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ORIGINAL ARTICLE

Genetic structure, morphological variation, and gametogenic peculiarities in water frogs (*Pelophylax*) from northeastern European Russia

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Abstract

The edible frog, *Pelophylax esculentus*, is a hybrid form that reproduces via clonal propagation of only one of the parental genomes through generations of hybrids while the genome of other parental species is eliminated during gametogenesis. Such reproductive ability requires hybrids to coexist with one of the parental species or rarely both parental species causing the formation of so-called population systems. Population systems and reproductive biology of water frogs from the east of the range remained partially unexplored. In this study, we investigated the distributions, population systems, genetic structure, types of gametes, and morphological variability of water frogs of the genus *Pelophylax* from the northeastern parts of their ranges (Mari El Republic and adjacent territories, Russia). We examined 1,337 individuals from 68 localities using morphological traits combined with DNA flow cytometry and a multilocus approach (fragments of a nuclear and two mitochondrial genes). We revealed five types of population systems: "pure" populations of the parental *P. ridibundus* (R) and *P. lessonae* (L), mixed populations of parental species (R-L) along and with their hybrids (R-E-L), as well as mixed populations of *P. lessonae* and *P. esculentus* (L-E). However, the "pure" hybrid (E) and the mixed *P. ridibundus* and *P. esculentus* (R-E) population systems were not found. All hybrids studied by DNA flow cytometry were diploid. Analysis of gametogenesis showed that the majority of hybrid males, as well as hybrid females from the L-E system, produced gametes with the *P. ridibundus* genome. However, in the R-E-L system, hybrid females were usually sterile. The reproduction of hybrids in such systems is primarily based on crosses of *P. esculentus* males with *P. lessonae* females. Molecular analysis showed the presence of mitochondrial and nuclear DNA

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introgression of the Anatolian marsh frog (*P.* cf. *bedriagae*) into both *P. ridibundus* and *P. esculentus*. The observations of alleles and haplotypes of *P.* cf. *bedriagae* in *P. ridibundus* and *P. esculentus* individuals from the same localities suggest de novo formation of local hybrids. However, the presence of the Balkan marsh frog (*P. kurtmuelleri*) haplotypes in local hybrids supports the hypothesis regarding the migration of old hemiclonal lineages from glacial refugia. Finally, the diagnostic value of various morphological characteristics was discussed.

KEYWORDS
amphibia, morphology, *Pelophylax esculentus* complex, population systems, Ranidae

1 | INTRODUCTION

Interspecific hybridization is a widespread phenomenon among animals. The characteristic of interactions among species (within genera) at the boundaries of their distributional ranges usually depends on their divergence time. As a rule, closely related species more easily hybridize than distant species. In cases when species are strongly diverged and differences between their genomes are already too large, hybridization becomes impossible (e.g., Dufresnes, Mazepa, et al., 2019; Dufresnes, Strachinis, et al., 2019). However, hybrids between distant species may successfully overcome this barrier when reproduced clonally. In this case, parental genomes of hybrids remain non-recombining. Such hybrids carry new characteristics that can be supported by adaptive selection. Often, polyploid individuals arise in hybrid lines (Hermaniuk et al., 2013; Litvinchuk et al., 2016, 2019). The reproduction of the clonal hybrids is associated with numerous difficulties, which can strongly reduce their fertility. Therefore, tetraploid lines, which are characterized by the transition from clonal to sexual reproduction, usually complete a cycle of reticulate evolution (Borkin & Darevsky, 1980).

Hybridization, asexual reproduction, and polyploidization are well-known attributes of Palaearctic water frogs of the genus *Pelophylax* Fitzinger, 1843 (Berger, 2008; Graf & Pelaz, 1989; Plötner, 2005; Polls, 1994) consisted of about 22 species (Frost, 2020). Therefore, this group has attracted the attention of many researchers as a fascinating model to study reticulate evolution (Borkin & Darevsky, 1980; Litvinchuk, Borkin, et al., 2016). Some species in this genus can easily hybridize with each other, leading not only to introgression of genetic material into the gene pool of various species but also to hybrids reproducing hemiclonally. Moreover, hemiclonal reproduction of such hybrids is frequently accompanied by polyploidization (Plötner, 2005). The Western Palearctic species (about 15 species) are usually divided into three groups (*P. saharicus*, *P. lessonae*, and *P. ridibundus*). The representatives of different groups can hybridize and form several hybridogenetic complexes: *P. esculentus*, *P. grafi*, *P. hispanicus*, and unnamed hybrid species with parental *P. kurtmuelleri* and *P. perezi* (Dufresnes et al., 2017). The *P. esculentus* complex is the most widespread and well-studied (Plötner, 2005). This complex consists of two parental species, the pool frog, *P. lessonae* (Camerano, 1882), and the marsh frog, *P. ridibundus* (Pallas, 1771), as well as their hybrid, the edible frog, *P. esculentus* (Linnaeus, 1758).

Pelophylax esculentus is characterized by a special mechanism of hemiclonal reproduction, known as hybridogenesis, during which one genome is eliminated from gonial cells, while the second duplicates and transmits to gametes (Tunner, 1974). During gametogenesis, the hybrids usually exclude the genome of one of the parental species and produce gametes with the genome of another; thus, hybrids require crosses with parental species to emerge and reproduce. Such a reproductive strategy resulted in a variety of population systems, where hybrids are able to reproduce with one of the parental species (Berger, 2008; Borkin et al., 1987, 2004; Dedukh et al., 2015; Hoffmann et al., 2015; Plötner, 2005; Raghianti et al., 2007; Vinogradov et al., 1988). Depending on the coexistence of these parental species hybrids, three types of mixed population systems can be revealed. In particular, the L-E system includes *P. lessonae* and *P. esculentus*, the R-E system consists of *P. ridibundus* and *P. esculentus*, and the R-E-L system unites both parental and hybrid species (Plötner, 2005; Rybacki & Berger, 2001; Uzzell & Berger, 1975). Moreover, throughout the distribution range, populations can be represented by hybrids with only one sex or include not only diploid but also triploid and, rarely, tetraploid individuals (Borkin et al., 2004, 2006; Doležálková-Kaštánková et al., 2018; Hoffmann et al., 2015; Plötner, 2005; Rybacki & Berger, 2001). The hybrid *P. esculentus* can also form "pure" population systems known mainly from the northwestern edge of the species range where hybrids live without parental species (Berger, 2008; Plötner, 2005).

Extensive data concerning the distribution of population systems and gametogenesis of hybrids in Western and Central Europe were previously published (Daf et al., 2006; Krizmanić & Ivanović, 2010; Mikulíček et al., 2014; Pagano et al., 2001; Rybacki, 1994a,b; Sas, 2010; Mayer et al., 2013; Tunner & Heppich-Tunner, 1992; Zavadil, 1994). However, the frog populations in the East European (Russian) Plain have not been thoroughly investigated.

Parental species, as well as their hybrids, are frequently observed across the Volga River drainage in the European part of Russia. However, the local population systems are characterized by some peculiarities that were collectively named "the Volga River paradox" (Borkin et al., 2003). First, polyploid individuals of *P. esculentus* were not observed in these populations. Second,

mixed R-E-L and R-L systems are considerably more common than in Western and Central Europe (Borisovsky et al., 2001; Borkin et al., 2002; Borkin et al., 2003; Ruchin et al., 2005; Lada et al., 2011; Svinin et al., 2013; Svinin et al., 2016). Third, *P. esculentus* is less frequently observed than in Western and Central Europe (Borkin et al., 2003). Fourth, introgression of alleles of the Anatolian marsh frog (*P.* cf. *bedriagae*) was widely detected in local populations of *P. ridibundus* and *P. esculentus* (Ermakov et al., 2013; Svinin et al., 2016; Ivanov et al., 2019), though the native range of *P.* cf. *bedriagae* covers western Iran, Turkey, southern Bulgaria, eastern Greece, the Caucasus, and the Crimea (Akin et al., 2010; Ermakov, Fayzulin, Zaks, et al., 2014; Ermakov, Fayzulin, Askenderov, et al., 2016; Ermakov, Simonov, et al., 2016; Fayzulin et al., 2017; Ivanov, 2019; Ivanov et al., 2015; Kukushkin et al., 2018; Plötner et al., 2012). It should be noted that *P.* cf. *bedriagae* was widely introduced to Italy, Belgium, France, Switzerland, Germany, and Russia (Bellati et al., 2019; Dubey & Dufresnes, 2017; Dubey et al., 2014; Dufresnes et al., 2018; Hoffmann et al., 2015; Holsbeek et al., 2009, 2008, 2010; Litvinchuk et al., 2020; Lyapkov et al., 2018; Vershinin et al., 2019). Therefore, the detailed distribution of the species in the Russian Plain has not been elucidated.

In this paper, we described the distribution, population systems, genetic structure, types of gametes, and morphological variability of water frogs of the *P. esculentus* complex in the northeastern parts of their ranges.

2 | MATERIALS AND METHODS

2.1 | Studied sites

During the period of 2008–2019, we examined 68 localities throughout the territory of the Mari El and Tatarstan republics, as well as adjacent territories in the Kirovskaya Oblast (= Kirov Province) in the northeastern part of European Russia (Figure 1; Table S1). The region is located at the northeastern border of the distributional ranges of all three species of the *P. esculentus* complex. Data concerning an additional 11 localities were taken from the literature (Efremov et al., 1984; Garanin, 2000; Kuzmin, 2012). The observed localities were predominantly situated on the watershed between the Volga and Kama rivers (the Volga River drainage). Several types of water bodies were identified: trenches, ponds, rivers, oxbows, sand quarries, water reservoirs, and lakes (Table S2). The studied territory is mostly

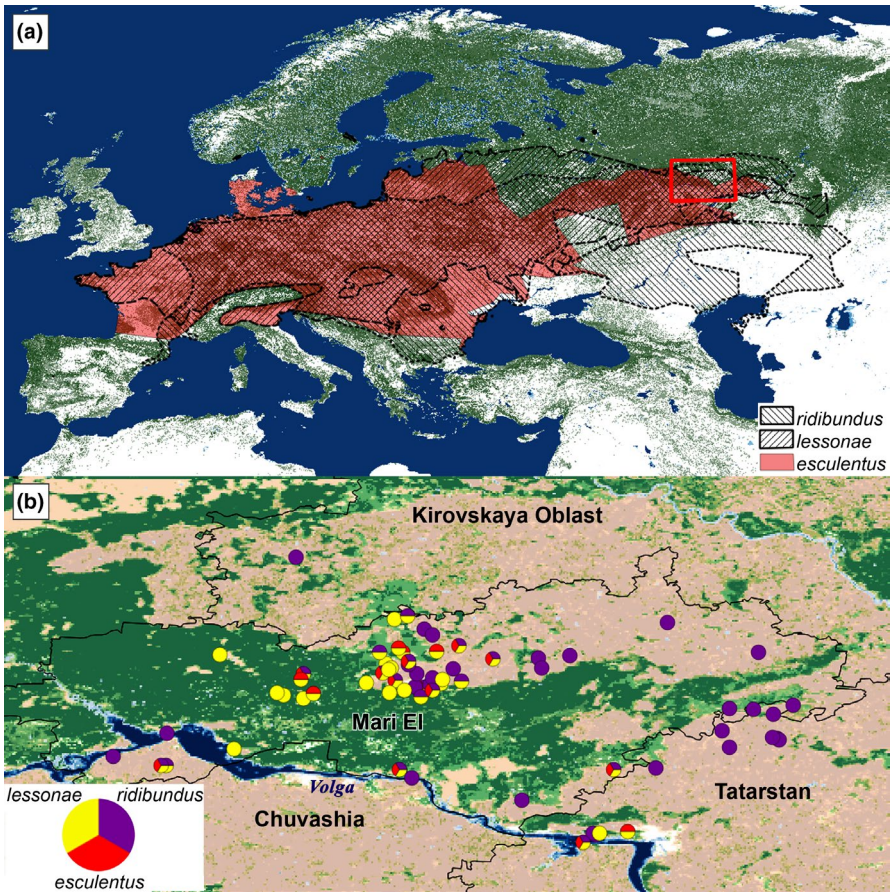

FIGURE 1 (a) Distributional ranges of three species of the *Pelophylax esculentus* complex in Europe; the studied area marked by red square. (b) Sample localities with identified frog species: red—*Pelophylax esculentus*, yellow—*Pelophylax lessonae*, and violet—*Pelophylax ridibundus*. Forests and cultivated lands are marked by green and rose, respectively

covered by deciduous broadleaf, coniferous, and mixed forests. The presence of surrounding forest vegetation was registered for each water body. Additionally, we estimated the percentage of forest vegetation and agricultural environments on a square land area of 1 km^2 in localities of water frogs using the QGIS Point Sampling Tool Plugin (https://plugins.qgis.org/plugins/pointsamplingtool/), which extracted data from the global 1 km consensus land-cover maps (Tuanmu & Jetz, 2014).

2.2 | Samples and methods of species identification

In total, 1,337 adult water frogs from 68 localities were identified based on external characteristics (Table 1; Table S1). Among all specimens, 556 were preserved in 70% ethanol and deposited to collections (97 in the herpetological collection of Mari State University, 459 in the herpetological collection of Institute of Cytology of the Russian Academy of Sciences) and 781 individuals were released to the field sites (Table S8). Among preserved individuals (n = 556), blood samples of 460 individuals (before their preservation) were studied by DNA flow cytometry for reliable determination of species. Individuals were euthanized by 1% solution of 3-aminobenzoic acid ethyl ester (MS 222). All procedures were carried out to minimize suffering. Among these specimens, 222 individuals were measured and used in the analysis of morphometric characters, 162 individuals were additionally identified with the use of multiplex PCR, and the type of gametes was studied for 195 individuals (Table 1).

2.3 | Species identification by DNA flow cytometry

We analyzed 460 specimens of three species using DNA flow cytometry (238 were studied for the first time; Table S4). This method enables the precise identification of species and ploidy of hybrids and parental species from the *P. esculentus* complex (Borkin et al., 1987; Vinogradov et al., 1990). The details of the method were previously published (Borkin et al., 1987; Vinogradov et al., 1990, 1991).

2.4 | Morphological analysis

In total, 222 adults from 18 localities, which were previously studied by DNA flow cytometry, were used for detailed morphometric treatment (Tables S1 and S3). We analyzed seven morphological characteristics in specimens preserved in 70% ethanol: *L.* is body length (from tip of snout to center of cloacal opening); *Lt.c.*—head width (distance between posterior edge of jaw articulations); *F.*—femur length (from center of cloacal opening to distal end of the femur bone); *T.*—tibia length (from knee to heel); *C.s.*—length of tarsus; *D.p.*—length of the first toe; and *C.int.l.*—length of internal metatarsal tubercle. Measurements were made with a digital caliper to the nearest 0.1 mm for each specimen by the first author. Based on these parameters, nine ratios (indices) were calculated (L./Lt.c., L./F., L./T., L./C.s., L./D.p., L./C.int.l., F./T. T./C.int.l., and D.p./C.int.l.). In addition, we used two multiplicative indices. Tarashchuk's (1989) index was calculated as $Tar = T.^2 \times D.p./C.int.l.^2 \times C.s.$ Hemmer's (1979) index (following Korshunov, 2010) was estimated according to the formula Hem = D.p./C.int.l. + T./C.int.l.

2.5 | Statistical analysis of morphological characteristics

Statistical analysis was performed using standard procedures (Sokal & Rohlf, 1981). The Levene and Brown–Forsythe tests were applied for comparison of variances. We used the Fisher test with Bonferroni correction for the post hoc comparison of disparate variances. We applied two-way ANOVA for comparison of means. The Sheffe test was used for post hoc comparisons. The Mann–Whitney test was used for comparisons of morphological indices between sexes and species. For the test, the natural logarithm conversion was made for all not normally distributed morphometric indices. Determination of most diagnostic characteristics was evaluated using principal component analysis (PCA). The level of morphological differences between species was estimated by squared Mahalanobis distances in canonical discriminant analysis following Peskov et al. (2009). Analysis was performed with the use of Statistica 8.0 (StatSoft Inc.).

TABLE 1 Sample size, methods of diagnostics of water frog species

Methods	*N* (localities)	*n* (individuals)	RR	RL	LL	Suppl. Inform.
Diagnostics by morphological traits	68	1,337	587	210	540	Table S1
Morphometry	18	222	57	67	98	Table S3
DNA flow cytometry	28	460	129	169	162	Table S4
Multiplex PCR, markers *COI* and *SAI-1*	18	162	66	86	10	Table S5
Sequences of a fragment of the *ND2* gene	13	57	28	29	—	Table S6
Allozyme analysis of sperm	20*	195*	2	33	6	Table S7
DNA flow cytometry of sperm			20	69	9	
Allozyme analysis of oocytes			9	11	54	

Note: Genotypes of *Pelophylax ridibundus* are designated as RR, *Pelophylax esculentus* as RL, and *P. lessonae* as LL. * The number is given for all samples for which gametes were studied.

2.6 | Species identification by multiplex PCR method and sequencing a fragment of the *ND2* gene

Pieces of femur muscle or toe tips were fixed by 96% ethanol and stored in 70% ethanol were used as tissue samples. In total, 162 specimens (10—*P. lessonae*, 66—*P. ridibundus*, 86—*P. esculentus*) were analyzed (Table S5). The identification of alleles of the intron-1 of the nuclear *serum albumin* (*SAI-1*) and the subunit 1 of the mitochondrial *cytochrome-c-oxidase* (*COI*) gene fragments of water frog species was performed using the methods described by Ermakov et al. (2019). The annealing temperature, PCR product length, and primer structure are given in Table 2. The DNA was extracted by the standard salt-extraction method (Aljanabi & Martinez, 1997). The PCR mixture (25 μl) contained 50–100 ng of DNA, 0.5 μM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, 2.5 μl 10 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), and 2 units of Taq polymerase (Thermo Scientific). In each mtDNA and nDNA PCR mixtures, primer concentrations were equal. PCR was performed at 94°C for 30 s, 60 and 62°C (for *SAI-1* and *COI*, respectively) for 30 s, and 72°C for 30 s (30 cycles). Notable differences between amplified species-specific fragments (80–306 bp) enabled us to visually identify species and their hybrids after electrophoresis of PCR products in polyacrylamide gels. Species identification of 161 individuals was performed by both DNA flow cytometry and multiplex PCR methods. It should be noted that the method does not allow distinguishing alleles and haplotypes of closely related *P. ridibundus* and *P. kurtmuelleri*.

Additionally, selective sequencing was used to verify the primary identification results. The subunit 2 of mitochondrial *NADH dehydrogenase* (*ND2*) gene (1,038 bp) in 57 specimens (28—*P. ridibundus*, 29—*P. esculentus*) was sequenced (Table S6). Before the primers were removed, the individual amplicon *ND2* had the length 1,168 bp. After primer trimming, the obtained *ND2* complete sequences had a length of 1,038 bp. Sequencing of the *ND2* gene was performed on an ABI 3500 automatic sequencer (Applied Biosystems) using the BigDye® Terminator 3.1 (Applied Biosystems) kit and the same primers that were used for amplification. The *ND2* gene sequence was amplified with use of the universal primer ND2L1 5′-AAG CTT TTG GGC CCA TAC CCC-3′ (Meyer, 1993) and developed the specific primer ND2H1 5′-GCA AGT CCT ACA GAA ACT GAA G-3′. The following amplification conditions were used: initial denaturation for 1 min at 95°C followed by 32 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, and final extension for 5 min at 72°C. The PCR mixture proportions were the same as for amplification of the *COI* gene fragment. The sequences obtained have been deposited in GenBank (nos. MN808383-MN808439; Table S6).

The nucleotide sequences were aligned both with BioEdit (Hall, 1999) software and manually. We used MEGA v. 7.0. software (Kumar et al., 2016) for data processing. For constructing the phylogenetic tree, the maximum-likelihood (ML) method was used. The most appropriate DNA substitution model for the datasets was established using jModelTest 2.1.10 (Posada, 2008). The ML trees were created with the Tamura–Nei model with the frequency of invariable sites set at 0.5570 (TN93 + I) (–lnL = 4,471.99, BIC = 13,692.06, AICc = 9,723.51). Node support values in phylogenetic trees were estimated according to bootstrap support (200 replicates). Haplotype networks were constructed using the median-joining method in PopART software (Leigh & Bryant, 2015). Haplotype diversity (*h*) and nucleotide diversity (π) within each species were calculated in DnaSP v. 5.10.01 (Librado & Rozas, 2009).

2.7 | Identification of gamete types in hybrids

Gamete types were determined in 111 individuals of hybrid *P. esculentus* (84 males and 11 females; see Table S7). As a control, we additionally analyzed gametes of 32 *P. ridibundus* (22 males and nine females) and 69 *P. lessonae* (15 males and 54 females) individuals. To identify the genome composition of gametes, we applied two methods: DNA flow cytometry and allozyme analysis. Cell suspension obtained from testes of adult animals was analyzed. To obtain the sperm suspension, testes were dissected in a drop of the Versene solution (Biolot, St. Petersburg), and then, the suspension was selectively examined under a light microscope (phase contrast). Sperm of 69 *P. esculentus* males (including a hermaphrodite), 20 *P. ridibundus* males, and nine *P. lessonae*

TABLE 2 Primers of multiplex PCR test systems for identification of water frog species

Primer	Position	Sequence (5′-3′)	Annealing temperature, °C	PCR product length, bp	Specificity
COIR-Pu	624-601	CCTGCRGGATCAAAAAATGTTGT	63.6	–	All three species
COIF-Pl	329-349	GAACTGTGTACCCCCCACTAG	63.7	294	*P. lessonae*
COIF-Pr	409-429	GCTGGGGTTTCATCAATTCTG	61.8	214	*P. ridibundus*
COIF-Pb	183-204	CTTTGGAAATTGACTCGTGCCA	63.8	440	*P.* cf. *bedriagae*
SA1F-Pu	25-59	CCATACAAATGTGCTAAGTAGGTT	61.3	–	All three species
SA1R-Pl	140-119	TACCGTACCGATATTTGTATGC	60.2	109	*P. lessonae*
SA1R-Pr	245-221	GATACAAATGATACATTCCCACCT	61.0	210	*P. ridibundus*
SA1R-Pb	450-429	TTGTTCCCTATACTAAGGTCAC	59.3	415	*P.* cf. *bedriagae*

males were analyzed by DNA flow cytometry (see details in Biriuk et al., 2016). Sperm suspension of 33 males of *P. esculentus*, 2 *P. ridibundus*, and 6 *P. lessonae* was analyzed by allozyme analysis (18 *P. esculentus* were studied by both DNA flow cytometry and allozyme analyses). The suspension (stored at −80°C) was used as a tissue sample and vertical polyacrylamide (6%) gel electrophoresis was performed using Tris-citric pH 8.0 buffer. The Ldh-A (lactate dehydrogenase-1) locus with species-diagnostic polymorphism was visualized by the standard technique (Shaw & Prasad, 1970). The same method was used for the analysis of genome composition in oocytes of 11 *P. esculentus*, 9 *P. ridibundus*, and 54 *P. lessonae* (for details, see Uzzell et al., 1980).

3 | RESULTS

3.1 | Distribution of species and population systems

Among 1,337 individuals of water frogs determined by morphological traits, 587 (44%) were *P. ridibundus*, 540 (40%) were *P. lessonae*, and 210 (16%) were *P. esculentus*. We registered the pool frog in 37 localities (54%), the marsh frog in 47 localities (69%), and hybrids in 18 (27%) localities. *Pelophylax lessonae* was predominantly found in ponds located in a forest. The species inhabits forest biotopes more often than others (41%; Table 3). *Pelophylax ridibundus* is usually observed in open water bodies and rivers that flow down through forests on agricultural land (68%). Hybrid frogs were registered in the transition zone of these biotopes (Figure 1). We detected hybrids co-occurring with *P. lessonae* individuals (L-E systems) and with both parental species (R-E-L systems). The L-E systems were considerably more common in forest (70%; Table 3) than the R-E-L systems (25%).

Marsh frogs inhabited dams on rivers (27%), rivers (27%), ponds (23%), lakes (7%), carriers (5%), and oxbows (4%). Among these bodies of water, artificial and natural bodies of water accounted for 67% and 33%, respectively. Both pool and edible frogs preferred artificial water bodies (76 and 68%, respectively) and mainly occurred in ponds (49% and 40%). Parental species were found to co-occur syntopically (mixed R-L and R-E-L populations) in various types of water bodies.

Population systems with only one parental species (68%) were most frequent. Pure *P. ridibundus* populations occurred in 44% (n = 30) localities, and pure *P. lessonae* populations occurred in 22% (n = 15). We found only 4 (6%) localities, including both parental species without hybrids (mixed R-L systems). Population systems including hybrids were observed in 19 localities (28%): L-E (n = 6; 9%) and R-E-L (n = 13; 19%). We did not find the E and R-E population systems.

3.2 | Genome size variation

After measuring the ploidy level by DNA flow cytometry, we found that all studied water frogs were diploids (Table S4). The mean genome size (the nuclear DNA content, 2C) in *P. ridibundus* (n = 125) was 16.22 ± 0.01 pg (15.89–16.52), *P. esculentus* (n = 176) was 15.05 ± 0.01 pg (14.81–15.61), and *P. lessonae* (n = 167) was 13.82 ± 0.01 pg (13.62–14.10). The genome size values among species did not overlap.

TABLE 3 Percentage (mean ± standard deviation and range) of forest vegetation and agricultural land in areas (1 km^2) associated with water frog population systems

Population system	n	Forest	Agricultural
R	30	11.7 ± 23.2 (0–82)	73.5 ± 35.3 (0–100)
R-L	4	27.0 ± 48.2 (0–99)	69.8 ± 46.8 (0–100)
R-E-L	13	25.4 ± 35.6 (0–100)	54.2 ± 41.4 (0–100)
L	15	46.4 ± 41.5 (0–100)	46.9 ± 43.0 (0–100)
L-E	6	69.8 ± 32.9 (14–100)	17.8 ± 27.2 (0–66)
P. lessonae	38	40.9 ± 40.6 (0–100)	47.2 ± 41.8 (0–100)
P. esculentus	19	39.4 ± 39.9 (0–100)	46.7 ± 40.6 (0–100)
P. ridibundus	43	15.8 ± 27.8 (0–100)	67.7 ± 37.8 (0–100)

3.3 | Genetic structure

According to the multiplex PCR test system, all pool frogs (n = 10) and most marsh frogs (53 out of 66 individuals) had species-specific markers for both mtDNA and nDNA (L/LL and R/RR consequently). Other marsh frogs had introgressive genotypes, including mtDNA of *P.* cf. *bedriagae* in the *P. ridibundus* nDNA background (B/RR—7 individuals) or had their own mtDNA but were heterozygous by nDNA (R/RB—6 individuals). In a total sample of marsh frogs, mtDNA and nDNA of *P.* cf. *bedriagae* had relatively small contributions (0.11 and 0.05%, respectively).

In edible frogs, we found five out of six possible combinations of mtDNA and nDNA of parental species, except genotype L/BL (Table 4). Most *P. esculentus* (74 out of 86) had genotypes L/RL or R/RL. Others (n = 12) carried genes of *P.* cf. *bedriagae* (B/RL, R/BL, B/BL). In R-E-L systems, mtDNA haplotypes of Anatolian and Central European marsh frogs (R or B—79%) were prevalent, while in L-E systems, edible frogs had predominantly mtDNA of the pool frog (L—79%).

The study of the nuclear *SAI*-1 marker showed the presence of alleles of *P.* cf. *bedriagae* in six marsh frogs from Medvedevo only (13% for the locality and 5% for the species in total). In *P. esculentus*, alleles of *P.* cf. *bedriagae* were revealed in two individuals (2% for the species) from Medvedevo (13%) and Chermyshevo (3%). The mitochondrial DNA of *P.* cf. *bedriagae* was more frequent. We found it in 11% individuals of the marsh frogs (four localities; Table 4; Suppl. Table 5) and 11% edible frogs (three localities). Haplotypes of *P. lessonae* were detected in 47% individuals of *P. esculentus* (Table 4).

The results of species identification using both DNA flow cytometry and multiplex PCR methods for the same individuals (n = 161) coincided completely.

TABLE 4 Genetic features of the edible frog *Pelophylax esculentus* according to the multiplex PCR data

Population system	Number of populations	*n*	*COI* mtDNA				
			L	R		B	
			SAI–1 nDNA				
			RL	RL	BL	RL	BL
R-E-L	7	48	10	26	1	10	1
L-E	4	38	30	8	–	–	–
Total	11	86	40 (47%)	34 (39%)	1 (1%)	10 (12%)	1 (1%)

Note: B, mtDNA haplotypes and nDNA alleles of *P.* cf. *bedriagae*; L, *P. lessonae*; R, *P. ridibundus*.

TABLE 5 Genetic diversity in marsh and edible frogs from northeastern European Russia

Types of mtDNA	*n*	*H*	*h* ± *SD*	$\pi \pm SD$	*S*/η	*K*
Marsh frogs						
ridibundus	21	1	–	–	–	–
cf. *bedriagae*	7	3	0.524 ± 0.209	0.0019 ± 0.0009	7/7	2.00
Edible frogs						
ridibundus	16	4	0.350 ± 0.148	0.0016 ± 0.0011	13/13	1.62
cf. *bedriagae*	5	3	0.700 ± 0.218	0.0021 ± 0.0008	5/5	2.20
lessonae	8	1	–	–	–	–

Note: h, haplotype diversity; *H*, number of haplotypes; *K*, mean number of nucleotide substitutions; *n*, sample size; S, total number of polymorphic positions; η, total number of mutations; π, nucleotide diversity (per site).

The phylogenetic analysis based on the mitochondrial *ND2* gene fragment showed that the local *P. ridibundus* mtDNA was closely related to *P. kurtmuelleri*. The second cluster formed *P.* cf. *bedriagae*, and *P. lessonae* was most distant (Figure 2). Sequencing of the *ND2* gene of mtDNA in 28 individuals of the marsh frog and 29 individuals of the edible frog revealed a low level of genetic diversity (Table 5). Genetic variability was lacking in *P. ridibundus* (*n* = 21) and *P. esculentus* with mtDNA of the pool frog (*n* = 8). These frogs had one of the most frequent Europe-specific haplotypes (Figure 2), which were found in genetic pools of marsh and edible frogs across all their ranges. Intermediate values of haplotype diversity, low frequency of nucleotide diversity, and positive results of the Fs test (which showed a deficit of haplotypes in investigated population systems) were observed in marsh frogs with mtDNA of *P.* cf. *bedriagae* (*n* = 7) and edible frogs with haplotypes of *P. ridibundus* (*n* = 16) and *P.* cf. *bedriagae* (*n* = 5). One individual of the edible frog from Chermyshevo had genetic markers of three taxa. This frog had mtDNA of the Balkan water frog (*P. kurtmuelleri*) and nuDNA alleles of *P.* cf. *bedriagae* and *P. lessonae*.

3.4 | Gamete type variation in hybrids

Based on DNA flow cytometry data (Table 6), the proportion of haploid sperm in a mixture of cells obtained from testes of *P. esculentus* was 37% on average (*SD* = 26%, range 0%–85%, *n* = 69), *P. ridibundus* was 64% (*SD* = 28%, range 0%–94%, *n* = 20), and *P. lessonae* was 87% (*SD* = 4%, range 77%–91%, *n* = 9). The majority of studied hybrid males (from both the L-E and R-E-L population types) and one hermaphrodite from Myamikeevo (from the R-E-L population) produced sperm with the genome of *P. ridibundus* (*n* = 52; 75%). Three hybrid males (4%) yielded sperm with the *P. lessonae* genome, two hybrid males (3%) produced sperm with intermediate (presumably non-clonal) genomes with the nuclear DNA content average between parental species, and 11 males (16%) did not have sperm (and other haploid cells). Among these presumably sterile individuals, only four (36%) had normal-sized testes. A hybrid male (1%) from Chermyshevo (the R-E-L population) produced a mixture of sperm cells containing intermediate and *P. ridibundus* genomes (25% and 75% of analyzed haploid cells, respectively).

Similar data were obtained after allozyme analysis. The majority (*n* = 9; 60%) of hybrids produced sperm with the genome of *P. ridibundus* (Table 6). Four males of *P. esculentus* (27%) had gametes with the *P. lessonae* genome. Sperm of two hybrids (13%) from Nol'ka (the L-E population) gave allozyme spectra characteristic of both parental species.

In contrast to males, hybrid females produced different types of gametes in the studied populations (Table 6). In the R-E-L system (Chermyshevo), the majority of females (*n* = 7; 88%) produced oocytes with a mixture of two parental genomes. Only one *P. esculentus* female (13%) gave gametes with the *P. lessonae* genome. In the L-E system (Kuguvan), all three studied hybrid females produced gametes with the *P. ridibundus* genome.

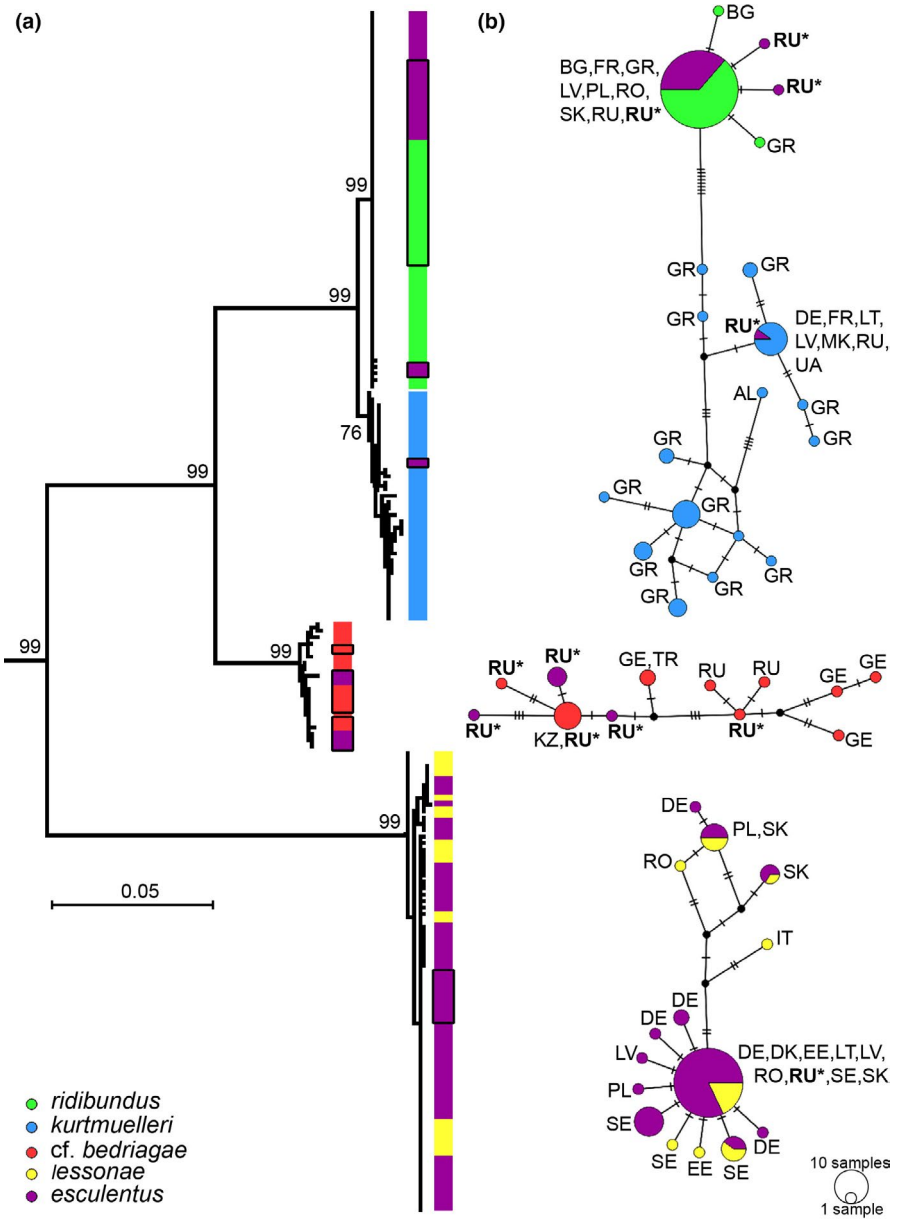

FIGURE 2 Phylogenetic tree inferred by ML analysis (a) and haplotype network (b) using a fragment of the *ND2* gene sequences for 57 individuals from Mari El Republic (shown in black borders in phylogenetic tree and designated as symbol RU* in haplotype networks) and 130 individuals from various European populations represented in GenBank. Bootstrap values over 70% are shown next to branches. The out group is not shown. Countries are shown as two-letter codes (ISOs)

3.5 | Variation of morphological characters

The means of all 11 morphological indices for *P. esculentus* were intermediate between parental species, but the limits overlapped (Table 7).

Two-way ANOVA showed the absence of sexual dimorphism for all studied indices. However, significant species-specific differences were revealed for all indices (two-way ANOVA; $p < .001$). The post hoc comparison of all indices showed that differences between parental species were significant ($p < .001$). After comparison of hybrids with parental species, we detected four insignificant differences (L./Lt.c., L./F., L./C.s., and L./D.p.) between *P. ridibundus* and *P. esculentus*, as well as two indices (L./F. and L./C.s.) between *P. lessonae* and *P. esculentus*.

A comparison of variance for indices L./C.int.l., D.p./C.int.l., T./C.int.l., *Hem*, and *Tar* showed significant differences ($p < .001$). A comparison of variance for all three species using Levene and Brown–Forsythe tests showed significant differences ($p < .001$) for five indices: L./C.int.l., D.p./C.int.l., T./C.int.l., *Hem*, and *Tar*. The post hoc pairwise comparison of variances by the Fisher test (with the Bonferroni correction for *p*-value) revealed the following differences between variances. Males and females of *P. esculentus* differed from females of *P. ridibundus*. The hybrids also had equal variances with both males and females of *P. lessonae*. Sexual dimorphism in body size (pairwise comparison by Fisher's test with the Bonferroni correction) for all three species was not revealed.

TABLE 6 Gamete types produced by water frogs from studied population systems

Gamete type	*Pelophylax ridibundus*	*Pelophylax lessonae*	*Pelophylax esculentus*	
			L-E	R-E-L
Males				
DNA flow cytometry				
R	20 (1.00)[a]	—	34 (0.87)	17 (0.59)
L	—	9 (1.00)	1 (0.03)	2 (0.07)
E (intermediate)	—	—	1 (0.03)	1 (0.03)
R + E	—	—	—	1 (0.03)
Sterile	—	—	3 (0.07)	8 (0.28)
Total	20	9	39	29
LDH electrophoresis				
R	2 (1.00)	—	9 (0.64)	—
L	—	6 (1.00)	3 (0.21)	1 (1.00)
mixture (R + L)[b]	—	—	2 (0.14)	—
Total	2	6	14	1
Females				
LDH electrophoresis				
R	9 (1.00)	—	3 (1.00)	—
L	—	54 (1.00)	—	1 (0.13)
E[c]	—	—	—	7 (0.87)
Total	9	54	3	8

[a]Number of individuals (share of the total in the group).

[b]Mixture of haploid spermatozoa bearing *Pelophylax ridibundus* chromosomes and haploid spermatozoa bearing *P. lessonae* chromosomes.

[c]The Ldh-A spectra of both parental species; according to Dedukh et al. (2019), these oocytes contained chromosomes of both parental species (conditionally named here as the E type), sometimes, a portion of oocytes from one female contained the mixture of haploid E- and R-oocytes or haploid E- and diploid RL-oocytes.

The results of the PCA demonstrated good separation of the three species by the first principal component (PC1), which explains 53% of the total variance (Figure 3, Table 8). The first axis (PC1) had high loadings for five indices: L./C.int.l., T./C.int.l., D.p./C.int.l., *Hem*, and *Tar*. All these characteristics are connected with the length of the inner metatarsal tubercle. The second component (PC2) explained 26% of the total variance.

We calculated Mahalanobis distances between species and found that *P. esculentus* was more similar to *P. ridibundus* than to *P. lessonae* for most simple indices: L./Lt.c., L./T., L./C.s., L./D.p., and F./T. (Table 9). All these indices are not connected with the length of the inner metatarsal tubercle. However, if indices connected with *callus internus* length (L./C.int.l., D.p./C.int.l., T./C.int.l.), *P. esculentus* proved to be more similar to *P. lessonae* than to *P. ridibundus*. These results were similar in both males and females (Table 9).

4 | DISCUSSION

After a 12-year study, we summarized data on the distributions of water frog species and their population systems in the eastern parts of their ranges, genetic structure, types of gametes produced by hybrids, and peculiarities of morphology. The concordance between the molecular structure of *P. ridibundus* and the hybridogenous *P. esculentus* was tested, as well as the morphological peculiarities of frogs from different population system types and genotypes in the studied territory.

4.1 | Morphological peculiarities

For a long time, zoologists were searching for diagnostic morphological traits for reliable identification of water frog species. Significant differences between parental species by means of indices selected in the current work were revealed in numerous studies (Berger, 1966; Borisovsky et al., 2000; Gubanyi & Korsos, 1992; Günther, 1975; Lada, 2012; Lada et al., 1995; Nekrasova & Morosov-Leonov, 2001; Okulova et al., 1997; Peskov et al., 2009; Plötner et al., 1994; Ruchin et al., 2005). The characteristics and indices for the hybridogenetic *P. esculentus* were intermediate between parental species. However, the limits of these values in hybrids overlapped with those of parental species (Berger, 1966; Borisovsky et al., 2001;

TABLE 7 Variability of body length and morphological indices in water frog species studied by DNA flow cytometry and/or molecular *COI* and *SAI-1* markers analysis

Indices	*Pelophylax ridibundus* (*n* = 57)		*Pelophylax esculentus* (*n* = 67)		*Pelophylax lessonae* (*n* = 98)	
L.	75.1 ± 1.36 10.26	59.0–100.0	*73.9 ± 1.05* 8.60	58.0–95.5	*61.6 ± 0.74* 7.35	48.0–79.0
L./Lt.c.	*2.60 ± 0.02* 0.13	2.36–2.95	*2.64 ± 0.01* 0.12	2.42–2.90	*2.76 ± 0.01* 0.13	2.52–3.15
L./F.	*2.00 ± 0.01* 0.10	1.81–2.32	*2.06 ± 0.01* 0.09	1.87–2.27	*2.10 ± 0.01* 0.10	1.89–2.36
L./T.	*1.89 ± 0.01* 0.07	1.74–2.02	*2.03 ± 0.01* 0.08	1.88–2.33	*2.19 ± 0.01* 0.10	1.97–2.48
L./C.s.	*3.59 ± 0.02* 0.17	3.16–4.00	*3.64 ± 0.02* 0.16	3.35–4.10	*3.76 ± 0.02* 0.21	3.28–4.28
L./D.p.	*7.08 ± 0.07* 0.54	5.78–8.40	*7.22 ± 0.06* 0.47	6.42–8.33	*8.24 ± 0.07* 0.70	6.78–10.50
L./C.int.l.	*18.19 ± 0.26* 1.97	13.28–23.75	*15.00 ± 0.13* 1.06	13.20–17.69	*13.09 ± 0.08* 0.78	11.25–15.00
F./T.	*0.95 ± 0.01* 0.04	0.84–1.02	*0.99 ± 0.01* 0.04	0.91–1.10	*1.05 ± 0.00* 0.04	0.89–1.15
D.p./C.int.l.	*2.58 ± 0.04* 0.33	1.96–3.31	*2.08 ± 0.02* 0.16	1.68–2.40	*1.60 ± 0.01* 0.12	1.28–1.82
T./C.int.l.	*9.61 ± 0.13* 1.01	7.60–12.75	*7.40 ± 0.06* 0.46	6.60–8.68	*5.99 ± 0.04* 0.38	5.11–6.91
Hem	*12.20 ± 0.17* 1.29	9.60–16.00	*9.48 ± 0.07* 0.57	8.48–11.03	*7.58 ± 0.05* 0.45	6.38–8.69
Tar	*47.60 ± 1.38* 10.43	27.50–77.50	*27.80 ± 0.41* 3.40	21.40–37.70	*16.50 ± 0.20* 1.97	11.20–19.60

Note: n is number of specimens. First column for species: numerator, mean value ± standard error of mean, denominator, standard deviation; second column: limits. Italicized values are significant ($p < 0.001$).

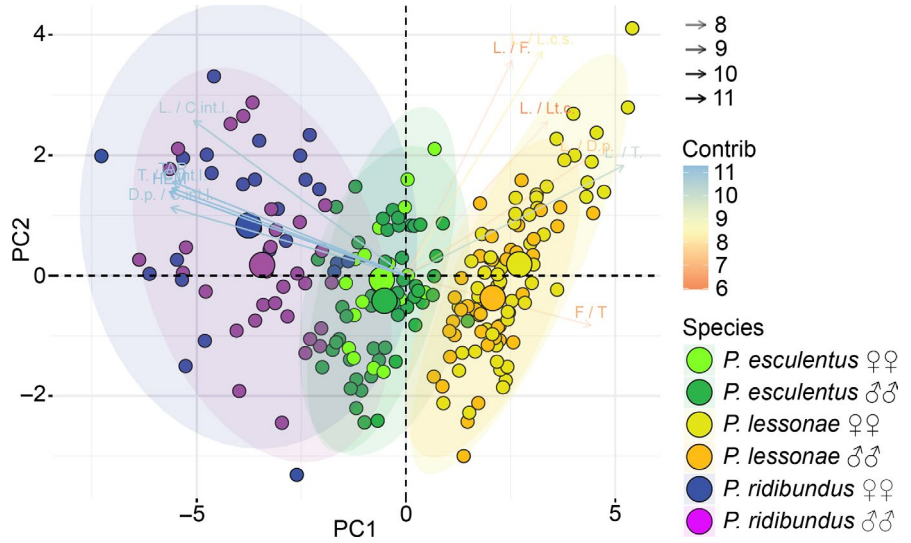

FIGURE 3 Principal component analysis results: scatterplot for three water frog species in the space of the first and second principal components (PC1 and PC2; morphological indices) and contributions of indices to the principal components (gradient colors of vectors)

Gubanyi & Korsos, 1992; Lada et al., 1995; Ruchin et al., 2005; and others). According to the Mahalanobis squared distances calculated by us, *P. esculentus* was more similar to *P. ridibundus* than to *P. lessonae*. In previous papers (Borisovsky et al., 2000; Nekrasova & Morosov-Leonov, 2001; Reminnyi & Matviychuk, 2018; Tarashchuk, 1985, 1989), the multiplicative index *Tar* was considered to be the most reliable for species identification in the *P. esculentus* complex. We found that the limit values of the index in parental

TABLE 8 Factor loadings of analyzed morphometric indices on the first two principal components for water frog species (*Pelophylax ridibundus*, *Pelophylax esculentus* and *Pelophylax lessonae*)

Index	PC1	PC2
L./Lt.c.	−0.295	**0.662**
L./F.	−0.084	**0.739**
L./T.	−0.626	**0.701**
L./C.s.	−0.178	**0.823**
L./D.p.	−0.487	**0.646**
L./C.int.l.	**0.967**	−0.033
F./T.	−0.729	0.242
D.p./C.int.l.	**0.930**	−0.294
T./C.int.l.	**0.953**	−0.244
Hem	**0.957**	−0.257
Tar	**0.959**	−0.226
Expl.Var	5.830	2.894
Prp.Total	0.530	0.263

Note: Value loadings with strong impact on the principal components are shown in bold.

TABLE 9 Squared Mahalanobis distances between three species of water frogs. R—*Pelophylax ridibundus*, E—*Pelophylax esculentus*, and L—*Pelophylax lessonae*

	Males (n = 123)			Females (n = 99)		
Indices	E-R	E-L	R-L	E-R	E-L	R-L
L./Lt.c.	**0.13**	0.88	1.68	**0.06**	0.88	1.41
L./F.	0.48	**0.01**	0.62	0.29	**0.23**	1.04
L./T.	**3.56**	3.12	13.34	**2.22**	3.06	10.48
L./C.s.	**0.14**	0.23	0.73	**0.03**	0.47	0.73
L./D.p.	**0.07**	4.00	4.65	**0.05**	2.58	3.35
L./C.int.l.	6.01	**2.05**	15.08	6.35	**3.30**	18.80
F./T.	**0.72**	1.94	5.02	**1.73**	2.93	9.17
D.p./C.int.l.	6.00	**5.57**	23.13	**5.78**	6.65	24.84
T./C.int.l.	11.98	**4.17**	30.27	13.76	**6.95**	40.27
Hem	11.61	**5.01**	31.89	12.68	**7.67**	40.08
Tar	11.50	**3.60**	27.97	12.68	**4.52**	32.33

Note: Lowest values are shown in bold.

species and *P. esculentus* overlapped slightly. Perhaps, high variability of marsh frogs in the region is caused by the presence of alleles of two genetically distant species (*P.* cf. *bedriagae* and *P. ridibundus*). The combination of indices together with some external morphological features (coloration of vocal sacks, inner metatarsal tubercle shape, and relative hind limb length) enables the reliable identification of water frog species. Correct diagnostics were made in 99% of cases: only four individuals of 460 *P. esculentus* were determined incorrectly, while parental species were always correctly identified.

Sexual dimorphism by morphometric characteristics in water frogs has been discussed in many studies. However, the differences between males and females can vary geographically (Aleksandrovskaya, 1981; Caune, 1987; Lada, 2012). For example, significant sexual differences in morphological traits were found for *P. ridibundus* (and *P.* cf. *bedriagae*) in Kazakhstan, Armenia, Ukraine, and Russia (Aleksandrovskaya, 1981; Lada, 2012; Nekrasova & Morozov-Leonov, 2001; Terentjev, 1943), for *P. esculentus* in France, Latvia, Ukraine (Caune, 1987; Lada, 2012; Mikitinez & Suryadna, 2007; Regnier & Neveu, 1986), and, finally, for *P. lessonae* was registered more often than in previous two species and observed in France, Poland, Latvia, Hungary, Ukraine, and Russia (Berger, 1966; Caune, 1987; Gubanyi & Korsos, 1992; Lada, 2012; Okulova et al., 1997; Regnier & Neveu, 1986). In the northeastern part of European Russia, we did not reveal sexual dimorphism in all indices for the three species. However, the dimorphism would be expressed by a separate comparison of females and males of *P. lessonae*. The Mann–Whitney test showed differences in six indices. In contrast, multiple comparisons using two-way ANOVA showed that these differences become insignificant.

We did not find any significant difference between *P. esculentus* samples from two various types of population systems: the L-E (Kuguvan) and R-E-L (Chermyshevo) systems. No differences in body proportions were registered between *P. lessonae* samples from these two population systems, as well. Importantly, these localities are separated both by a relatively large distance (100 km) and by the Volga River, which forms a physical barrier. Moreover, both populations exist in different environmental conditions (small ponds in the forest in Kuguvan and large open artificially impounded water bodies in Chermyshevo). Genetically different variants (with the presence of alleles and haplotypes of *P.* cf. *bedriagae*) of hybrids from these populations, as well as hybrids producing different types of gametes, also demonstrated no differences in body proportions.

4.2 | Distribution of population systems, molecular structure, and gamete types

The distribution of water frog species, types of population systems, and hybrid gametogenesis in the studied region are highly similar to adjacent territories in the East European Plain, namely, in Nizhegorodskaya Oblast (= Nizhny Novgorod Province) and the republics of Udmurtia, Chuvashia, and Mordovia (Borisovsky et al., 2001; Borkin et al., 2002; Ruchin et al., 2005, 2010). This finding indicates the common features of water frogs in the studied region, making populations from the Volga River drainage distinct from hybrids from other regions of Europe.

First, we and other researchers (Borkin et al., 2003) noted that hybrids in the Volga River drainage are not as numerous as in Western and Central European populations. In the studied localities, hybrids were found in 26% of the analyzed populations (Table S1), but compared to parental species, they were notably rare and observed only in 16% of all water frog individuals. A lower occurrence of *P. esculentus* was previously observed in adjacent territories. For example, in the Udmurtia and Mordovia republics, as well as Ivanovo and Nizhegorodskaya Oblasts, hybrids were

found in 5%–9% of the studied populations (Borkin et al., 2002; Ruchin et al., 2005). Such a low occurrence of hybrids in local populations could be explained by the load of ineffective reproduction of hybrids that have numerous gametogenesis disorders and a high proportion of sterile hybrids, while hybridogenetic hybrid frequency is low (Dedukh et al., 2019; Litvinchuk et al., 2016). This finding may be attributable to introgression of *P.* cf. *bedriagae* alleles into the genetic pool of *P. ridibundus* (Fayzulin et al., 2018; Svinin et al., 2016). However, it should be noted that the number of hybrid males lacking or strongly reduced (<3 mm in length) testes in the studied region (18%; Table S7) was obviously lower in diploids from most populations inhabiting Eastern Europe (57%; Litvinchuk, 2018). Therefore, most likely, the reduced reproductive ability of local hybrids is due to decreased fertility of hybrid females.

All observed hybrids were diploids, and no triploids were revealed. Earlier triploids were also not found in adjacent regions (Borkin et al., 2002, 2003). The nearest triploids were observed approximately 1,000 km from this location to the south in the Severskiy Donets River drainage in eastern Ukraine and adjacent Rostov Oblast of Russia (Biriuk et al., 2016; Borkin et al., 2004, 2006; Dedukh et al., 2017, 2015). However, in studied by us localities, the possibility of triploid emergence is high, as hybrid females are able to produce diploid gametes (Dedukh et al., 2019). According to earlier results, the formation of diploid eggs produced by diploid hybrid females often leads to the emergence of triploids in the majority of Central European systems (Berger, 2008; Plötner, 2005). However, the absence of triploids in natural populations suggests their mortality in initial stages of development or undiscovered mechanisms of post- or prezygotic elimination.

According to our analysis, we detected five types of population systems in the studied territory. In particular, we observed population systems, including parental species only (R and L systems), a mixture of parental species without hybrids (R-L systems), two parental species with hybrids (R-L-E systems), and *P. lessonae* individuals co-occurring with *P. esculentus* (L-E). We did not detect pure E and R-E population systems.

In the L-E and R-E-L systems, hybrids and parental individuals were represented by both sexes, which were usually presented in approximately the same proportion. Nevertheless, in some populations of *P. esculentus* (for example, the L-E system in Kuguvan), the number of males (80%) strongly exceeded the number of females.

The prevalence of the R-E-L type among mixed population systems in the studied region could be explained by the syntopical occurrence of two parental species in the same water bodies formed after deforestation of surrounding territories. Migration of a parental species into the preferable biotopes of others can also lead to their hybridization. Interestingly, we found the dominance of sterile hybrid females in R-E-L systems in comparison with L-E, which allows us to suggest that the primary hybrid progeny (with disturbed gametogenesis) predominantly present in R-E-L systems (Dedukh et al., 2019; present paper). In L-E systems, hybrids are represented by clonal lineages, mostly producing gametes with the *P. ridibundus* genome. The primary hybrids from the nearest R-E-L systems originally migrated to such L-E populations.

There are two hypotheses about the origin of local hybridogenetic *P. esculentus* in the studied territory. The first hypothesis suggests that the hybrids can emerge "de novo" after crosses of parental species. Alternatively, hybridogenetic hybrids could obtain this region from glacial refugia and colonize the Volga River drainage together with parental species. The sporadic appearance of alleles and haplotypes of *P.* cf. *bedriagae* in the gene pool of *P. ridibundus*, and co-occurring hybrid is a good marker indicating the "de novo" origin of local *P. esculentus*. However, the presence of *P. kurtmuelleri* haplotypes in a local hybrid can support the hypothesis about the migration of old hemiclonal lineages from more southwestern glacial refugia.

The effect of *P.* cf. *bedriagae* genome on selective genome elimination in *P. esculentus* has not been determined; however, our study showed that hybrids with alleles of *P.* cf. *bedriagae* can be found. Perhaps hybrids between *P. lessonae* and pure *P.* cf. *bedriagae* are sterile. In our study, we analyzed individuals of *P. ridibundus* and *P. esculentus* with an undetermined frequency of *P.* cf. *bedriagae* alleles. Crosses between some other species of water frogs with *P. ridibundus* (*P. shqipericus* × *P. ridibundus* and *P. epeiroticus* × *P. ridibundus*) led to progeny unable to reproduce via hybridogenesis (Hotz et al., 1985).

In the study area, we revealed that the majority of individuals produced gametes with the *P. ridibundus* genome. Such gametes are widespread for hybrid frogs from L-E systems in Central Europe, providing reproduction and maintenance of such systems (Ragghianti et al., 2007). However, we found that some *P. esculentus* produced gametes with the *P. lessonae* genome. Such hybrids are common for southern parts of Eastern Europe populated by R-E systems (Biryuk et al., 2016; Dedukh et al., 2015, 2017; Doležálková-Kaštánková et al., 2018). However, the prevalence of hybrids producing gametes with the *P. ridibundus* genome cannot contribute to continued maintenance of R-E systems in Volga River drainage. Hybrids from both the L-E and R-E-L systems were able to produce not only clonal gametes with one of the parental genomes but also gametes with intermediate between parental species the nuclear DNA content (presumably, non-clonal), as well as a mixture of these and clonal gametes.

Molecular studies helped us to disclose the role of species in the functioning of population systems. Previously, we failed to obtain progeny from hybrid females (Dedukh et al., 2019), but the presence of *P. ridibundus* mtDNA in hybrid females from L-E systems (Kuguvan; 10-year study) indicates their previous participation in the reproduction of hybrids. Widespread unidirectional mitochondrial DNA transfer from *P. lessonae* to *P. ridibundus* through hybrids was found in Western and Central Europe (Plötner et al., 2008). Although the transfer from *P.* cf. *bedriagae* to *P. lessonae* was recently found in a water frog population from the Volga River drainage in Mordovia Republic (Ivanov et al., 2019), this transfer is still a highly rare phenomenon. The lack of introgression from *P. lessonae* to *P. ridibundus* can be explained by the fact that local hybrid males and females rarely interbreed and/or that progeny of such interhybrid crosses is unviable.

4.3 | Conservation status of *P. esculentus* populations

In some regions of the Volga River drainage (Samara, Tambov, and Kursk Oblasts, as well as republics of Udmurtia and Mordovia), the edible frog is under protection (see review in Lada, 2012). However, the conservation status of this frog depends on understanding the taxonomic status of *P. esculentus*. If edible frogs are hybrids formed "de novo," their conservation status should be reviewed because hybrids can permanently emerge as a result of human activity (deforestation and creation of artificial water bodies). On the other hand, if hybrids represent long-aged hemiclonal lineages that colonize new territories from glacial refugia, protection of such populations is necessary.

Regardless, studies of population systems from the northeastern part of the *P. esculentus* complex distribution seem to be of considerable interest as a model for the study of the first steps of reticulate speciation and analysis of the effect of *P.* cf. *bedriagae* gene introgression on hemiclonal reproduction of *P. esculentus*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. Studied localities and sample size of water frogs.

Table S2. Types of biotopes and population systems of water frogs.

Table S3. Morphological traits of studied individuals.

Table S4. Genome size and ploidy level of studied individuals.

Table S5. Results of genetic (*COI* and *SAI-1*) identification of water frogs from various population systems.

Table S6. GenBank numbers of sequences of the mitochondrial *ND2* gene in studied by us water frog specimens (a) and published previously (b).

Table S7. Types of gametes produced by parental species and hybrids: A. Males. B. Females.

Table S8. List of studied individuals of water frogs.

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