent of the parental species (Christiansen et al. 2005, Christiansen 2009, Christiansen & Reyer 2009). Triploids are, however, rare in central Europe (Hoffmann et al. 2015, Pruvost et al. 2015). The syntopic occurrence of all three taxa of the complex is relatively rare, probably due to their different habitat preferences. While *P. ridibundus* prefers larger and well-oxygenated, often man-made water bodies, *P. lessonae* is associated with smaller, more densely vegetated and natural marsh habitats. *Pelophylax esculentus* is ecologically plastic but often inhabits similar habitats to *P. lessonae*, on which it is reproductively dependent in most locations (Mikulíček et al. 2015).

Since hybridogenetic hybrids are reproductively dependent on parental species, they have a significant impact on their ecology and evolution (Janko et al. 2023). One important consequence of hybridization and hybridogenesis in the water frog complex is introgression, i.e., hybrid-mediated gene flow between parental species. The mode of hybridogenetic gametogenesis prevents significant introgression of nuclear genomes but allows efficient introgression of mitochondrial DNA (mtDNA) (Uzzell et al. 1977, Spolsky & Uzzell 1986). In western and parts of central Europe, up to 30% of *P. ridibundus* individuals carry *lessonae* mtDNA, with introgression rates decreasing towards the east (Plötner et al. 2008, Mikulíček et al. 2014). Mitochondrial introgression is mediated by hybrid individuals carrying *lessonae* mtDNA, which form R gametes and interbreed either with each other or with *P. ridibundus* individuals (Vorburger 2001, Vorburger et al. 2009). These types of crosses result in *P. ridibundus* offspring with *lessonae* mtDNA; thus, mtDNA introgression is unidirectional, from *P. lessonae* to *P. ridibundus*.

As knowledge of the genetic diversity of water frogs increases, haplotypes and alleles of the taxon *Pelophylax kurtmuelleri* have been discovered in central, western and eastern Europe (Dubey et al. 2014, Kolenda et al. 2017, Dufresnes et al. 2018, Svinin et al. 2021). The taxonomic status of *P. kurtmuelleri* is controversial (Speybroeck et al. 2020) due to low mtDNA divergence and shared interspecific polymorphism with *P. ridibundus* (Lymberakis et al. 2007, Akin et al. 2010, Hofman et al. 2015, Papežík et al. 2023). *Pelophylax kurtmuelleri* is considered to be either a separate species, subspecies or a diverged evolutionary lineage of *P. ridibundus* (Dufresnes et al. 2024). The center of its distribution is the southwestern Balkans, from where individuals may have reached other parts of Europe either as a result of unintentional human introduction (western Europe) or natural dispersal after the last Ice Age (Kolenda et al. 2017, Papežík et al. 2023, Litvinchuk et al. 2024). Hybridization with central European species, especially *P. ridibundus*, may have led to the introgression of this Balkan endemic's genes into local populations of water frogs.

Although research on the hybridization and hybridogenesis of water frogs has been ongoing since the mid-1960s (e.g., Berger 1967, 1968, Tunner 1974), many areas of Europe are still understudied. In our study, we focused on populations inhabiting the eastern part of Slovakia, where research has not yet been carried out. We used several genetic markers to answer the following questions. 1) What is the taxonomic composition, sex ratio and ploidy level in populations of water frogs in eastern Slovakia? 2) What is their morphological and genetic variability? 3) Which parental genome is more influenced by clonality and how do hybrids reproduce? 4) What is the rate of mtDNA introgression in populations? and 5) Are mitochondrial haplotypes of *P. kurtmuelleri* present in eastern Slovakia?

Material and methods

Sampling

In total, 279 samples of water frogs were collected during three seasons (May–July 2016, May–August 2017 and May–October 2018) at 21 sites in eastern Slovakia (Table 1, Fig. 1). Frogs were caught mostly during the night in a net or by hand. Five morphometric traits were measured in captured individuals using a calliper with an accuracy of 0.02 mm (L – snout-to-vent length, F – femur length, T – tibia length, CINT – metatarsal tubercle length, DP – length of the first toe on the hind limb) and the four indices were calculated (L/T, L/CINT, DP/CINT T/CINT; adapted from Günther 1990, Plötner 2005). In addition, qualitative traits such as the shape of the metatarsal tubercles, the presence or absence of yellow pigment on the thighs and flanks, the presence or absence of ventral marbling and the color of the vocal sacs in males were recorded. Sex was determined based on the presence of vocal sacs and nuptial pads in males. Individuals were considered adults if their snout-to-vent length was greater than 55 mm in *P. ridibundus* and 45 mm in *P. lessonae* and *P. esculentus* (Mikulíček et al. 2015). Males with this body length have visible vocal sacs. In all frogs, dorsal, ventral and lateral sides were photographed. A tissue sample was collected from the tip of the third finger of the forelimb and was preserved in

Table 1. List of sampling sites of water frogs in eastern Slovakia with site abbreviations in brackets, geographical coordinates, number of sampled individuals (N), habitat type, number of individuals of each species (M – males, F – females, JUV – juveniles) and percentage of *P. lessonae*-specific (L) mtDNA found in *P. ridibundus* (RR) and *P. esculentus* (LR). A – artificial, N – natural, NC – not calculated.

Locality	Latitude	Longitude	N	Habitat type	*P. ridibundus*			*P. esculentus*			*P. lessonae*			L–mtDNA in RR (%)	L–mtDNA in LR (%)
					M	F	JUV	M	F	JUV	M	F	JUV		
Oreské (OR)	48.8490	21.9048	23	water reservoir (A)	4	19	0	0	0	0	0	0	0	8.7	–
Baňa (BA)	48.7642	21.9319	5	material pit (A)	2	3	0	0	0	0	0	0	0	20.0	–
Janík (JA)	48.5443	20.9903	11	water reservoir (A)	4	4	3	0	0	0	0	0	0	18.2	–
Vranie jazero (HMR)	48.6876	21.2940	17	oxbow lake (N)	11	2	4	0	0	0	0	0	0	0	–
Nad jazerom (JMR)	48.6904	21.2864	10	gravel pit (A)	7	1	2	0	0	0	0	0	0	0	–
Krásna (KR)	48.6636	21.3152	21	gravel pit (A)	9	3	9	0	0	0	0	0	0	0	–
Ždaňa (ZD)	48.6100	21.3498	22	oxbow lake (N)	7	6	9	0	0	0	0	0	0	0	–
Perín–Chym (PCH)	48.5421	21.1445	10	water reservoir (A)	3	7	0	0	0	0	0	0	0	10	–
Senné (SEN)	48.6783	22.0569	16	water reservoir (A)	4	9	1	0	2	0	0	0	0	35.7	NC
Rakovec n. Ondavou (RO)	48.7655	21.7920	14	water reservoir (A)	0	8	0	0	6	0	0	0	0	0	66.7
Seňa (SE)	48.5643	21.2740	4	water reservoir (A)	3	1	0	0	0	0	0	0	0	0	–
Turniansky rybník (TU)	48.6046	20.8638	1	water reservoir (A)	0	0	1	0	0	0	0	0	0	NC	–
Vojany (V)	48.5693	21.9720	1	water depression (N)	0	0	0	0	1	0	0	0	0	–	NC
Nová Vieska pri Bodrogu (NV)	48.4171	21.8159	19	oxbow lake (N)	0	0	0	0	16	3	0	0	0	–	94.1
Rad (R)	48.4633	21.8539	18	oxbow lake (N)	0	0	0	2	14	0	1	1	0	–	75.0
Boťany (BO)	48.4410	22.0985	9	oxbow lake (N)	0	0	0	0	8	1	0	0	0	–	100
Streda nad Bodrogom (SB)	48.3877	21.7747	6	oxbow lake (N)	0	0	0	0	4	1	0	0	1	–	100
Somotor (SO)	48.3870	21.8406	26	water depression (N)	0	0	0	12	10	0	3	1	0	–	76.5
Klin nad Bodrogom (KB)	48.3819	21.7250	21	oxbow lake (N)	0	0	0	0	13	8	0	0	0	–	81.0
Strážne (ST)	48.3800	21.8551	21	water depression (N)	0	0	0	3	12	0	4	2	0	–	100
Kamenec (KA)	48.3554	21.7801	4	drainage channel (A)	0	0	0	0	0	0	4	0	0	–	–
Total			279		54	63	29	17	86	13	12	4	1		

96% ethanol for subsequent molecular analyses. Individuals were thus also marked to avoid recapture. A drop of blood was taken from the cut finger and was used to prepare a blood smear. The blood smears were stained with Giemsa in a lab and then examined under a light microscope (Leica DM500).

DNA extraction, microsatellite and mtDNA amplification

A total DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Microsatellite genotypes were used as a complementary method for species identification, estimation of genetic diversity and clonality. Microsatellites were amplified in three multiplex PCR reactions using a Type-it Microsatellite PCR Kit (Qiagen). The first multiplex consisted of loci RlCA1b5 (Garner et al. 2000), Ga1a19 (Christiansen 2009), RlCA2a34 (Christiansen & Reyer 2009), Rrid013A (Hotz et al. 2001), Res17 (Zeisset et al. 2000), Rrid064A (Christiansen & Reyer 2009), the second multiplex amplified loci Re1Caga10 (Arioli et al. 2010), Res22 (Zeisset et al. 2000), RlCA18 (Garner et al. 2000) Rrid082A (Hotz et al. 2001), Rrid169A (Christiansen & Reyer 2009) and RlCA1b6 (Arioli et al. 2010), and the third multiplex loci RlCA5 (Garner et al. 2000), Ga1a23 (Christiansen & Reyer 2009), Rrid059A (Hotz et al. 2001), RlCA1a27 (Christiansen & Reyer 2009), Rrid135A (Christiansen & Reyer 2009) and Pper3.22 (Sánchez-Montes et al. 2016). The composition of the PCR mixtures is given in Table S1. PCR program was as follows: initial denaturation for 5 min at 95 °C, 30 cycles of denaturation for 30 s at 95 °C, annealing for 90 s at 60 °C and extension for 60 s at 72 °C, and final extension at 60 °C for 30 min (adapted from Christiansen & Reyer 2009).

Microsatellite fragments were scored in Geneious Prime software (v. 2021.1.1).

The type of mitochondrial DNA (*ridibundus*-specific or *lessonae*-specific mtDNA) was identified by PCR amplification of the NADH dehydrogenase 2 (ND2) gene followed by the restriction fragment length polymorphism (RFLP) analysis. The PCR mixture consisted of 1.125 µl of DreamTaq buffer with 1.5 mM $MgCl_2$, 0.45 µl of dNTPs (concentration of each 10 mM), 0.054 µl of DreamTaq™ DNA polymerase (Thermo Fisher Scientific), 5.971 µl of ddH_2O, 0.2 µl of each primer at a concentration of 10 mM (primers L2 and H2 according to Plötner et al. 2008), and 2 µl of DNA. The PCR program was as follows: initial denaturation for 2 min at 94 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C and extension for 60 s at 72 °C, final extension at 72 °C for 10 min. PCR products were digested with AluI restriction enzyme (Thermo Fisher Scientific) at 37 °C for 3 h. The RFLP mixture consisted of 5 µl of PCR product, 0.37 µl of AluI restriction enzyme, 1 µl of 10× Tango buffer and 9.13 µl of ddH_2O. Restriction fragments were visualized on a 2% agarose gel. In *P. ridibundus*-specific mtDNA, AluI recognized a restriction site in the ND2 gene, resulting in two RFLP fragments of 300 and 450 bp; in *P. lessonae*-specific mtDNA, the ND2 amplicon was not digested (Plötner et al. 2008). We were unable to determine the mtDNA type of one *P. ridibundus* and seven hybrids. We calculated the percentage of *lessonae*- and *ridibundus*-specific mtDNA in all populations, except those where only one or two individuals were captured.

For the analysis of mtDNA diversity, we amplified a 1038 bp-long fragment of ND2 using primers L1 and H2 according to Plötner et al. (2008). The PCR mixture composition and PCR program were the same as described above, except for the annealing temperate, which was 60 °C. PCR products were commercially sequenced in Macrogen Europe (Amsterdam, The Netherlands). Sequences were checked and aligned in Geneious Prime software (v. 2021.1.1). Sequences of recorded unique haplotypes were deposited in GenBank under accession numbers PV177127–PV177145.

Determination of ploidy level

Ploidy level in hybrids was determined using erythrocyte size and the gene-dose effect in species-specific single nucleotide polymorphic (SNP) sites in the ubiquinol-cytochrome c reductase subunit 1 (uqcrfs1) gene. Triploid

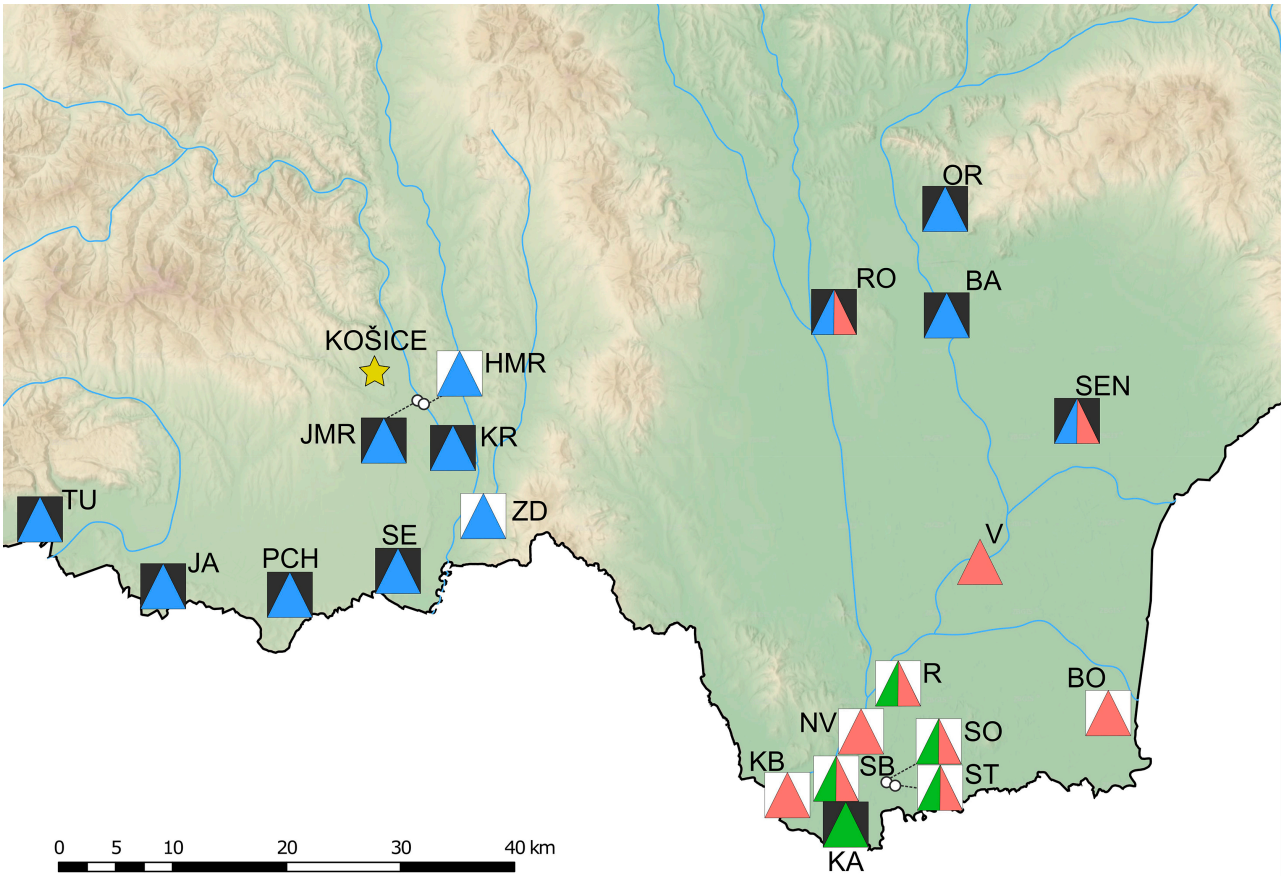

Figure 1. Taxonomic composition of water frog populations and habitat type: Blue triangle – *Pelophylax ridibundus*, green triangle – *P. lessonae*, red triangle – *P. esculentus*, black square – artificial water bodies, white square – natural water bodies. For site codes see Table 1.

individuals have larger erythrocytes (erythrocyte length is more than 27 µm) than diploid individuals (Günther 1977). Eighty of the hybrid frogs were examined for erythrocyte size; in the others, blood smears were of poor quality. The average erythrocyte size was calculated for each individual by measuring 15 randomly selected blood cells. If average value was equal or more than 26 µm, or blood smears were of poor quality, we used uqcrfs1 sequences for ploidy level determination based on the gene dose effect (Tecker et al. 2017). RRL triploids exhibit significantly higher *ridibundus*-specific peaks in electropherogram than LLR triploids and vice versa. In diploid hybrids, *ridibundus*- and *lessonae*-specific peaks exhibit equal height. A 441-bp long fragment of the uqcrfs1 gene was amplified according to Tecker et al. (2017). PCR mixture was composed of 1.5 µl of DreamTaq buffer with 1.5 mM $MgCl_2$, 0.3 µl of dNTPs (concentration of each10 mM), 0.1 µl of DreamTaq™ DNA polymerase (Thermo Fisher Scientific), 10.5 µl of ddH_2O, 0.3 µl of each primer at a concentration of 10 mM (for primer sequences see Tecker et al. 2017), and 2 µl of DNA. The PCR program was as follows: initial denaturation for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 62 °C and extension for 60 s at 72 °C, final extension at 72 °C for 5 min. PCR products were sequenced in Macrogen Europe (Amsterdam, The Netherlands). Sequences and gene dose effects at selected SNPs were analyzed in Geneious Prime software (v. 2021.1.1).

Morphological analyses

Morphometric indices were tested for normality using the Shapiro-Wilk test. Due to the nature of the data, differences between species in four indexes (L/T, L/CINT, T/CINT, DP/CINT) were examined using the nonparametric Kruskal-Wallis test with subsequent pairwise comparisons using stat_compare_means() function.

We log-transformed morphometric data for multivariate analysis. To mitigate the potential influence of sexual dimorphism, we created separate datasets for males and females. To assess the separation between species in multivariate space, we performed Principal Component Analysis (PCA) and Discriminant Analysis of Principal Components (DAPC).

The association between selected qualitative morphological traits and species was tested using the Chi-square test. The nature of the association and contributions to the chi-square score were then visualized by plotting standardized Pearson's residuals between expected and observed values.

All statistical analyses and data visualizations were performed in the R statistical environment, version 4.1.3 (R Core Team 2022) using the adegenet package (Jombart 2008), corrplot package (Wei & Simko 2021), ggplot2 package (Wickham 2016), ggpubr package (Kassambara 2023) and vegan package (Oksanen et al. 2024).

Genetic analyses

Microsatellite loci showed species-specific amplification or species-specific sets of alleles that allowed the assignment of each individual to one or the other parental species or to a group of hybrids. This preliminary assignment of individuals to taxa was confirmed by ordination methods Principal Coordinate Analysis (PCoA) implemented in GenAlEx v. 6.51 (Peakall & Smouse 2006) and PCA and DAPC implemented in the adegenet package (Jombart 2008). DAPC was performed to infer genetic differences in a multivariate space with a priori defined clusters corresponding to parental species and their hybrids. Each individual was assigned to the inferred cluster based on morphology and a species-specific pattern in microsatellites. The optimal number of PCs retained for DAPC was k-1, where k is the number of clusters. This criterion captures the maximum variability among populations while being less sensitive to unintended interpretations of population structure (Thia 2023). We followed the recommended standard reporting for DAPC suggested by Miller et al. (2020). The graphical output was edited by the ggplot2 package.

For the estimation of genetic diversity and clonality, parental species and parental genomes of hybrids (R and L) were analyzed separately. In *P. ridibundus* and R genome of hybrids, 13 loci were analyzed (Ga1a19, Re1Caga10, Rrid169A, RlCA1b5, Rrid064A, Res22, Rrid082A, Res17, Rrid013A, RlCA1b6, Rrid059A, Rrid135A, and Pper3.22). In *P. lessonae* and L genome of hybrids, the analyses were based on 10 loci (Ga1a19, RlCA18, RlCA2a34, RlCA1b5, Rrid013A, RlCA1b6, RlCA5, Rrid059A orig., RlCA1a27, Ga1a23). Species-specific loci and alleles are presented in Table S2.

Genetic diversity was estimated using the average number of alleles per locus (Na), observed (Ho) and unbiased expected heterozygosity (uHe). The rate of clonality in the R and L genomes was estimated using three approaches – multilocus genotypes (MLGs), genotypic diversity (Div) and multilocus linkage disequilibria (LD).

A multilocus genotype is defined as the identical combination of alleles found in microsatellite loci. A few combinations and high frequencies of these combinations may indicate clonal reproduction. Conversely, sexual reproduction with periodic recombination produces unique genotypes. However, the same MLG may also be found in two or more unrelated sexual individuals if the discriminatory power of the molecular markers used is low. Therefore, we first calculated the probability of identity (PI), an estimate of the probability that two individuals randomly chosen from a population have the same MLG on a set of markers (Pruvost et al. 2015). PI reached 2.0×10^{-9} in *P. ridibundus* and 7.5×10^{-7} in *P. lessonae*, indicating a low probability of two individuals of the parental species sharing the same MLG.

The genotypic diversity (Div) is defined as the probability that two individuals taken at random from a population have different genotypes. This value is zero if every individual is the same, and one if every individual is different.

Multilocus disequilibrium (LD) is a score of non-random association of alleles at microsatellite loci and varies on a scale from zero to one. Low LD values are associated with a high recombination rate and thus indicate a low level of clonality. Contrarily, high LD values may be associated with clonal inheritance, as clonal genomes are inherited en bloc, i.e., without recombination.

Parameters of genetic diversity and MLGs were calculated using the GenAlEx v. 6.51 package (Peakall & Smouse 2006, 2012). Genotypic diversity and multilocus LD were calculated in the program Multilocus 1.3 (Agapow & Burt 2001). For the analysis of mitochondrial diversity, we incorporated GenBank sequences from Plötner et al. (2008) in the *P. lessonae* ND2 dataset, and sequences from Papežík et al. (2023) in the *P. ridibundus* dataset. These sequences were used to determine the affiliation of collected individuals with established lineages. ND2 sequences were also used to detect *P. kurtmuelleri* haplotypes. For this purpose, haplotype networks were constructed using the Parsimony Network Analysis and TCS algorithm proposed by Clement et al. (2000) in PopArt 1.7 (http://popart.otago.ac.nz). To further investigate mitochondrial diversity, we performed PCA using the adegenet package within the R statistical environment (R Core Team 2022). Haplotype diversity (Hd) and nucleotide diversity (π) statistics with corresponding standard deviation (SD) values were calculated for the *ridibundus*-specific mitochondrial genome present in *P. ridibundus* and hybrids, and for the *lessonae*-specific mitochondrial genome present in *P. lessonae*, hybrids and *P. ridibundus* (introgressed mitogenome). The DnaSP v. 6 program was used to calculate Hd and π (Rozas et al. 2017).

Results

Species identification, sex ratio and ploidy level

Microsatellite analyses identified 146 individuals of *P. ridibundus*, 17 individuals of *P. lessonae* and 116 *P. esculentus* hybrids (Table 1).

PCA, DAPC and PCoA based on microsatellite genotypes assigned individuals to distinct clusters corresponding to parental species and hybrids (Figs 2, S1A, B). Only in PCA were two individuals of *P. lessonae* not assigned to the species-specific cluster: instead, they showed affinity to the hybrid cluster (Supplement, Fig. S1A). The sex ratio in *P. ridibundus* (53.8% of females, 46.2% of males, N = 117,

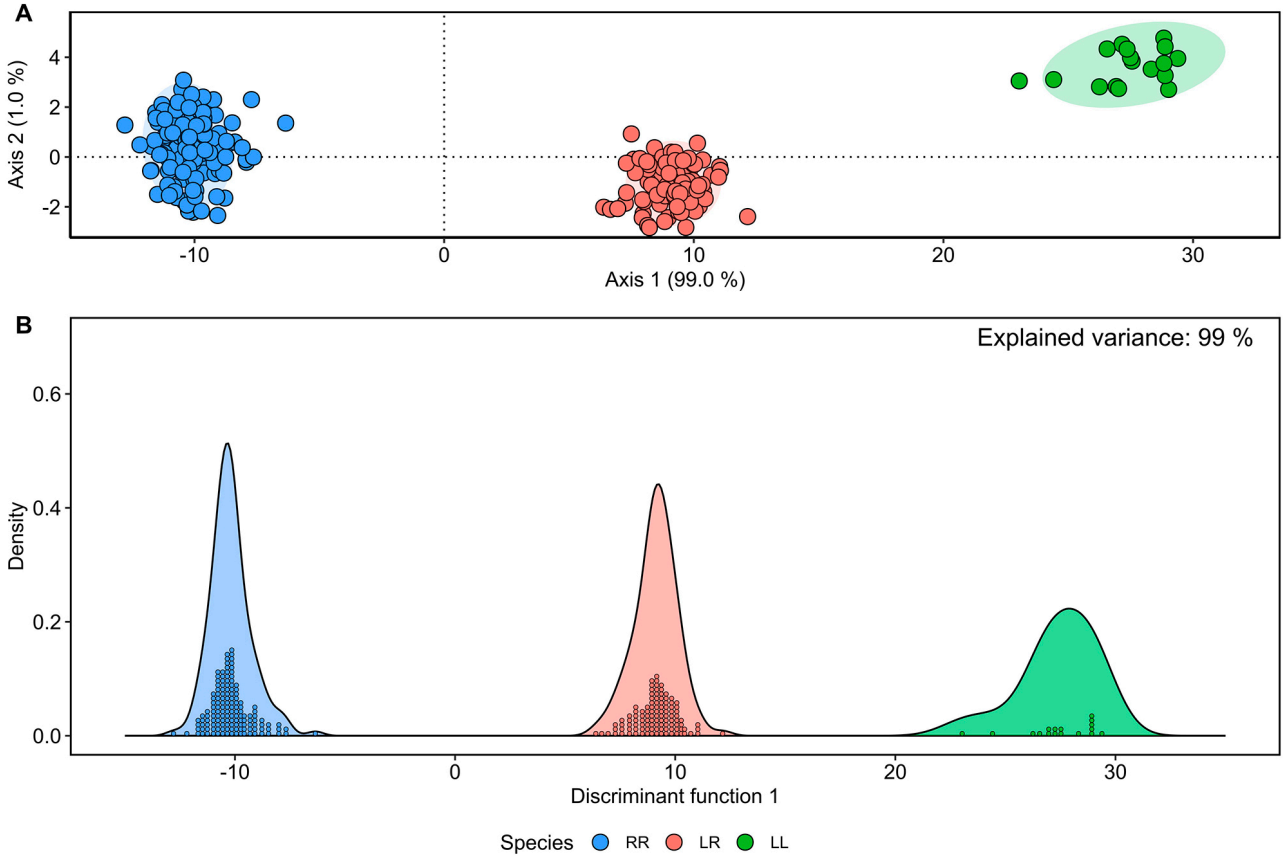

Figure 2. Clustering of water frog individuals based on microsatellite genotypes: (A) Discriminant analysis of principal components (DAPC); (B) discriminant function 1 from DAPC with histograms of individuals explaining the majority of the variance. RR – *Pelophylax ridibundus*, LR – *P. esculentus*, LL – *P. lessonae*.

Fisher's exact test, p = 0.695) and *P. lessonae* (25% of females, 75% of males, N = 16, Fisher's exact test, p = 0.273) did not differ from the expected equal ratio. In hybrids, females significantly predominated (83.3% of females, 16.7% of males, N = 102, Fisher's exact test, p < 0.0001).

The size of erythrocytes in hybrids showed a unimodal distribution and corresponded to diploid genotypes (N = 80, mean = 23.278 µm, SD = 1.039, min/max = 20.895 / 25.889 µm; Supplement, Fig. S2). Similarly, sequences of the uqcrfs1 gene showed the presence of only diploid individuals; electropherogram peaks at species-specific positions were of the same height and showed no gene-dose effect.

Taxonomic composition of populations

Pelophylax ridibundus lived either alone or in two localities together with *P. esculentus* (RO and SEN) (Table 1, Fig. 1). Sites inhabited by this species mainly included artificial water bodies (e.g., water reservoirs, gravel pits, material pits) in or near human settlements. On the contrary, hybrids were frequently observed in coexistence with *P. lessonae* in the southernmost part of eastern Slovakia, where both species inhabited mainly natural water bodies (e.g., oxbow lakes, water depressions) or drainage channels with abundant submerged and riparian vegetation. At three sites (BO, KB, NV) we found hybrids without parental individuals, but due to the limited number of captured frogs, we cannot exclude the presence of one or the other parental species.

Morphological variability

The clustering of individuals based on morphometric data was not as pronounced as for microsatellites. In males, there was partial overlap between *P. lessonae* and hybrids, but both were separated from *P. ridibundus*. In females, differences in morphometrics between representatives of the complex were even smaller. The hybrid group occupied an intermediate position and overlapped with both parental species (Fig. 3, 4, 5, S3).

All four indices (L/T, L/CINT, T/CINT, DP/CINT) showed highly significant differences between species (p ≤ 0.001), and only the comparison of L/CINT between *P. lessonae* and *P. esculentus* showed lower statistical significance (p ≤ 0.05) (Fig. 4). Qualitative morphological traits also showed significant species differences (Chi-squared = 438.33, df = 14, p < 2.2e-16). Yellow pigment on hind limbs and lateral sides, and metatarsal tubercle size showed the strongest species correlations (Fig. 5). Yellow pigment, white vocal sacs and a high metatarsal tubercle were positively correlated with *P. lessonae*, dark grey vocal sacs, ventral marbling on the belly and a low metatarsal tubercle were positively correlated with *P. ridibundus*. Hybrids showed a positive correlation with yellow pigment and a medium-sized metatarsal tubercle.

Genetic variability and clonality

Genetic variability expressed as a percentage of polymorphic loci (P), average number of alleles per locus (Na), observed

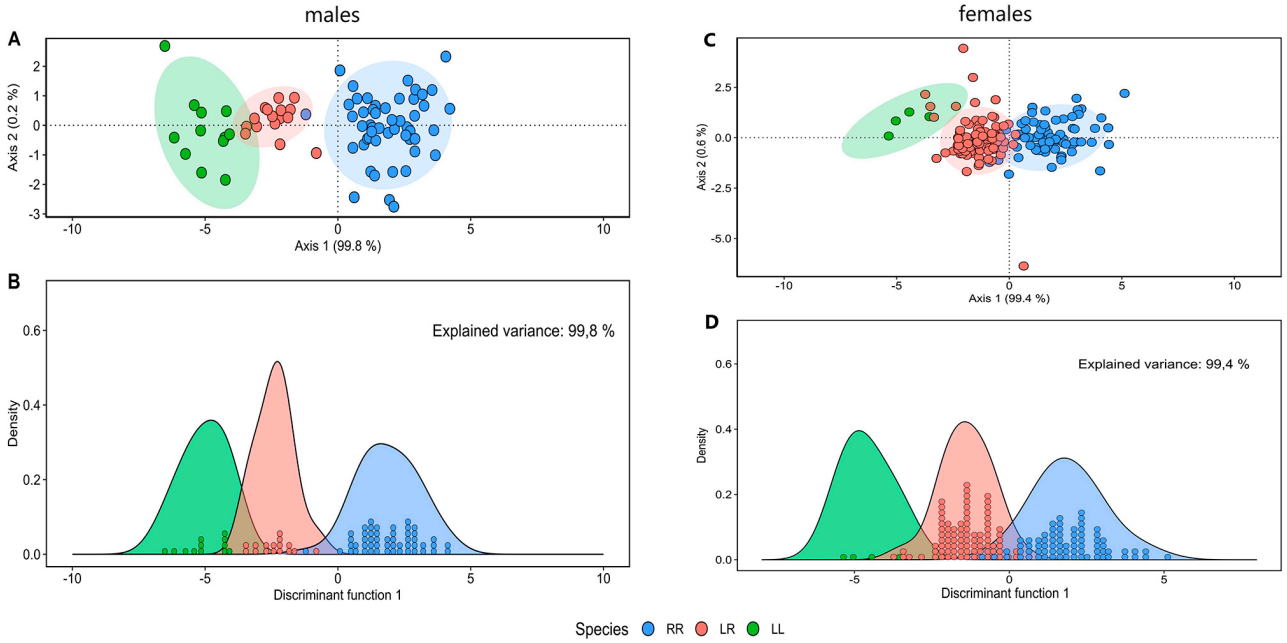

Figure 3. Morphological variation of water frogs. (A) Males and (C) females: clustering in multivariate space based on discriminant analysis of principal components (DAPC). (B) Males and (D) females: discriminant function 1 from DAPC with histograms of individuals explaining the majority of the variance RR – *Pelophylax ridibundus*, LR – *P. esculentus*, LL – *P. lessonae*.

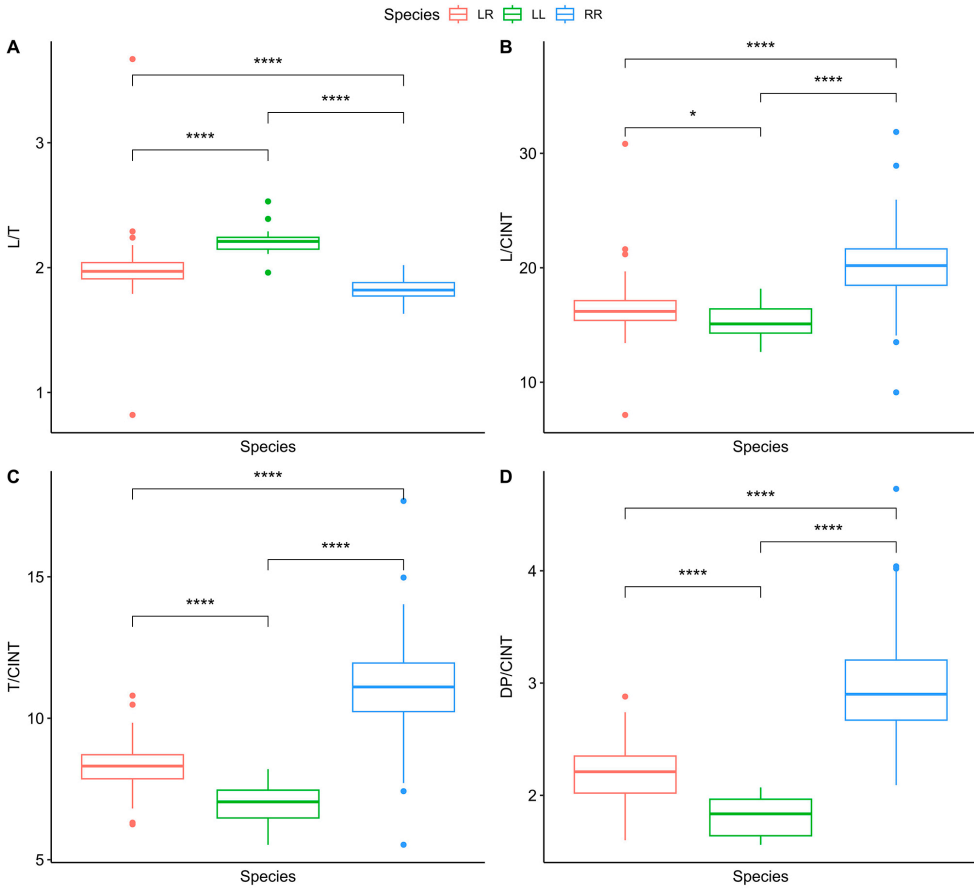

Figure 4. Comparison of four morphometric indices between all representatives of the *Pelophylax esculentus* complex. For indices abbreviations see Materials & methods. RR – *P. ridibundus*, LR – *P. esculentus*, LL – *P. lessonae*. Asterisks indicate the significance level between the compared species: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

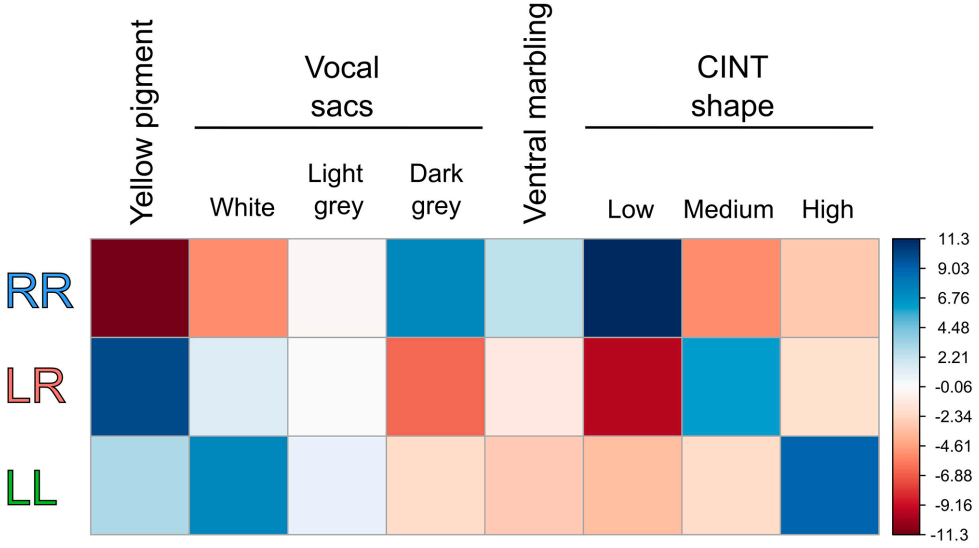

Figure 5. Correlations between water frog species and selected qualitative traits. RR – *Pelophylax ridibundus*, LR – *P. esculentus*, LL – *P. lessonae*; blue shades – positive correlations, red shades – negative correlations, yellow pigment – yellow pigment in the hind legs and lateral sides, CINT shape – shape (low, medium, high) of the metatarsal tubercle.

Table 2. Parameters of genetic variability in parental species *Pelophylax ridibundus*, *P. lessonae* and the R and L genomes of hybrids *P. esculentus*. N – number of individuals, P – percentage of polymorphic loci, Na – average number of alleles per locus, Ho – observed heterozygosity, uHe – unbiased expected heterozygosity. Observed heterozygosity was not analyzed (na) in a haploid genome of hybrids.

Species/genome	N	P (%)	Na	Ho	uHe
P. ridibundus	146	100.0	7.538±1.124	0.477±0.071	0.530±0.071
P. lessonae	17	80.0	5.200±1.200	0.418±0.103	0.470±0.116
P. esculentus R genome	116	92.3	3.231±0.579	na	0.252±0.073
P. esculentus L genome	116	90.0	6.900±1.567	na	0.473±0.112

(Ho) and unbiased expected heterozygosity (uHe) were slightly higher in *P. ridibundus* than in *P. lessonae* (Table 2). Values of genetic variability in the R genome of the hybrids were approximately lower by half compared to both *P. ridibundus* and the L genome. Contrarily, the genetic variability in the L genome and the *P. lessonae* gene pool were comparable (uHe) or only slightly higher in the hybrids (Na).

Both parental species exhibited exclusively unique multilocus genotypes (MLGs), consistent with their sexual mode of reproduction. Unique MLGs were also present in the L genome of the hybrids. In contrast, 27 MLGs out of 116 were unique in the R genome, the rest of 89 hybrids possessed 13 MLGs (MLG-A 4 individuals, MLG-B 4 individuals, MLG-C 6 individuals, MLG-D 8 individuals, MLG-E 2 individuals, MLG-F 6 individuals, MLG-G 3 individuals, MLG-H 6 individuals, MLG-I 2 individuals, MLG-J 3 individuals, MLG-K 40 individuals, MLG-L 2 individuals, MLG-M 3 individuals).

Multilocus linkage disequilibrium (LD) in the L genome of the hybrids was low and non-significant (LD = 0.001, p = 0.416), and the genotypic diversity index (Div) reached the maximum value of 1.000. Multilocus LD was significantly higher in the R genome (LD = 0.156, p = 0.001) and Div reached the value of 0.837.

Introgression of mtDNA and haplotype diversity

All individuals of *P. lessonae*, 86.2% of *P. esculentus* and 8.3% of *P. ridibundus* had *lessonae* mtDNA. The incidence of *lessonae* mtDNA varied among populations; from 66.7% to 100% in hybrids and from 0% to 35.7% in *P. ridibundus* (Table 1, Fig. 6A). Introgression of *lessonae* mtDNA into the *P. ridibundus* gene pool was observed in pure *P. ridibundus* populations (OR, BA, JA, PCH) as well as in the syntopic occurrence with hybrids (SEN). In the other five pure populations of *P. ridibundus* (HMR, JMR, KR, ZD, SE), introgression was not observed.

Six *ridibundus*-specific haplotypes (rid1–rid6) were identified in 128 ND2 sequences, with only two were shared with

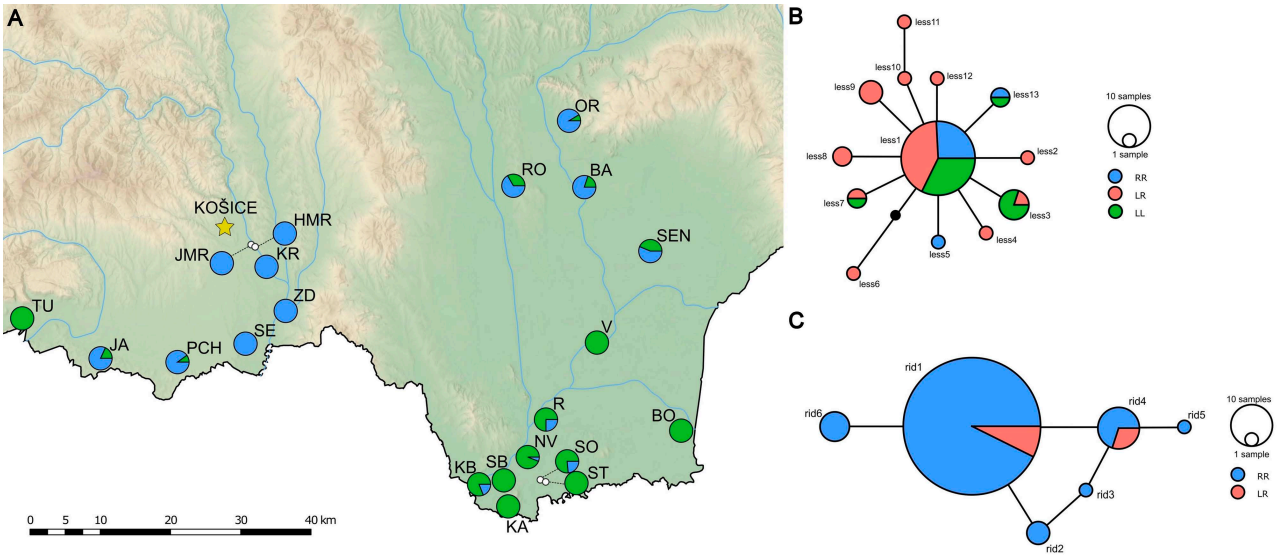

Figure 6. Distribution of mitochondrial lineages and TCS haplotype networks based on the variation of the mitochondrial ND2 gene in water frog populations in eastern Slovakia. (A) Blue color – *Pelophylax ridibundus* mtDNA, green color – *P. lessonae* mtDNA. For site codes see Table 1. (B) TCS haplotype network of *P. lessonae*-specific haplotypes. (C) TCS haplotypes network of *P. ridibundus*-specific haplotypes. Diameter of circles indicates frequency of haplotypes. The coloration indicates which species of the *P. esculentus* complex possess each haplotype. A black circle indicates missing or unsampled haplotype; RR – *P. ridibundus*, LR – *P. esculentus*, LL – *P. lessonae*.

P. esculentus (rid1, rid4; Hd = 0.436±0.133, π = 0.043±0.013%), others were present only in *P. ridibundus* (Hd = 0.266±0.053, π = 0.031±0.007%). In the *lessonae*-specific mtDNA, 13 haplotypes were detected in 52 ND2 sequences. In *P. ridibundus*, three different *lessonae*-specific haplotypes were identified (less1, less5, less13; Hd = 0.378±0.181, π = 0.039±0.020%) with only less5 being unique for this species (but detected in only one individual). In hybrids, 11 *lessonae*-specific haplotypes (less1, less2, less3, less4, less6, less7, less8, less9, less10, less11, less12; Hd = 0.748±0.088, π = 0.108±0.021%) were detected, with one (less1) shared with both parental species and the other two (less3, less7) shared with *P. lessonae* only. In *P. lessonae*, four haplotypes (less1, less3, less7, less13; Hd = 0.575±0.115, π = 0.063±0.016%) were found, but none of them were unique for this parental species. (Fig. 6B). No haplotypes specific to *P. kurtmuelleri* were detected.

Discussion

Species identification

The occurrence of species-specific microsatellite alleles in the *P. ridibundus* and *P. lessonae* genomes, with an application of ordination methods (PCA, PCoA, DAPC), make microsatellites reliable markers for the identification of central European water frog species (Hoffmann et al. 2015, Pruvost et al. 2015, this study). Despite being more efficient than morphological markers, their analysis is usually costly and time-consuming. Therefore, the use of classical morphological (Berger 1968, Günther 1990, Plötner et al. 1994, Svinin et al. 2021) and bioacoustic (Günther 1990, Schneider 1992, Plötner 2005) markers represents an alternative to genetic methods, especially in cases where species identification needs to be performed directly in the field. Although commonly used indices (L/T, L/CINT, T/CINT, DP/CINT) significantly differed among species, some hybrid individuals had an unclear intermediate position between parental species in ordination plots, particularly in females. Nevertheless, a combination of quantitative and qualitative morphological traits increased the assignment of individuals to the correct taxon (cf., Breka et al. 2020). The qualitative traits whose values were most highly correlated with individual species of water frogs, and thus are reliable for species identification, were the presence/absence of yellow pigment on the thighs and flanks, the shape of the metatarsal tubercles, and the color of the vocal sacs in males. All these characteristics have traditionally been used for identifying Central European *Pelophylax* species (e.g., Günther 1990), and our study confirmed their effectiveness. However, accurate morphological identification requires previous field experience.

Genotypic composition and ecological patterns of water frog populations

In the investigated localities of eastern Slovakia, we found both parental species and hybrids that were exclusively diploid. In addition to pure populations of *P. ridibundus*, we recorded syntopic occurrence of hybrids with one or other parental species, as well as populations in which only hybrids were detected. However, due to low population density or challenging terrain that hindered capture, the number of individuals obtained at some sites was insufficient to determine their population system. This is particularly the case at sites where only hybrids were recorded and sites where hybrids were found together with *P. ridibundus* individuals. Based on genetic analyses, it appears that the hybrids in the populations studied produce predominantly clonal R gametes and are thus reproductively dependent on the parental species *P. lessonae* (see below). It can therefore be assumed that even at syntopic sites of hybrids with *P. ridibundus* and at sites where only hybrids were captured, *P. lessonae* or both parental species can occur in low abundance. A similar composition of populations with low abundance of *P. lessonae* was found, for example, in western Slovakia (Mikulíček et al. 2015) or eastern Hungary (Herczeg et al. 2017).

The high abundance of *P. ridibundus* and hybrids, along with the low abundance of *P. lessonae* in the sample, likely reflects the ecological conditions at the sampled sites and may also be influenced by the ongoing expansion of *P. ridibundus* in Europe and its increasing presence in habitats typically occupied by *P. lessonae* (Kaufmann et al. 2015, Joško & Pabijan 2021, Dufresnes et al. 2024). Of the 21 sites, almost half represented artificial, large, deep and open water bodies, which *P. ridibundus* strongly prefers and where it also reaches its highest dominance and frequency (e.g., Pagano et al. 2001, Mikulíček et al. 2015). Natural, more vegetated and often temporary water bodies located mainly in the southernmost part of eastern Slovakia hosted all individuals of *P. lessonae* and the vast majority of ecologically generalist hybrids. These findings are similar to the results of Mikulíček et al. (2015) for water frog habitat preferences in western Slovakia, where *P. lessonae* had a relatively high frequency but low dominance in marshes and sandpits. *Pelophylax ridibundus* reached the highest frequency and dominance in large fishponds and gravel pits but also, together with *P. esculentus*, in oxbow lakes. A similar situation was reported by Joško & Pabijan (2021) for water frogs captured in eight pond complexes and one natural oxbow pond along the upper Vistula River valley in southern Poland. They recorded nearly equal numbers of *P. ridibundus* individuals and hybrids, while *P. lessonae* accounted for only approximately 7%, despite its historical dominance in the region (Joško & Pabijan 2021). Similarly, Herczeg et al. (2017) reported a predominance of *P. ridibundus* being captured mainly in irrigation canals and fishponds. Only one individual of *P. lessonae* was captured in a marshland with a temporary water level.

The once common species *P. lessonae* is becoming rare in some parts of Europe, with an apparent decline in the abundance of its populations (e.g., Kaufmann et al. 2015, Dufresnes et al. 2020). The causes of these changes may be different, but one of them is the loss of natural wetland habitats preferred by this species and their human-mediated conversion to artificial, larger and open water bod-

ies (e.g., canals or gravel pits). These, in turn, are suitable for *P. ridibundus*, which may thus occupy new sites at the expense of *P. lessonae*. Therefore, such habitat alterations can significantly affect the original taxonomic structure of water frog populations and cause *P. lessonae* to become a much more vulnerable and rare species than it was in the past (e.g., Ferreira & Beja 2013, Jośko & Pabijan 2021).

Sex ratio in parental species and hybrids

The sex ratio in parental species did not differ from the expected 1:1 ratio but in hybrids, females significantly predominated (83.3% of females) in all types of populations in eastern Slovakia. These results are in concordance with other studies that found a predominance of hybrid females in Pannonian populations (Berger et al. 1988, Gubányi & Creemers 1994, Sas 2010, Mikulíček et al. 2015, Herczeg et al. 2017). It is supposed that the sex ratio in water frogs is genetically determined and follows the XX/XY system with females being homogametic (Berger et al. 1988). For size-related behavioral reasons, primary hybridizations are expected between females of the larger species *P. ridibundus* (genotype R^XR^X) and males of the smaller species *P. lessonae* (genotype L^XL^Y) (Berger 1970, Plötner et al. 2008). Hybrid progeny thus should be of both sexes (R^XL^X, R^XL^Y). In the L-E population system, which is the most common in central Europe, hybrids eliminate the L genome and clonally inherit the R genome. Mating between hybrid females and *P. lessonae* males leads to progeny of both sexes. However, mating between hybrid males producing R sperm with linked X chromosome (R^X) and *P. lessonae* females producing L^X eggs results in hybrid females only (Berger et al. 1988). This mechanism, however, could not explain such a high occurrence of hybrid females in Pannonian populations as well as in populations of eastern Slovakia. Therefore, Berger et al. (1988) proposed that XX and many XY hybrid genotypes could be females. An alternative explanation could be related to Haldane's rule, according to which hybrids of the heterogametic sex are preferentially rare, sterile or inviable (Coyne & Orr 2004), or a more complicated sex determination system in hybridogenetic hybrids (Spasic-Boskovic et al. 1999, Mikulíček et al. 2015).

Clonality and genetic variability

All three approaches for the detection of clonality (MLGs, LD, Div) clearly showed that the L genome of hybrids is recombined, whereas the R genome is likely to be inherited clonally in most hybrid lineages. If we consider the R genome as clonal, then 13 microsatellite loci revealed 40 R hemiclones (27 were unique, 13 occurred in two or more hybrids). This high hemiclonal diversity is most likely the result of multiple primary hybridization events between the parental species, made possible by their sympatric occurrence in the study area.

Hybridogenetic hybrids are known to act as sexual parasites because they use gametes from their sexual host (usually parental species) for their own reproduction. In eastern Slovakian populations, it seems that hybrid *P. esculentus* produce predominately clonal R gametes and use *P. lessonae* as a host species. *Pelophylax ridibundus* does not seem to be involved in hybrid reproduction, even in localities where hybrids with *P. ridibundus* have been found. Thus, the populations studied do not differ from the vast majority of water frog populations in central Europe which genetically belong to the L-E population system (Uzzell & Berger 1975, Günther 1983, 1990, Graf & Polls Pelaz 1989, Plötner 2005). However, occasional clonal inheritance of the L genome or both parental genomes and the use of *P. ridibundus* as a host species, which have been documented in some populations of water frogs (Biriuk et al. 2016, Doležálková-Kaštánková et al. 2021, Dedukh et al. 2022, Skierska et al. 2023), cannot be excluded, although it is less likely in eastern Slovakia.

Genetic diversity in the mitochondrial ND2 gene was higher for the *lessonae*-specific mtDNA (13 haplotypes) compared to the *ridibundus*-specific mitochondrion (6 haplotypes). No haplotypes specific to *P. kurtmuelleri* have been detected. Differences in diversity between *P. ridibundus* and *P. lessonae* mitochondrial genome may reflect historical processes, such as the postglacial history of local populations related to the dispersal of frogs from glacial refugia and the recolonization of central Europe (Hewitt 1999, 2000, 2004, Hoffmann et al. 2015). Elucidation of this hypothesis would require sampling on a wider geographic scale and the inclusion of additional populations, especially from Pannonia and the Balkans.

Significant differences in the genetic diversity were found between the R and L genomes of hybrids in microsatellites and reflect the mode of reproduction of these hybridogenetic frogs. The L genome showed genetic diversity values comparable to the parental species *P. lessonae* which reflects the regular backcrosses of hybrids with this parental (host) species. In the R genome of hybrids, the genetic variability is approximately half that of the parental species *P. ridibundus*. Since the R genome of hybrids is clonal, its variability reflects the degree of primary hybridization between the parental species. The R genome is "frozen" in hybridogenetic lineages and cannot be regularly enriched from the sexual *P. ridibundus* gene pool, as is the case of the L genome. Similar results were also reached by Pruvost et al. (2015) analyzing populations of water frogs in western Slovakia and by Herczeg et al. (2017) analyzing populations in eastern Hungary.

Introgression of mtDNA

Plötner et al. (2008) discovered massive unidirectional introgression of *lessonae* mtDNA into the *P. ridibundus* gene pool (33.7% of 407 *P. ridibundus*) exclusively within the range of hybrids which possessed mostly *lessonae* mtDNA (90.4% of 335 individuals). In Eastern and Southeastern Eu-

rope, *P. ridibundus* exclusively carried *ridibundus* mtDNA irrespective of the occurrence of *P. esculentus* or *P. lessonae* (Plötner et al. 2008). Furthermore, Spolsky & Uzzell (1984) recorded a similar massive presence of *P. ridibundus* individuals with *lessonae* mtDNA (42%) in central Poland. Both Plötner et al. (2008) and Spolsky & Uzzell (1984) hypothesize that this type of mtDNA introgression can occur only when *P. esculentus* females with *lessonae* mtDNA form R eggs and mate either with hybrid males (producing R sperm) or *P. ridibundus* males. So, hybrids might serve as a vehicle for interspecific introgression. In southern Poland, Jośko & Pabijan (2021) recorded *lessonae* mtDNA in all hybrids, including those living in R-E populations. However, they recorded a lower rate (18.1%) of *lessonae* mtDNA introgression in *P. ridibundus* compared to previously mentioned studies. Furthermore, they found a positive relationship between the proportion of *P. ridibundus* with *lessonae* mtDNA and the proportion of *P. esculentus* at a site. Introgressed individuals were rare or absent in pure *P. ridibundus* populations but more frequent in other population types. The rate of mtDNA introgression in *P. ridibundus* found in our study (8.3%) was in agreement with the findings of Mikulíček et al. (2014) in western Slovakia, where only 4.9% of 426 *P. ridibundus* samples had the *lessonae* mitogenome. The introgression occurred in populations with a syntopic occurrence of hybrids or in pure *P. ridibundus* populations connected to such syntopic populations via gene flow. In our study, introgressed individuals of *P. ridibundus* occurred syntopically with hybrids in one population but were also found in four of the nine pure populations of *P. ridibundus*. It cannot be ruled out that these populations are, or in the recent past have been, connected to populations in which *P. ridibundus* occurs syntopically with hybrids and where introgression is ongoing.

Hybrids and introgressed individuals of *P. ridibundus* share some mitochondrial haplotypes, which indicates that the introgression is relatively recent. If introgression had taken place in the distant past or in a different geographic location, we would expect to see a representation of distinct and divergent haplotypes between the species (cf., Hofman et al. 2012). In the Mikulíček et al. (2014) study, the authors also documented the sharing of the same *lessonae* mitochondrial haplotypes by introgressed *P. ridibundus* from western Slovakia. Furthermore, Hofman et al. (2012) found out that the introgressed *lessonae* mtDNA on a *P. ridibundus* background differed from other *lessonae* mtDNA haplotypes by only a few substitutions in central and western Poland. All these findings support the hypothesis of a very recent introgression event in which a female *P. esculentus* must have participated.

Further, our study confirmed the massive presence of *lessonae* mtDNA in hybrid lineages (86.2%), which is consistent with other studies (90.4% – Plötner et al. 2008, 84% – Mikulíček et al. 2014, 100% – Jośko & Pabijan 2021). Primary hybridization leading to hybridogenetic lineages is expected to occur between females of the larger species *P. ridibundus* and males of the smaller species *P. lessonae* (Berger 1970, Plötner et al. 2008). Since mtDNA is maternally inherited, hybrids resulting from such mating must have *ridibundus* mtDNA. Subsequent mating between *P. lessonae* females and *P. esculentus* males must have resulted in hybrid offspring with the *lessonae* mitogenome. Hybrid males are relatively rare in gynogenetic and hybridogenetic vertebrates and, in addition to water frogs, have been documented in asexual complexes of fish genera *Carassius* (Zhou et al. 2000), *Squalius* (Cunha et al. 2008), and *Hypseleotris* (Schmidt et al. 2011). The importance of *P. esculentus* males in interspecific mtDNA introgression highlights the fact that hybrid males, in general, can play an important evolutionary role in gynogenetic and hybridogenetic complexes.

Our study focusing on morphological, genetic and ecological aspects of *Pelophylax* water frogs did not yield any surprising results that would overwrite previous knowledge of this group of amphibians. However, it filled a gap in research and provided new data on habitat preferences, morphological and genetic variation, mode of reproduction, and degree of interspecific gene flow in eastern Slovakian populations. Our study confirmed distinct habitat preferences of parental species, clonal inheritance of the *ridibundus* genome in hybridogenetic lineages, the use of *P. lessonae* as a host species, and unidirectional and recent introgression of the mitochondrial genome, in which hybrids are important mediators. These results thus fit into the overall picture we have of *Pelophylax* water frogs in central Europe.

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Supplementary data

The following data are available online:

Supplementary Table S1. The composition of three multiplex PCR mixtures for microsatellite amplification.

Supplementary Table S2. Species-specific loci and alleles of three representatives of the *Pelophylax esculentus* complex.

Supplementary Figure S1. Multivariate analyses of water frogs based on microsatellite data.

Supplementary Figure S2. A histogram showing the frequency of *Pelophylax esculentus* individuals in a particular erythrocyte size category.

Supplementary Figure S3. Multivariate Principal Component Analysis of water frog males and females based on morphometric data.