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SPECIES AND HYBRID COMPOSITION AND GENETIC DIVERSITY OF WATER FROGS (*PELOPHYLAX ESCULENTUS* COMPLEX) IN WESTERN UKRAINIAN HEMICLONAL POPULATION SYSTEMS

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Background. Two species of water frogs, *Pelophylax ridibundus* and *Pelophylax lessonae*, and their hybrid, *Pelophylax* kl. *esculentus*, are widespread in Ukraine. The purpose of this study was to investigate the population structure of various types of hemiclonal population systems (HPS) of water frogs formed due to the coexistence of frogs in the same territory. In Ukraine, a hybridization centre with the spread of triploid individuals of hybrid nature has been identified. Triploid hybrids are an intriguing research subject due to the diverse hypotheses about their origin and role in HPS. Outside the hybridization centre in Kharkiv Region, triploids are not commonly found. In our study, we describe the initial findings of triploid specimens in Lviv Region and analyze the genetic structure of the HPS where such individuals were detected.

Methods. In total, 193 specimens of green frogs were collected between 2011 and 2015. Here we present population structure analysis which was conducted using two microsatellite loci, *Rrid059A* and *RICA1b5*. A wide range of software programs were utilized for processing the genetic analysis data, including GenePop 4.7.5, Micro-Checker and NewHybrids 1.1.

Results. Three types of hemiclonal population systems were identified: R-E type in Perekalky and Lake Pisochne, L-E type in Lake Luky, and R-E-L type in Nyzhankovychi, Velykyi Lyubin, Zhovtantsi and Cholgyni. Additionally, population systems with hybrids of mixed ploidy (diploids and triploids) were found in Perekalky, Velykyi Lyubin and Zhovtantsi.

Conclusions. Genetic diversity analysis revealed variations in the number of alleles per population. *P. ridibundus* individuals exhibited higher genetic diversity compared to *P. lessonae* individuals, whereas hybrids showed intermediate genetic diversity. Further investigations of the localities where potential triploids were detected are necessary to assess the survival and reproductive potential of hybrid individuals and determine all types of hybrids and individuals of both marsh and pool frogs.

Keywords: water frogs, *Rrid059A*, *RICA1b5*, NewHybrids, hemiclonal population systems (HPS)

INTRODUCTION

The *Pelophylax esculentus* complex in Ukraine consists of two parental species: the marsh frog (*Pelophylax ridibundus*) and the pool frog (*Pelophylax lessonae*). Additionally, this complex includes the edible frog (*Pelophylax* kl. *esculentus*), which is a viable hemiclonal hybrid of the parental species (Berger, 1964; Berger & Berger, 1994; Pysanets', 2007). These species coexist and interact at various levels, forming a hemiclonal population system (HPS) where recombinant and clonal genomes are transmitted across generations (Biriuk *et al.,* 2015). In some regions, there are local groups consisting exclusively of hybrid individuals, which can be found in several European countries, including Ukraine (Plötner, 2005).

Hybrids within the *Pelophylax esculentus* complex can be classified into two distinct groups based on their origin. The first group consists of *de novo* hybrids which result from crossbreeding between parental species. The second group comprises backcross hybrids which arise from reverse crossbreeding between hybrids and parental species. The reproduction of *P.* kl. *esculentus* involves partial elimination of genetic material from one of the parents. This means that the genome of the second parental species is inherited in a semi-clonal manner. The characteristics of hybrids can vary depending on their geographical location: they may be diploid or triploid, exclusively female or male, or exhibit an equal sex ratio like the parental species (Doležálková *et al.,* 2016; Rybacki & Berger, 2001; Tunner & Kárpáti, 1997; Uzzell *et al.,* 1976). Hybrids can form gametes of both parental species (R, L or at the same time R and L). This process can result in the elimination of genes from both the pool frog and the marsh frog (Dedukh *et al.,* 2015, 2017; Reyer *et al.,* 2015). When hybrid frogs are crossed with each other and produce gametes of the same parental species, there is a possibility of hybridolysis. This phenomenon leads to the transfer of different clonal genomes of the same species into one individual. Noteworthy, to reproduce the parent species, the presence of exactly two genomes of the same species is necessary. Individuals formed through hybridolysis typically exhibit reduced viability (Dedukh *et al*., 2017), which can be attributed to changes (mutations) that accumulate in the clonal genome during transmission from generation to generation without recombination (Doležálková-Kaštánkova *et al*., 2018). Alternatively, it may result from the introgression of mitochondrial DNA from another species (Bohling *et al*., 2013; Hill, 2019). Individuals with two clonal genomes have low viability and often perish during the pre-reproductive period, a process known as postzygotic elimination. Crossbreeding between hybrids produces viable offspring, and there are genetic differences between hybrids of the first generation and backcross hybrids. These differences are reflected in various types of population systems and the sexual structure of hybrids across different geographic localities (Stöck *et al.,* 2022).

The presence of triploid (3n) individuals in the hybrid population of water frogs is a commonly observed phenomenon (Berger & Berger, 1994; Dedukh *et al.,* 2017). Triploids are formed through fertilization events involving a diploid egg cell and a haploid spermatozoon (Hoffmann *et al.,* 2015; Pidancier *et al.,* 2003), a haploid egg cell and a diploid spermatozoon (although less frequent and less studied) (Plötner, 2005; Tunner & Kárpáti, 1997), or potentially through genome doubling of a haploid egg cell after fertilization (Biriuk, 2015). Triploid hybrids possess either two *P. lessonae* genomes and one *P. ridibundus* genome (genotype LLR) or one *P. lessonae* genome and two *P. ridibundus* genomes (genotype LRR) (Plötner, 2005). They can produce both haploid and diploid gametes, thereby eliminating the minority genome (Stöck *et al.,* 2022). Despite the presence of triploidy, sexually mature triploids have comparable lifespans and slightly faster growth rates than diploid hybrids (Meleshko, Korshunov, & Shabanov, 2014).

The occurrence of triploidy in *Pelophylax* kl. *esculentus* is most commonly reported in Western and Central Europe, spanning from France to Sweden and Poland, with additional records in Slovakia and Hungary (Holsbeek & Jooris, 2010). In Ukraine, researchers from Kharkiv have identified a specific region in the Siversky Donets River basin as the sole hybridisation centre of water frogs in the country, which is currently the subject of active research (Biriuk *et al.,* 2015; Dedukh *et al.,* 2017; Kryvoltsevych *et al.,* 2022; Stöck *et al.,* 2022).

Identifying diploid and triploid water frogs solely based on their external characteristics is a complex task, but it can be reliably accomplished in laboratory settings. One accessible approach involves measuring the size of erythrocytes, as it is closely linked to ploidy. The boundary between diploids and triploids is determined by the length of erythrocytes, typically falling within the range of 26 to 28 µm (Bondarieva *et al.,* 2012; Ogielska-Nowak, 1978; Plötner, 2005). Additionally, triploids exhibit a reduced erythrocyte count due to their enlarged size, along with a decrease in haemoglobin content (Christiansen, 2005). However, to determine ploidy more accurately, other sophisticated molecular methods can be employed. Overall, the taxonomic classification of water frog groups in natural habitats presents difficulties due to their morphometric and phenetic similarities (Hyne *et al.,* 2009). However, by utilizing genetic techniques like microsatellite DNA analysis, RADseq etc., it becomes possible to precisely determine the taxonomic affiliation or ploidy of an individual frog (Ambu & Dufresnes, 2023; Cuevas *et al.,* 2022; Hofman *et al.,* 2012).

Modern molecular methods are now employed to study the genetic population structure of water frog hybridization sites in Europe. These studies have revealed the clonal transmission of different types of genomes within various hemiclonal population systems (Biriuk *et al.,* 2015; Dedukh *et al.,* 2013*,* 2015*,* 2017; Doležálková *et al.,* 2016; Dufresnes *et al.,* 2018; Herczeg *et al.,* 2017; Quilodran *et al.,* 2015). Some studies have also focused on investigating the population structure of the edible frog (Christiansen, 2009) and populations with the presence of parental species alongside exclusively male individuals of hybrid forms (Pruvost *et al.,* 2015). Such population systems have been documented in several countries, including the Czech Republic (Doležálková *et al.,* 2016), Germany (Uzzell *et al.,* 1976), Hungary (Tunner & Kárpáti, 1997), and Poland (Rybacki & Berger, 2001).

Genetic markers, such as nuclear microsatellite loci, are valuable tools in assessing individual genotypes, genetic diversity within populations, and population differentiation. Despite the widespread availability of molecular methods in zoology, there is still untapped research potential in this field within Ukraine, particularly regarding the genetic diversity of water frog groups in western Ukraine. Expanding the use of genetic tools and conducting further investigations will enhance our understanding of the genetic structure and evolutionary dynamics of water frog populations in the region. This knowledge is vital for comprehending the species' ecology, conservation, and management, providing valuable insights for future research and conservation efforts aimed at preserving these frogs and their habitats.

The main objectives of the present study were:

1. **To assess the population composition** at the analyzed localities, specifically focusing on the distribution of hybrid individuals and the composition of parental species. It was anticipated that localities with hybrids would exhibit the presence of at least one parental species or triploid hybrids, which serve as donors of the genome (Hoffmann *et al.,* 2015).
2. **To evaluate the genetic diversity of parental species and hybrids** in the analyzed population systems using microsatellite DNA analysis, taking into account the geographical locations of the localities. A correlation between the genetic diversity of the population systems and the geographic coordinates of the localities was expected. Based on previous research by A. Hoffmann (2015), a decrease in diversity from west to east and from south to north was anticipated. The samples from the Shatsky National Nature Park (Stakh *et al.,* 2018) were expected to demonstrate the lowest diversity due to glacial retreat and range expansion, as described in the literature (Hoffmann *et al.,* 2015).
3. **To assess the hybrid composition of the population systems**, including the presence of various hybrids such as F1, F2, backcrosses between marsh frogs and pool frogs, and hybrids with different ploidy levels (Hoffmann *et al.,* 2015; Stakh *et al.,* 2018).

MATERIALS AND METHODS

The study focused on two species of water frogs: the marsh frog (*Pelophylax ridibundus*) and the pool frog (*Pelophylax lessonae*). Additionally, we investigated the result of hybridization between these two species, known as the edible frog (*Pelophylax* kl. *esculentus*). Amphibians were collected from 2011 to 2015. The captured frogs were placed in pre-moistened bags made of breathable material (such as satin, muslin, cotton, etc.).

Localities for collecting amphibians. The amphibians were collected from seven localities within the administrative boundaries of two regions in Ukraine – Lviv and Volyn Regions (**Fig. 1**) and were more extensively described in previous works (Stakh *et al.,* 2014, 2018). In this work, we chose the water bodies in Nyzhankovychi village (49°40′34″ N, 22°48′30″ E), Cholgyny (49°55′14″ N, 23°26′11″ E), Perekalky (50°11′11″ N, 24°23′41″ E), Zhovtantsi (49°59′33″ N, 24°14′18″ E), Velykyi Lubin (49°43′26″ N, 23°44′01″ E), Lake Pisochne (51°34'13" N, 23°54'11" E) and Lake Luky (51°34'28" N, 23°50'49" E).

The localities of Nyzhankovychi, Cholgyni, Velykyi Lyubin, and Zhovtantsi are artificial water bodies. Perekalky represents a channel for the discharge of cooling waters from the Dobrotvir Thermal Power Plant. Lakes Pisochne and Luky are located in Volyn Region and are natural water bodies.

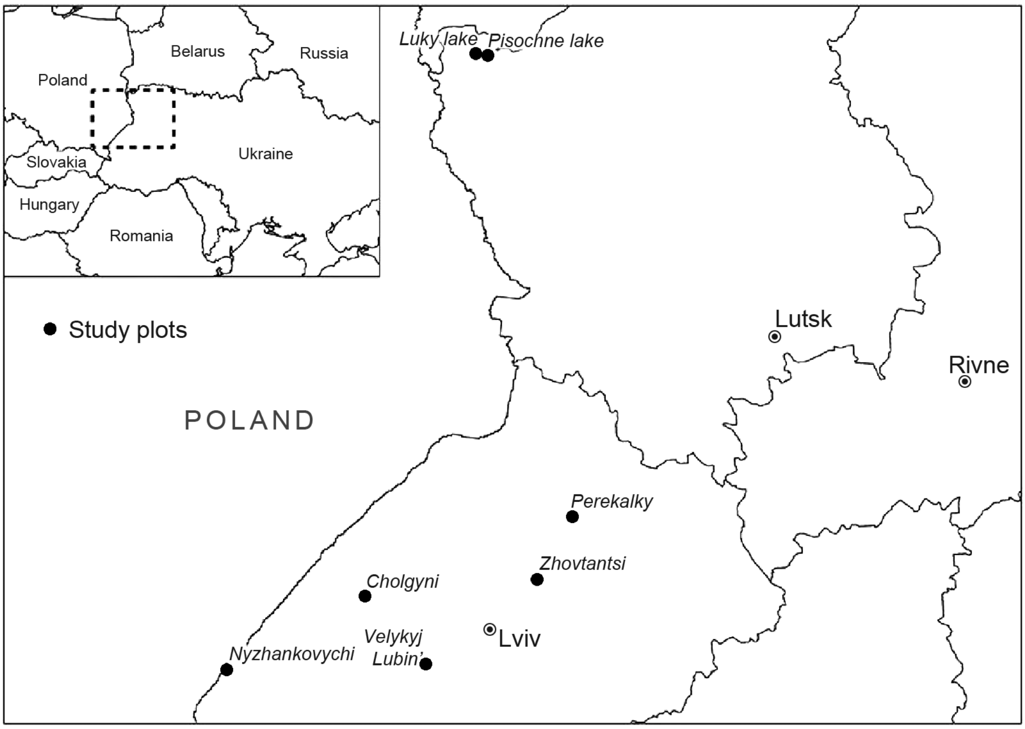

Fig. 1. Localization of the investigated water bodies

Sample Collection and Types of Hemiclonal Population Systems. A total of 193 water frog specimens were collected between 2011 and 2015. In the field, the taxonomic classification of each individual was determined based on a set of characteristics described in our previous works (Stakh *et al.,* 2014, 2018). Samples of oral epithelium were collected using cotton swabs following the appropriate methods (Ambu & Dufresnes, 2023; Pidancier *et al.,* 2003) and placed in 1.7- or 2-mL tubes. The samples were stored in freezer chambers at -18 °C and transported to the laboratory under low-temperature conditions using specialized refrigerated bags. DNA extraction was successful for 174 specimens (*P. ridibundus* – 68, *P. lessonae* – 53, *P. esculentus* – 53).

The type of hemiclonal population system (HPS) was determined based on the species present in each locality. The species composition was assessed using cumulative information on the genotypic structure of LL, RR, RE, LLR, and LRR individuals.

The selection of microsatellite loci. A detailed description of the DNA extraction method, PCR amplification of microsatellite sequences, electrophoresis of PCR products, and result analysis were described in previous studies (Stakh *et al.,* 2014, 2018).

When assessing the genetic structure of water frog HPS, the analysis of microsatellite DNA is commonly used as a relatively accessible method (Dufresnes *et al.,* 2017; Hotz & Uzzel, 1982; Kaeuffer *et al.,* 2007; Quilodran *et al.,* 2015; Smouse *et al.,* 2017; Yin *et al.,* 2018). To accurately evaluate the diversity of clonal individuals, it is necessary to use microsatellite markers with high polymorphism and even distribution (Doležálková-Kaštánkova *et al.,* 2018). For the group of water frogs, several species-specific loci have been described (Doležálková *et al.,* 2016; Dufresnes *et al.,* 2018;

Garner *et al.,* 2000; Herczeg *et al.,* 2017; Holm, 1979; Hotz & Uzzel, 1982; Pruvost *et al.,* 2013; Zeisset *et al.,* 2000). In this study, we present the results of population structure analysis for 174 individuals using two loci, *Rrid059A* and *RICA1b5*. The analysis of the first 91 individuals is described in detail in our previous works (Stakh *et al.*, 2014, 2018), and here we supplement the analysis of the results.

Measurement of erythrocyte size. In 2019, the ploidy analysis of water frogs from Perekalky (24 specimens) and Zhovtantsi (42 specimens) was conducted to analyze the erythrocyte size. For the measurement of erythrocyte size, a blood smear was prepared for each individual. A drop of blood was applied to a glass slide and quickly spread into a thin layer using the edge of another glass slide. The erythrocyte smear was then dried (Bondarieva *et al.,* 2012; Ogielska-Nowak, 1978). Subsequently, the smear was photographed under a microscope with an object micrometer using a USB camera. The length of the major axis of 20 erythrocytes was measured using the PdfXChange Viewer software. The measurement results were converted into micrometres and recorded in a database. In triploid individuals, the erythrocytes were slightly larger (26–28 μm) than those of diploid individuals (<26 μm) (Christiansen, 2005; Ogielska-Nowak, 1978; Plötner, 2005).

Processing of the results of microsatellite DNA analysis. The obtained multilocus individual genotypes of water frogs were examined for the presence of gene linkage groups using the GenePop 4.7.5 software (Raymond & Rousset, 1995a; Raymond & Rousset, 1995b; Reyer *et al.,* 2015; Rousset & Raphaël, 2007; Rousset, 2008), as well as for the presence of hidden null-alleles using the Micro-Checker software (Van Oosterhout *et al.,* 2004).

Population structure analysis was conducted using Bayesian methods (employing the MCMC algorithm) in the NewHybrids 1.1 (Anderson & Thompson, 2002). The NewHybrids 1.1 program incorporates species-specific alleles as initial conditions (Dufresnes *et al.,* 2017; Falush *et al.,* 2007; Herczeg *et al.,* 2017; Pritchard *et al.,* 2000). The NewHybrids1.1 accurately identifies parental species (P) and hybrids resulting from their crossbreeding (F1), as well as hybrids crossing with each other (F2) or with parental species (Bx). Classification is based on genotypic frequencies (**Table 1**). The program determines pure species based on unique alleles (Anderson & Thompson, 2002). Therefore, using the NewHybrids 1.1 program, an assessment of individual hybridity in HPS was conducted based on individual genotypes at two diagnostic loci, *RICA1b5* and *Rrid059A*. These loci were chosen because they contain unique alleles that are fixed (frequency equals 1.00) in *P. lessonae*. Calculations were performed with 20,000 generations (MCMC). All triploids were deleted before the analysis.

Table 1. **Genotypic frequency classes used by NewHybrids 1.1 for detecting hybrids of different origins**

Classes	Frequency			
Sp_1	1.00	0.00	0.00	0.00
Sp_2	0.00	0.00	0.00	1.00
F1	0.00	0.50	0.50	0.00
F2	0.25	0.25	0.25	0.25
1_Bx	0.50	0.25	0.25	0.00
2_Bx	0.00	0.25	0.25	0.50

Literature analysis of the genetic diversity of water frogs indicates the presence of species-specific alleles (**Table 2**) (Herczeg *et al.,* 2017; Hotz & Uzzel, 1982; Stakh *et al.,* 2014) at the loci used, namely *Rrid059A* and *RICA1b5*, which are fixed for each species (frequency equals 1.00). When comparing data collected by different laboratories, it is important to consider discrepancies in the interpretation of microsatellite allele sizes, as well as the fact that different software used in the instruments may have an error of +/- 2 nucleotides during the interpretation of electrophoresis results of amplified DNA fragments (Delmotte *et al.,* 2001). That is why **Table 2** includes both literature data (Herczeg *et al.,* 2017; Hotz & Uzzel, 1982) and our data (Stakh *et al.,* 2014, 2018).

Table 2. **Species-specific alleles of the analyzed loci *Rrid059A* and *RICA1b5***

Species	*RICA1b5*	*Rrid059A*
Pelophylax ridibundus	135, 137, 139, null allele	113, 115, 133, 136, 137, 138
Pelophylax lessonae	120, 122, 123	101, 103

RESULTS

Task 1. *Evaluation of the population composition of samples.*

Three types of hemiclonal population systems were identified: R-E type in Perekalky and Lake Pisochne, L-E type in Lake Luky, and R-E-L type in Nyzhankovychi, Velykyi Lyubin, Zhovtantsi and Cholgyni (**Fig. 1**). Additionally, hemiclonal population systems with hybrids of mixed ploidy (diploids and triploids) were found in Perekalky, Velykyi Lyubin and Zhovtantsi. Pure parental populations weren't found in the investigated localities.

Task 2. *Assessment of Genetic Diversity.*

The analysis of genetic variability in the analyzed populations of water frogs using microsatellite sequences of nuclear DNA identified 12 alleles at the *Rrid059A* locus, ranging in size from 103 to 149 base pairs (bp). The allele of 103 bp was found exclusively in pool frogs and edible frogs. The frequency of the 103 bp allele (**Table 3**) increases with the population of pool frogs and hybrids, which is consistent with the literature (Herczeg *et al.,* 2017; Hotz & Uzzel, 1982; Hotz *et al.,* 2001). H. Hotz & T. Uzzel (1982) describe the allele of 137 bp as characteristic of marsh frogs (**Table 3**), and the locus is generally associated with *P. ridibundus*.

We used the locus *RICA1b5* as one of the species-specific loci (**Table 2**) (Herczeg *et al.,* 2017; Pruvost, Hoffmann & Reyer, 2013). In our samples, the alleles at this locus range from 123 to 141 bp (**Table 4**) (Stakh *et al.,* 2014). The allele 123 bp is found only in samples with individuals of *P. lessonae* and hybrids since the locus is specific to the pool frog (Herczeg *et al.,* 2017) (**Table 2**).

Individuals of *P. lessonae* showed lower genetic diversity in the alleles of the studied loci, with a total of 9 alleles. On the other hand, *P. ridibundus* exhibited higher genetic diversity with 14 alleles. The genetic diversity of hybrid individuals was intermediate, with 10 alleles across the two loci.

Task 3. *Evaluation of hybrid composition in population systems.*

At the outset, it is important to emphasize that the NewHybrids program predicts whether an individual belongs to a certain species or different types of hybrids. Therefore, we provide the percentage probabilities of the assignment of each individual to specific classes.

Table 3. **Frequencies of alleles at the microsatellite locus *Rrid059A* in representatives of water frog species from different localities**

Rrid059A	Cholgyni (No 49)	Luky (No 18)	Nyzhankovychi (No 45)	Perekalky (No 16)	Pisochne (No 16)	Velykyi Lyubin (No13)	Zhovtantsi (No 8)	Grand Total
103	0.56	0.72	0.11	0.16	0.38	0.50	0.25	0.38
125	0.13	0.17	0.27	0.22	0.06	0.04	0.13	0.17
127	0.03	0.00	0.06	0.00	0.09	0.00	0.00	0.03
133	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
135	0.02	0.00	0.12	0.00	0.06	0.04	0.00	0.05
137	0.06	0.03	0.10	0.06	0.41	0.19	0.06	0.11
139	0.14	0.08	0.19	0.38	0.00	0.12	0.19	0.16
141	0.02	0.00	0.02	0.00	0.00	0.00	0.00	0.01
143	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
145	0.00	0.00	0.02	0.00	0.00	0.00	0.13	0.01
147	0.03	0.00	0.06	0.19	0.00	0.12	0.25	0.06
149	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.01

Table 4. **Frequencies of alleles at the microsatellite locus *RICA1b5* in representatives of water frog species from different localities**

RICA1b5	Cholgyni (No 49)	Luky (No 18)	Nyzhankovychi (No 45)	Perekalky (No 16)	Pisochne (No 16)	Velykyi Lyubin` (No 13)	Zhovtantsi (No 8)	Grand Total
123.00	0.56	0.64	0.14	0.44	0.41	0.58	0.38	0.42
137.00	0.09	0.19	0.00	0.34	0.09	0.15	0.06	0.11
139.00	0.28	0.17	0.86	0.22	0.50	0.27	0.56	0.45
141.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.02

The graph in **Fig. 2*A*** presents taxon composition in the Nyzhankovychi samples. The locality is characterized by the presence of two parent species (No 135, 137, 145 are *P. lessonae* with a probability of 98 %), different generation hybrids (individuals 136, 144, 146–149), and at least two backcrosses with *P. ridibundus* (individuals 139, 141). No triploid hybrids were detected.

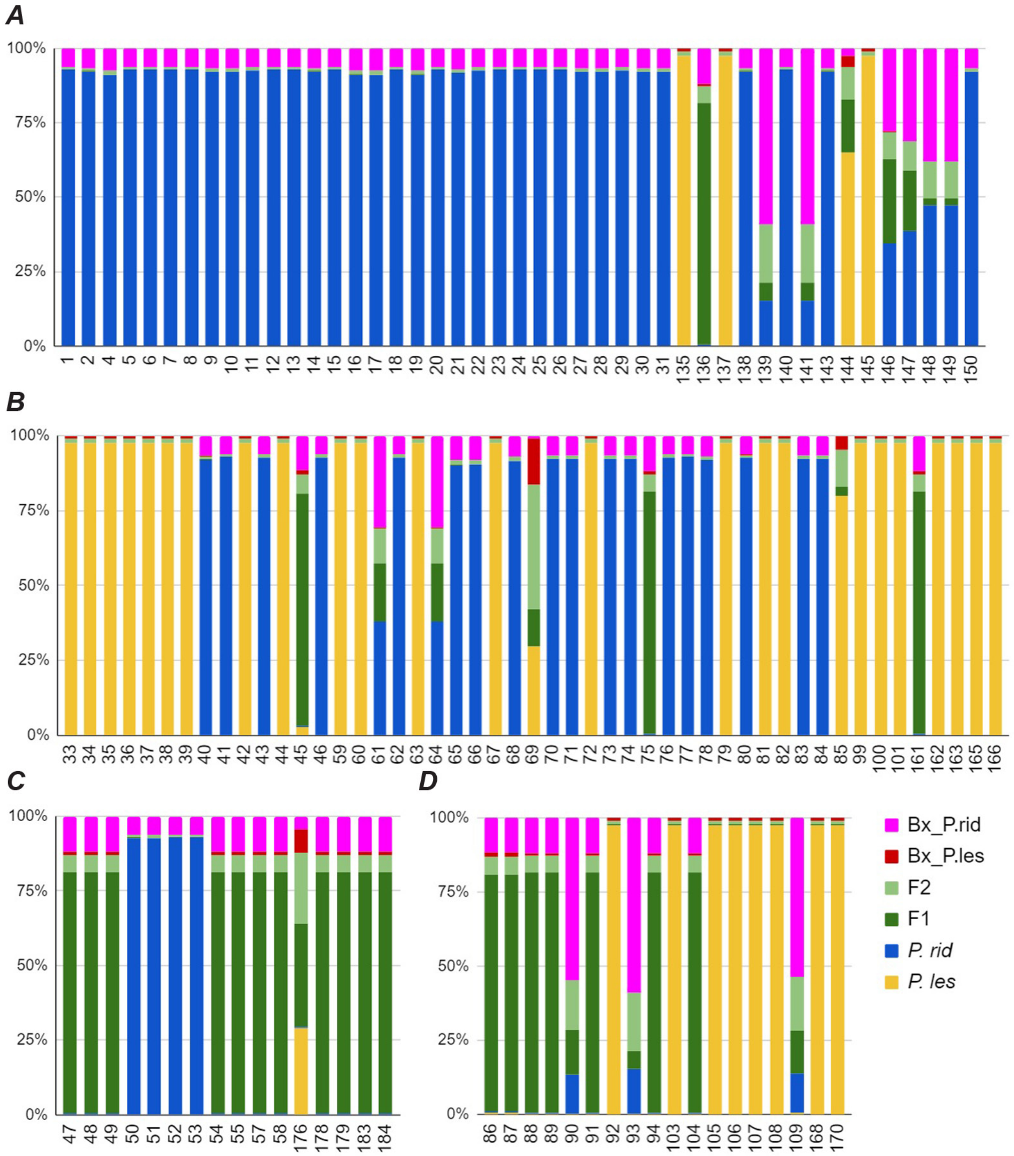

Fig. 2. The hybrid composition of the analyzed population systems was determined using NewHybrids 1.1 utilizing the results of the analysis of the *Rrid059A* and *RICA1b5* loci of individuals collected from various water bodies: ***A*** – Nyzhankovychi village; ***B*** – Cholgyni village; ***C*** – Pisochne Lake; ***D*** – Luky Lake. Different colours on the graph represent the assigned classes (P. rid. – marsh frog; P. les. – pool frog; F1 – first-generation hybrids; F2 – second-generation hybrids; B_x P.r. – backcrosses with marsh frog; B_x P.l. – backcrosses with pool frog)

Despite the presence of hybrids in <u>Cholgyni</u> (**Fig. 2*B***), the majority of them consist of pure parental species. For example, individuals No 33–39, 42, 44, 59, 60, 63, 67, 72, 79, 81, 82, 99–101, 162–166 are *P. lessonae* with a probability of 97 %. The locality is characterized by the presence of first-generation hybrids (individuals No 45, 75 and 161 with a probability of more than 77 %) and two backcrosses with *P. ridibundus* (No 61, 64). No triploid hybrids were detected.

The hybrid composition of the water frog population in Lake <u>Pisochne</u> differs from the ones analyzed in this study by the presence of exclusively F1 hybrids (individuals 47–49, 54–58, 178–184 with a probability of more than 81 %) (**Fig. 2*C***). This is an interesting feature, as individuals of the pool frog were not found, which is necessary for the formation of first-generation hybrids. There is a 93 % probability that individuals No 50–53 are *P. ridibundus*. No triploid hybrids were found.

In the <u>Luky</u> locality, individuals of the pool frog (No 92, 103, 105–108, 168, 170 with a probability of 98 %), F1 hybrids (No 86-89, 91, 94, 104), and backcrosses with *P. ridibundus* (individual No 90, 93 109) were found (**Fig. 2*D***). The marsh frog individuals were not found in this locality. All sampled individuals were of non-reproductive age. No triploid hybrids were found.

The main portion of hybrids in the locality of <u>Velykyi Lyubin</u> (**Fig. 3*A***) is the result of the first crossbreeding of parental forms (F1) (individuals No 206–209, 211, 213, 214, 216, 217). Four triploid hybrids were identified (No 207, 218, 219, and 221) and belong to the RRL type (**Table 5**).

The sample from the village of <u>Zhovtantsi</u> (**Fig. 3*B***) consists of both first-generation hybrids (F1) (individuals No 203, 204 with a probability of 80 %) and backcrosses with *P. ridibundus* (individuals No 197, 201 with a probability of 32.5 %) were found. Three triploid hybrids were also detected (No 198, 199, 200) and belong to the RRL type (**Table 5**).

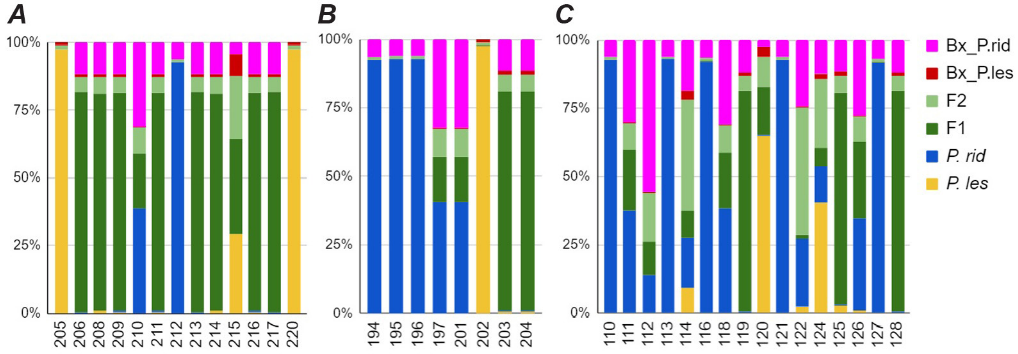

Fig. 3. The hybrid composition of the analyzed population systems was determined using NewHybrids 1.1 utilizing the results of the analysis of the *Rrid059A* and *RICA1b5* loci of individuals collected from various water bodies: ***A*** – Velykyi Lyubin; ***B*** – Zhovtantsi; ***C*** – Perekalky. Different colours on the graph represent the assigned classes (P. rid. – marsh frog; P. les. – pool frog; F1 - first-generation hybrids; F2 – second-generation hybrids; B_x P.r. – backcrosses with marsh frog; B_x P.l. – backcrosses with pool frog)

The sample from the village of <u>Perekalky</u> (**Fig. 3*C***) consists of 16 individuals, out of which 11 are hybrids. Three individuals, namely numbers 119, 125, and 128, have been identified as first-generation hybrids with a probability exceeding 78 %. These hybrids are the product of the crossbreeding of parental individuals. Additionally, individual number 112 has been identified as a backcross with *P. ridibundus*, with a probability of 56 %. Two individuals were also identified as triploids (No 123, 129) with an RRL genotype (**Table 5**).

Table 5. **Alleles of triploids at loci *Rrid059A* and *RICA1b5***

Individuals	Sex	Site	*Rrid059A*			Probable genotype	*RICA1b5*		
123	♀	Perekalky	139	103	-	RRL	139	137	123
129	♀	Perekalky	139	103	-	RRL	139	137	123
198	♂	Zhovtantsi	147	137	103	RRL	139	137	123
199	♂	Zhovtantsi	147	103	-	RRL	139	137	123
200	♂	Zhovtantsi	147	103	-	RRL	139	137	123
207	♂	Velykyi Lyubin	139	125	103	RRL	139	137	123
218	♂	Velykyi Lyubin	147	137	103	RRL	139	123	-
219	♀	Velykyi Lyubin	149	137	103	RRL	137	123	-
221	♂	Velykyi Lyubin	147	103	-	RRL	139	137	123

In 2019, additional sampling of water frogs from the localities of Perekalky and Zhovtantsi was conducted. Blood samples were collected from each individual for the analysis of erythrocyte size, which differs significantly between diploid and triploid water frogs (Bondarieva *et al.*, 2012; Ogielska-Nowak, 1978). Individuals with slightly larger erythrocytes than the diploid range were identified, but genetic analysis of microsatellite DNA was not performed.

DISCUSSION

Task 1. *Evaluation of the population composition in the samples.*

Pure parental populations were not found in the investigated localities. The majority of the analyzed localities are characterized by established hybridization (presence of backcrosses). A crucial task for the clonal genome is transitioning to sexual cells. In first-generation hybrids resulting from crosses between parent individuals, the formation of sexual cells is problematic. Many hybrids are infertile. Only individuals capable of producing viable sexual cells can pass on clonal genomes to the next generation. Clonal genomes that have undergone several generations are reproduced much more reliably. This indicates a unique form of genome evolution, characterized by the accumulation of adaptations for clonal transmission (Meleshko *et al.*, 2014). Most of the HPS that we analyzed have backcrosses, which indicates that hybrid frogs are capable of producing gametes and reproducing. Continuing the study of these HPS would be highly beneficial.

These are the first investigations of hemiclonal population systems using microsatellite analysis for samples from Velykyi Lyubin, Perekalky, and Zhovtantsi. Additionally, this work supplements our previous studies of HPS from Nyzhankovychi, Cholgyni, and Lakes Luky and Pisochne.

Task 2. *Assessment of genetic diversity.*

We expected to observe a subjection between the genetic diversity of HPS and the geographical coordinates of the localities. According to A. Hoffmann *et al.* (2015), diversity decreases from west to east and from south to north. In our case, the difference in geographical distance between the samples is not significant enough to draw obvious conclusions. Overall, samples located closer to the Carpathian Mountains (Nyzhankovychi, Cholgyni and Velykyi Lyubin) exhibit a higher diversity in terms of detected alleles.

Task 3. *Assessment of hybrid composition in population systems.*

The pool frog is less numerous in the HPS of Nyzhankovychi, and both morphometric and phenotypic characteristics make it difficult to distinguish individuals from those of the marsh frog or the edible frog. The results obtained in this study intrigued us, as we did not previously suspect the presence of the pool frog in this HPS (Stakh *et al.,* 2014, 2018).

It is evident that the hybridization of water frogs in Cholgyni (**Fig. 2*B***) is a relatively recent phenomenon, or crossbreeding between parental species does not always result in viable hybrid individuals (Berger & Berger, 1994; Plötner, 2005). The future fate of this hemiclonal population system depends on the genome transmitted semiclonally (possible clone genomes: (R), (L), and the simultaneous presence of both variants – (R) and (L)), the survival ability of individuals of the parental species whose genome is transmitted clonally (the phenomenon of hybrid breakdown), the viability of hybrid individuals, potential external admixtures, etc. (Kravchenko & Shabanov, 2008; Perez-Enriquez *et al.,* 2018; Plötner, 2005).

There is a probability that *P. lessonae* individuals inhabit Lake Pisochne, although they were not detected in our study. Another notable characteristic is the presence of only F1 generation hybrids. It is possible that we failed to capture F2 individuals or backcrosses. It is also possible that hybrids do not produce reproductive gametes capable of generating hybrids, or F1 hybrids may be unable to produce viable offspring and hence they perish at certain stages of development, and so on. Considering the fact that mature males and females were found (Stakh *et al.*, 2018), it is evident that a certain portion of hybrids reach sexual maturity. Further investigations are needed to explore the presence of pool frog individuals and other hybrid types in the locality.

The presence of backcrosses in Luky Lake indicates successful transmission of the clonal genome. Further research is needed to determine whether the backcrosses reach reproductive age. For successful reproduction and maintenance of hybrids in population systems, it is sufficient to have at least one parental species or triploid that are utilized by hybrids for sexual parasitism.

CONCLUSIONS

In our study, three localities with triploid hybrids were identified. Triploids were detected in Velykyi Lyubin, Zhovtantsi, and Perekalky (**Table 5**). A total of 9 triploid individuals were identified, but we cannot infer their population structure and origin due to insufficient sampling.

For more accurate conclusions about each HPS, it would be beneficial to have a significantly larger number of loci (Anderson & Thompson, 2002) that better reflect the genetic structure and encompass a greater overall diversity of alleles. Additionally, reanalyzing the amphibians from each locality by conducting various types of crosses and performing genetic analysis using more recent and reliable methods (Cuevas *et al.*, 2022; Stöck *et al.*, 2022) would also be advantageous. These methods will enable the determination of the genetic profile of hybrids and elucidate their origins and distribution within the population. Such studies will provide more detailed information about hybridization and the interaction of different species in population systems.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Animal Rights: This article does not contain any studies with animal subjects performed by the any of the authors.

AUTHOR CONTRIBUTIONS

Conceptualization, [V.S.; Iu.S.; I.Kh.]; methodology, [V.S.; Iu.S.]; validation, [Iu.S.; I.Kh.]; formal analysis, [Iu.S.; I.Kh.].; investigation, [V.S.; Iu.S.]; resources, [V.S.; I.Kh.]; data curation, [V.S.; Iu.S.]; writing – original draft preparation, [V.S.]; writing – review and editing, [V.S.; Iu.S.; I.Kh.]; visualization, [Iu.S.] supervision, [I.Kh.]; project administration, [V.S.]; funding acquisition, [I.Kh.; V. S.].

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ВИДОВИЙ І ГІБРИДНИЙ СКЛАД, ГЕНЕТИЧНЕ РІЗНОМАНІТТЯ ЗЕЛЕНИХ ЖАБ (*PELOPHYLAX ESCULENTUS* COMPLEX) У ГЕМІКЛОНАЛЬНИХ ПОПУЛЯЦІЙНИХ СИСТЕМАХ ЗАХОДУ УКРАЇНИ

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Вступ. На території України поширені два види зелених жаб *Pelophylax ridibundus* і *Pelophylax lessonae* та їхній гібрид – *Pelophylax* kl. *esculentus.* Метою роботи є дослідити популяційну структуру різного типу геміклональних популяційних систем (ГПС) зелених жаб, які утворені внаслідок співіснування жаб на спільній території. В Україні виявлено центр гібридизації із поширенням триплоїдних особин гібридної природи. Триплоїдні гібриди є цікавим об’єктом дослідження, оскільки гіпотези їхгього виникнення та ролі у ГПС є достатньо різні. На території України поза центром гібридизації в Харківській області триплоїди не трапляються настільки рутинно. В нашій роботі ми описуємо перші знахідки триплоїдів на території Львівської області й аналізуємо генетичну структуру ГПС, де і було виявлено таких особин.

Матеріали та методи. Загалом 193 особини зелених жаб було зібрано між 2011 та 2015 рр. Тут представлено аналіз популяційної структури з використанням двох мікросателітних локусів *Rrid059A* та *RICA1b5*. Для опрацювання даних генетичного аналізу у роботі використано широкий спектр програм, а саме GenePop 4.7.5, Micro-Checker та NewHybrids 1.1.

Результати. Результати виявили три типи геміклональних популяційних систем: тип R-E в Перекалках та озері Пісочне, тип L-E в озері Луки та тип R-E-L у Нижанковичах, Чолгинях, Великому Любіні та Жовтанцях. Крім того, виявлено системи з гібридами змішаної плоїдності (диплоїди та триплоїди): у Перекалках, Великому Любіні та Жовтанцях.

Висновки. Аналіз генетичного різноманіття показав варіації кількості алелів на популяцію. Особини *P. ridibundus* демонструють більшу генетичну різноманітність порівняно з особинами *P. lessonae*, тоді як гібриди відрізняються проміжним генетичним різноманіттям. Подальші дослідження локалітетів, де було виявлено потенційних триплоїдів, є необхідними для оцінки виживаності та репродуктивного потенціалу гібридних особин, а також для визначення усіх типів гібридів і особин жаби озерної та жаби ставкової.

Ключові слова: водяні жаби, *Rrid059A*, *RICA1b5*, NewHybrids, геміклональні популяційні системи (ГПС)

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