Zoodiversity, 59(5): 443–456, 2025

DOI 10.15407/zoo2025.05.443
UDC 597.8:575.222.7:576.35

WHETHER THE PRESENCE OF DI- AND TRIPLOID HYBRIDS, *PELOPHYLAX ESCULENTUS*, INFLUENCES GAMETOGENESIS OF THEIR PARENTAL SPECIES, *P. RIDIBUNDUS* (ANURA, RANIDAE)

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urn:lsid:zoobank.org:pub:8394E288-476C-4566-AB0B-F407F75324F4

Whether the Presence of Di- and Triploid Hybrids, *Pelophylax esculentus*, Influences Gametogenesis of Their Parental Species, *P. ridibundus* (Anura, Ranidae). Pustovalova, E., Strus, V., Suriadna, N., Biriuk, O., Biriuk, I., Shabanov, D. — Meiosis ensures efficient reproduction by the formation of viable gametes with a constant number of chromosomes. However, in natural

hybrid complexes, where parental and hybrid lineages coexist, the fidelity of this process may be challenged. To better understand how this process functions in natural populations, we investigated, for the first time, the features of spermatogenesis and assessed the level of abnormal meiotic metaphases in the parental species *Pelophylax ridibundus* and *P. lessonae* of the hybridogenetic *P. esculentus* complex from 11 locations in Ukraine. Using Giemsa and/or Ag-staining, we analysed 2,203 meiotic and 471 mitotic metaphases from 41 males. Most of the analysed spermatogonia and spermatocytes had chromosome numbers consistent with those forming viable gametes. The average level of aneuploidy in *P. ridibundus* males was 17% across all hemiclonal population systems analysed, which is significantly lower than in hybrids ($p < 0.05$). Furthermore, the lack of a significant difference ($p = 0.93$) in aneuploidy frequency among *P. ridibundus* from different population systems compared to the pure R-population suggests that the spermatogenesis of *P. ridibundus* is not affected by the presence of hybrids or the other parental species (*P. lessonae*). This cytogenetic robustness may contribute to the long-term stability of mixed populations, where *P. ridibundus* serves as a consistent source of haploid gametes necessary for the reproduction of *P. esculentus* hybrids.

Key words: meiosis, chromosome, water frog, bivalent, population system.

Introduction

Meiosis is a conserved process across various eukaryotic groups (Loidl, 2016; Sato et al., 2021), essential for producing viable gametes with a stable chromosome number to ensure reproduction. In species with constant chromosome numbers, interspecies hybridisation leads to hybrid formation, promoting speciation and evolution (Mallet, 2010; Abbott et al., 2013; Stöck et al., 2021; Marta et al., 2023). In such contexts, maintaining chromosomal integrity is crucial to ensure successful gametogenesis and offspring viability. However, chromosomal aberrations, such as translocations or deletions, can result in inviable gametes or malformed progeny (Savage, 2001; Raudsepp & Chowdhary, 2016).

Pelophylax esculentus complex represents a classic example of hybridisation. The *P. esculentus* complex consists of two parental species, *P. ridibundus* (Pallas, 1771) and *P. lessonae* (Camerano, 1882), whose interbreeding produces fertile hybrids (*P. esculentus* (Linnaeus, 1758)) with variable genome compositions and ploidy levels (Berger, 1977; Günther et al., 1979). These parental species share a karyotype of 26 homomorphic chromosomes (Heppich, 1978; Bucci et al., 1990; Suryadna, 2003; Dedukh et al., 2023), highlighting their genetic compatibility and enabling the hybridisation process. In hybrids, premeiotic elimination allows for the production of clonal gametes containing specific parental genomes, a phenomenon that often excludes parental species from hybrid-focused studies (Tunner, 1973; Uzzell et al., 1976; Dedukh & Krasikova, 2021).

Hybrid females typically produce haploid gametes of one parental genome (Dedukh et al., 2015, 2017, 2020), while hybrid males show amphigameticity, producing gametes with single or multiple genomes (Vinogradov et al., 1991; Doležálková-Kaštánková et al., 2021; Pustovalova et al., 2022 a, 2024). Unlike hybrid females, males exhibit many aberrant cells in their testes (Dedukh et al., 2015; Biriuk et al., 2016; Doležálková et al., 2016; Pustovalova et al., 2022 a). Meanwhile, parental females exhibit no evidence of aberrant gametes (Dedukh et al., 2013); however, limited data exist on parental male spermatogenesis and their aneuploidy rates (Günther,

1975; Suryadnaya, 2004), which are inferred mainly from artificial crossings and progeny analyses (Graf & Polls-Pelaz, 1989).

In hemiclonal population systems (HPS), hybrids coexist with parental species, contributing to genome recombination (Graf & Polls-Pelaz, 1989; Plötner, 2005; Dufresnes & Mazepa, 2020; Shabanov et al., 2020). L-E HPSs, consisting of *P. lessonae* and *P. esculentus*, are widespread across the European part of the continent, while R-E HPSs are more common in eastern Ukraine and parts of central Europe (Borkin et al., 2004; Doležálková-Kaštánková et al., 2018; Dufresnes & Mazepa, 2020; Fedorova & Pustovalova et al., 2025). In contrast, rare L-E-R and pure hybrid (E) HPSs are found primarily in central and northern Europe (Christiansen & Reyer, 2009; Plötner, 2005; Dufresnes & Mazepa, 2020). These hybrid systems likely originated from matings between *P. ridibundus* females and *P. lessonae* males, driven by size-based mate preferences favouring such pairings (Blankenhorn, 1977; Berger et al., 1988; Hermaniuk et al., 2020). As a consequence of these hybridisation events, hybrid gametes may contribute to the high rate (~40%) of undeveloped embryos often observed in hybrid–parental crosses (Doležálková-Kaštánková et al., 2022) due to aberrancies during spermatogenesis. Thus, we hypothesise that the level of aneuploid spermatocytes in parental males (*P. ridibundus* and *P. lessonae*) depends on the composition of their local HPS, with higher aneuploidy expected in systems where hybrid individuals are present due to potential genomic interactions and introgression effects.

This study examines: 1) the rate of aneuploidy in parental males from 11 Ukrainian populations analysing spermatocytes after Giemsa and/or Ag-staining; 2) whether aneuploid spermatocyte levels in parental males depend on their HPS context.

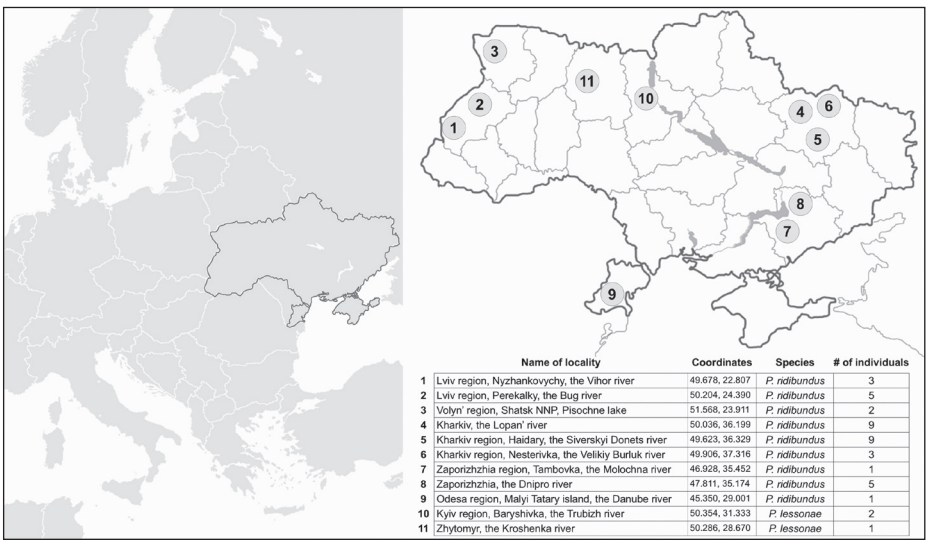
Material and Methods

Sampling and tissue fixation

Between 2000 and 2024, 38 adult *P. ridibundus* and three *P. lessonae* males were collected from 11 localities across Ukraine, covering habitats from the western Carpathian foothills to south-eastern steppe and coastal zones. Sampling regions included Lviv, Volyn', Zhytomyr, Kyiv, Kharkiv, Zaporizhzhia, and Odesa, spanning diverse river systems (e. g., Bug, Vihor, Siverskyi Donets, Dnipro, Danube) (Fig. 1; Table 1). This broad coverage reflects the ecological and population diversity of *Pelophylax* species across pure parental and hybridogenetic systems in eastern Europe.

Species were identified using morphological traits (Berger, 1977; Plötner, 2005) and further confirmed in selected individuals by DNA flow cytometry (Biriuk et al., 2016) or fluorescent *in situ* hybridisation (FISH) with species-specific probes (Dedukh et al., 2019) (Fig. 2).

Frogs were captured at night, temporarily housed under standard conditions, and fed cockroaches. Colchicine treatment (0.04%, 0.1 ml/10 g) preceded euthanasia with ethyl acetate (12–20 hours post-injection). Bone marrow, intestine, and testes were dissected for cytogenetic analysis. Tissue processing and chromosome preparation followed Pustovalova et al. (2022 b) and were approved by the Karazin National University Bioethics Committee (Approvals No. 4/16, 2016; No. 1/23, 2023), in line with ARRIVE guidelines.



	Name of locality	Coordinates	Species	# of individuals
1	Lviv region, Nyzhankovychy, the Vihor river	49.678, 22.807	*P. ridibundus*	3
2	Lviv region, Perekalky, the Bug river	50.204, 24.390	*P. ridibundus*	5
3	Volyn' region, Shatsk NNP, Pisochne lake	51.568, 23.911	*P. ridibundus*	2
4	Kharkiv, the Lopan' river	50.036, 36.199	*P. ridibundus*	9
5	Kharkiv region, Haidary, the Siverskyi Donets river	49.623, 36.329	*P. ridibundus*	9
6	Kharkiv region, Nesterivka, the Velikiy Burluk river	49.906, 37.316	*P. ridibundus*	3
7	Zaporizhzhia region, Tambovka, the Molochna river	46.928, 35.452	*P. ridibundus*	1
8	Zaporizhzhia, the Dnipro river	47.811, 35.174	*P. ridibundus*	5
9	Odesa region, Malyi Tatary island, the Danube river	45.350, 29.001	*P. ridibundus*	1
10	Kyiv region, Baryshivka, the Trubizh river	50.354, 31.333	*P. lessonae*	2
11	Zhytomyr, the Kroshenka river	50.286, 28.670	*P. lessonae*	1

Fig. 1. Localities and number of analysed *P. ridibundus* and *P. lessonae* individuals

Table 1. **Individual characteristics of each analysed water frog**

Frog ID	Locality, HPS type	Size, mm		# of analysed meiotic/ mitotic metaphases	% of meiotic metaphases with			% of mitotic metaphases with	
		Snout-vent length	Testes (left / right)		13 bivalents	13 univalents	Abnormal	26 chromosomes	13 chromosomes
1	2	3	4	5	6	7	8	9	10
				P. ridibundus					
126	Kharkiv, R-E	78.2	6.0 / 6.0	44 / 36	68	0%	32%	92%	8%
122	Kharkiv, R-E	78.2	5.5 / 6.0	85 / 26	87	1%	13%	73%	27%
125	Kharkiv, R-E	82.0	9.0 / 6.0	42 / 14	86	0%	14%	100%	0%
124	Kharkiv, R-E	78.5	6.5 / 6.5	40 / –	84	0%	16%	Not estimated	Not estimated
121	Kharkiv, R-E	84.6	5.0 / 4.0	66 / 16	91	0%	9%	63%	37%
127	Kharkiv, R-E	77.1	7.0 / 6.0	54 / –	89	0%	11%	Not estimated	Not estimated
128	Kharkiv, R-E	77.9	8.0 / 5.0	64 / –	97	0%	3%	Not estimated	Not estimated
129	Kharkiv, R-E	87.8	8.5 / 7.0	60 / –	93	0%	7%	Not estimated	Not estimated
130	Kharkiv, R-E	80.1	8.0 / 6.6	80 / –	98	0%	2%	Not estimated	Not estimated
11	Kharkiv Region, Donets, R-E*	61.7	6.0 / 6.0	30 / –	89	0%	11%	Not estimated	Not estimated
16	Kharkiv Region, Donets, R-E*	55.8	3.0 / 2.5	60 / 16	88	0%	12%	100%	0%
187	Kharkiv Region, Donets, R-E*	36.9	3.3 / 1.5	48 / 14	72	3%	28%	64%	36%
179	Kharkiv Region, Donets, R-E*	34.5	1.2 / 1.0	66 / 5	68	0%	32%	100%	0%
175	Kharkiv Region, Donets, R-E*	41.2	2.1 / 2.3	64 / 9	67	0%	33%	100%	0%
12	Kharkiv Region, Donets, R-E*	65.1	5.0 / 4.0	69 / –	89%	0%	11%	Not estimated	Not estimated

1	2	3	4	5	6	7	8	9	10
13	Kharkiv Region, Donets, R-E*	72.5	6.0 / 5.0	87 / –	96%	0%	4%	Not estimated	Not estimated
14	Kharkiv Region, Donets, R-E*	Not measured	4.0 / 4.0	85 / –	89%	0%	11%	Not estimated	Not estimated
17	Kharkiv Region, Donets, R-E*	Not measured	5.0 / 3.0	88 / –	88%	0%	12%	Not estimated	Not estimated
243	Kharkiv R., Nesterivka, R-E	Not measured	Not measured	16 / 38	44%	0%	56%	74%	12%
244	Kharkiv R., Nesterivka, R-E#	Not measured	Not measured	68 / 56	71%	10%	16%	48%	18%
245	Kharkiv R., Nesterivka, R-E#	Not measured	Not measured	14 / 19	43%	0%	50%	11%	11%
334	Lviv, Nyzhankovychy, L-E-R	86.8	7.9 / 5.1	75 / 11	80%	0%	20%	91%	9%
335	Lviv, Nyzhankovychy, L-E-R	86.4	8.1 / 7.2	86 / 20	83%	0%	17%	70%	15%
333	Lviv, Nyzhankovychy, L-E-R	92.2	7.5 / 6.2	80 / 13	96%	0%	4%	77%	23%
402	Lviv, Perekalky, R-E*	76.2	5.2 / 5.0	68 / 15	72%	0%	28%	87%	23%
401	Lviv, Perekalky, R-E*	70.9	5.0 / 5.3	58 / –	77%	0%	23%	Not estimated	Not estimated
404	Lviv, Perekalky, R-E*	68.1	4.2 / 5.0	78 / –	78%	0%	22%	Not estimated	Not estimated
405	Lviv, Perekalky, R-E*	72.7	5.3 / 4.9	91 / 16	91%	0%	9%	63%	25%
403	Lviv, Perekalky, R-E*	79.8	5.9 / 5.3	23 / 17	100%	0%	0%	47%	41%
416	Volyn', Shatsk, L-E-R	94.1	8.6 / 8.3	28 / 19	79%	1%	20%	58%	42%
417	Volyn', Shatsk, L-E-R	76.7	6.5 / 6.8	49 / 12	80%	3%	17%	50%	50%
201	Zaporizhzhia, R	Not measured	Not measured	91 / 29	77%	9%	14%	80%	10%
202	Zaporizhzhia, R	Not measured	Not measured	75 / 5	92%	0%	8%	40%	0%
203	Zaporizhzhia, R	Not measured	Not measured	93 / 13	97%	0%	3%	75%	15%
204	Zaporizhzhia, R	Not measured	Not measured	29 / 33	69%	10%	21%	79%	9%
205	Zaporizhzhia, R	Not measured	Not measured	11 / 4	27%	0%	73%	0%	0%
147	Zaporizhzhia, R	Not measured	Not measured	18 / 1	100%	0%	0%	0%	0%
151	Odesa, L-E-R	Not measured	Not measured	10 / 1	100%	0%	0%	0%	0%
				P. lessonae					
108	Kyiv, Baryshivka, L-E	Not measured	Not measured	6 / 2	100%	0%	0%	100%	0%
119	Kyiv, Baryshivka, L-E	Not measured	Not measured	1 / 11	–	–	–	100%	0%
325	Zhytomyr, L-E	Not measured	Not measured	3 / –	100%	0%	0%	Not estimated	Not estimated

Notes: Abnormal — a sum of aneuploid meiotic metaphase and metaphases with the absence of conjugation of chromosomes in bivalents; * indicates HPSs where triploid hybrids were detected; # indicates individuals exhibited minor diploid meiotic (26 bivalents) and mitotic (52 chromosomes) metaphases.

Fig. 2. Individuals identified as *Pelophylax ridibundus* (Zaporizhzhia, Dnipro River) based on their morphological traits

Giemsa and Ag-staining

Differential chromosome staining was used to detect nucleolar organiser regions (NORs; 18S and 28S rDNA sites) on mitotic chromosomes and nucleoli in interphase nuclei, facilitating ploidy determination (Sumner, 2002). AgNOR staining followed a simplified silver nitrate protocol (Macgregor & Varley, 1988; Howell & Black, 1980; Birstein, 1984), using 30% $AgNO_3$ and 20 mg/ml gelatin. Slides were incubated at +60 °C, briefly stained with 2% Giemsa, washed, and air-dried.

Fluorescent *in situ* hybridisation

FISH was performed on mitotic chromosomes (Choleva et al., 2023) from the bone marrow and intestine of eight *P. ridibundus* caught in 2024 (three from Nesterivka and five from Zaporizhzhia; Table 1, Fig. 3). Slides were treated with 0.005 % pepsin, dehydrated in an ethanol series, and air-dried. A hybridisation mixture containing 3 *ng/μL RrS*1 probe (Ragghianti et al., 1995), 50% formamide, 10 % dextran sulphate, 2× SSC, and salmon sperm DNA was denatured at +86 °C for 8 min and applied to

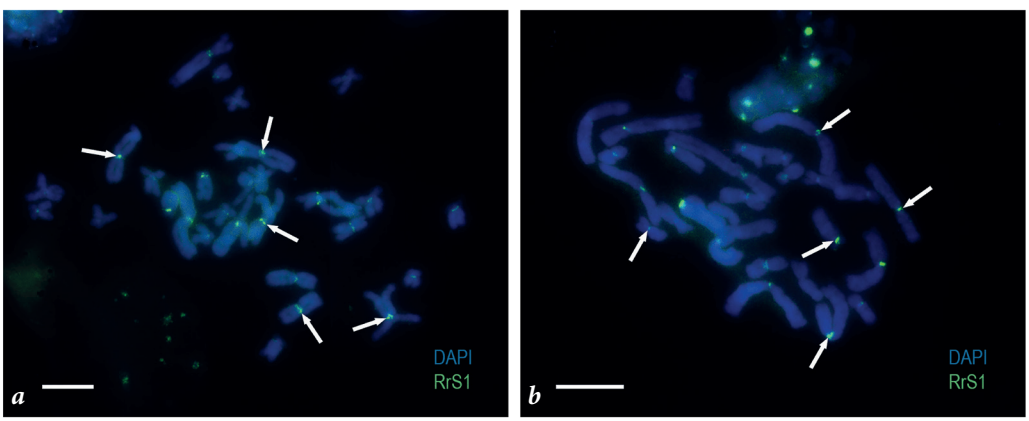


Fig. 3. Species identification using FISH with species-specific pericentromeric probe (*RrS1*) on somatic tissues from bone marrow of *P. ridibundus* individuals. *a* — male #203, *b* — male #244; arrows point on chromosomes of *P. ridibundus* labelled by green signal of *RrS1*. All frogs analysed (#243-245, 201-205) showed pericentromeric *RrS1* signals, identifying them as *P. ridibundus*. Scale bar 10 μm

slides denatured at +73 °C. Slides were incubated for 12 hours at room temperature. Biotin-labelled *RrS1* probe (Roche) was detected at +37 °C using streptavidin conjugated with Alexa 488 fluorochrome (Roche). Slides were washed in 4× SSC, dehydrated in an ethanol series, and mounted in Vectashield with 1 μg/ml DAPI (Vector, Burlingame, Calif., USA). The following primers for the *RrS1* probe were used according to Dedukh et al. (2019):

Forward: 5'-AAGCCGATTTTAGACAAGATTGC-3';
Reverse: 5'-GGCCTTTGGTTACCAAATGC-3'.

Image processing

Slides after stainings were photographed using a Leica DM2000 (DFC3000 G camera, V. N. Karazin Kharkiv National University), an Olympus BX51 (DP72 camera, State Museum of Natural History, Lviv), an Olympus Provis BX53 microscope (CCD camera DP30W, IAPG, Czech Republic), and a KONUS #5306 CAMPUS (DCM300 camera, Melitopol Institute of Ecology and Social Technologies, Kyiv). All mitotic and meiotic metaphases were imaged, manually scored for bi- and univalents, and processed using Adobe Photoshop and ImageJ. Metaphases with 13 bivalents (five large and eight small chromosomes) were classified as normal and indicative of viable gamete formation (Fig. 4, *a, b*). Cells with fewer (< 13; hypohaploid) or more (> 13; hyperhaploid) bivalents were considered aneuploid (Fig. 4, *c*). Metaphases showing disrupted chiasmata were also deemed non-viable due to likely meiotic

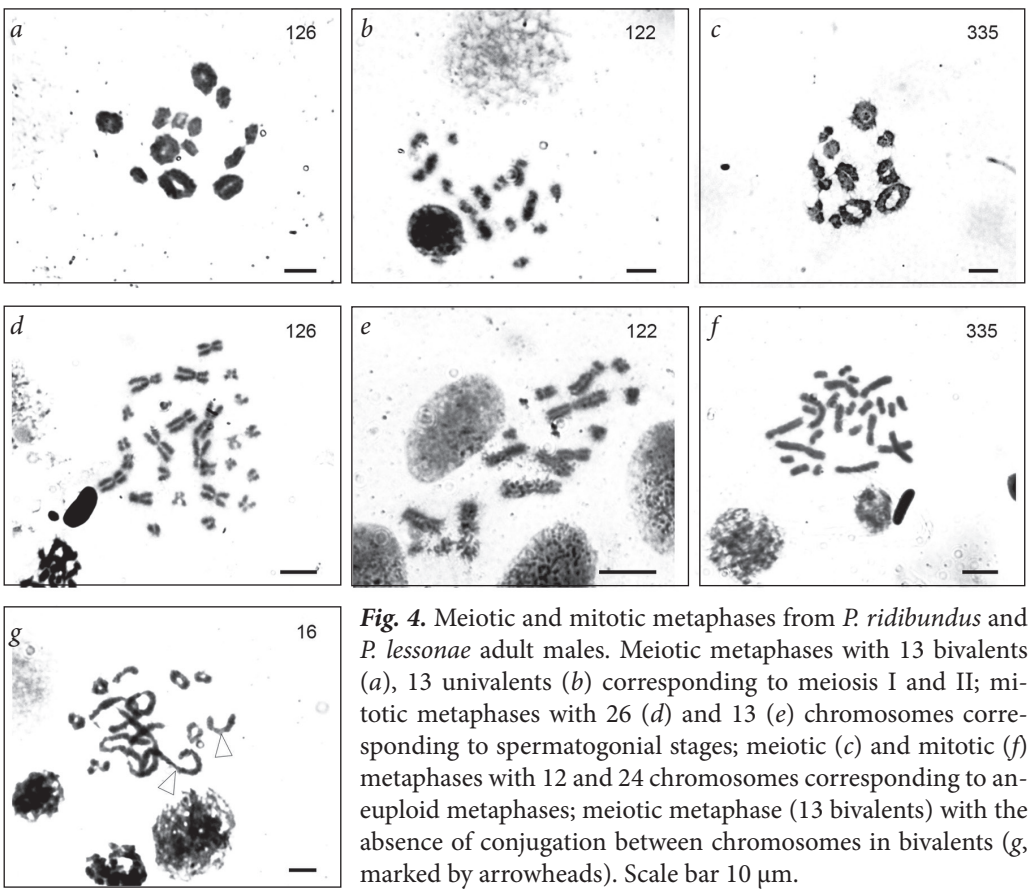


Fig. 4. Meiotic and mitotic metaphases from *P. ridibundus* and *P. lessonae* adult males. Meiotic metaphases with 13 bivalents (*a*), 13 univalents (*b*) corresponding to meiosis I and II; mitotic metaphases with 26 (*d*) and 13 (*e*) chromosomes corresponding to spermatogonial stages; meiotic (*c*) and mitotic (*f*) metaphases with 12 and 24 chromosomes corresponding to aneuploid metaphases; meiotic metaphase (13 bivalents) with the absence of conjugation between chromosomes in bivalents (*g*, marked by arrowheads). Scale bar 10 μm.

checkpoint failure (Fig. 4, *f*). Mitotic metaphases with 26 chromosomes corresponded to future meiotic metaphase I cells (Fig. 4, *d*), and those with 13 chromosomes represented metaphase II (Fig. 4, *e*). Other mitotic metaphases were classified as aneuploid and used as an approximate indicator of spermatogenic quality.

In the Kharkiv Region, 1,230 metaphases were examined from 21 *P. ridibundus* males (535 from nine individuals in Lopan', 597 from 12 in Siverskyi Donets, and 98 from Nesterivka). In Lviv, 559 metaphases were analysed from eight males (241 from Nyzhankovychy and 318 from Perekalky). Additional analyses included 77 metaphases from two males in Volyn' (Pisochne Lake) and 327 metaphases from seven males in Zaporizhzhia and Odesa (317 and 10, respectively). Notably, three metaphases with 26 bivalents were identified in two males from Nesterivka. For *P. lessonae* males from Kyiv and Zhytomyr, 10 metaphases were analysed. In 26 *P. ridibundus* and two *P. lessonae* males, a total of 471 mitotic metaphases (spermatogonial cells) were analysed: 92 from Kharkiv, 157 from the Kharkiv region (44 from Siverskyi Donets, 113 from Nesterivka), 92 from Lviv (44 from Nyzhankovychy, 48 from Perekalky), 31 from Volyn', 85 from Zaporizhzhia, one from Odesa, and 13 from Kyiv. Notably, 11 mitotic metaphases with 52 chromosomes were identified in two males from Nesterivka.

Statistical analysis

All analyses were performed using Jamovi software. The Mann–Whitney U test was used to compare median aneuploidy levels between individuals and among HPS types, as data were non-normally distributed. Differences in aneuploidy frequency and chromosomal abnormalities across regions were assessed using Chi-square (χ^2) tests. These approaches allowed us to detect significant variation in chromosomal stability among HPS contexts.

Results

Using Giemsa and/or Ag-staining, we analysed a total of 2,203 meiotic metaphases from 41 adult males, 38 *Pelophylax ridibundus* and three *P. lessonae*, sampled across nine hemiclonal population systems and two *P. ridibundus* populations in Ukraine (Fig. 1). HPS types were previously characterised based on genetic and ecological structure (Suriadna et al., 2020; Strus et al., 2023; Fedorova & Pustovalova et al., 2024). They included: 1) L–E, with diploid hybrids and *P. lessonae* (Localities 10, 11 in Fig. 1); 2) L–E–R, with diploid hybrids, *P. ridibundus* and *P. lessonae* (Localities 1, 3, 9 in Fig. 1); 3) R–E*, with di- and polyploid hybrids and *P. ridibundus* (Localities 2, 5 in Fig. 1); 4) R–E, with diploid hybrids and *P. ridibundus* (Localities 4, 6 in Fig. 1).

Testes from 28 *P. ridibundus* males were dissected, showing well-developed bilateral gonads with an average length of 5.8 mm (left) and 5.1 mm (right), without visible histological anomalies such as fibrosis, atrophy, or vacuolisation. Spermatogenic tissue was well-preserved, with mitotic and meiotic cells present in all examined samples. Ten *P. ridibundus* and three *P. lessonae* males were not measured due to preservation limits. Detailed specimen information, including locality, species ID, and testis size, is provided in Table 1.

The average frequency of aneuploid metaphases in *P. ridibundus* males was 20%, with variation across HPS types: 12% in the Kharkiv R–E HPS (Lopan'), 23% in the Kharkiv R–E HPS (Siverskyi Donets, 17%; Nesterivka, 40%), 20% in the Zaporizhzhia R population, 16% in the Perekalky R–E HPS, and 14% and 18% in the L–E–R HPSs of Nyzhankovychy and Shatsk, respectively. The most frequent form of meiotic abnormality was hypohaploidy, typically presenting as metaphase I cells with 12 bivalents and one univalent, suggesting nondisjunction or premeiotic chromosome loss.

Notably, disruptions in chiasma formation between homologous chromosomes were observed in the majority of abnormal metaphases (~90 % per individual), indicating synaptic instability or recombination failure (Fig. 4, G). These defects were especially pronounced in males from regions with known genomic introgression. In contrast, testicular sections showed abundant mitotic divisions with largely normal diploid karyotypes, supporting active spermatogenesis. Due to the low number of analysable metaphases in *P. lessonae*, aneuploidy frequency could not be reliably estimated for this species.

Discussion

In this study, we analysed abnormal meiotic metaphases in the testes of 38 *P. ridibundus* and three *P. lessonae* males using Giemsa and/or Ag-staining. The majority of meiotic and mitotic metaphases displayed chromosome numbers consistent with the formation of viable gametes, with no significant variation across individuals (Mann–Whitney U test, $p = 0.42$). Although earlier cytogenetic works (e. g., Günther, 1975) did not report aneuploidy frequencies in parental species, our findings allow a direct comparison with hybrids from the Siverskyi Donets River (Pustovalova et al., 2022 a), where aneuploidy was significantly more frequent in hybrids than in *P. ridibundus* (29% vs. 17%; χ^2, $p = 0.01$). This aligns with previous reports linking hybrid origin to gametogenic instability (Dedukh et al., 2015; Biriuk et al., 2016; Svinin et al., 2021).

Moreover, elevated embryo mortality observed in hybrid crosses compared to parental ones (Doležálková-Kaštánková et al., 2022) supports the conclusion that gametogenesis in parental species is more stable. This cytogenetic stability may contribute to the long-term persistence of parental lineages in mixed hybridogenetic population systems.

Although karyotype variability in *P. ridibundus* has been previously suggested to result from hybrid influence (Vegerina et al., 2014; Suriadna, 2023), our analysis did not reveal significant differences in aneuploidy rates between males from different hemiclonal population system types (L–E–R, R–E, R–E with triploids) and the genetically pure R-population ($p = 0.93$). This suggests that the presence of hybrids or *P. lessonae* does not compromise the meiotic chromosome integrity of *P. ridibundus*. Unfortunately, data for *P. lessonae* were limited due to its declining abundance in European population systems (Kolenda et al., 2024), precluding statistical comparison.

Spermatogonial mitotic activity was observed in 28 of 38 *P. ridibundus* males, confirming active spermatogenesis and the absence of gonadal degeneration (Ogiel-

ska & Bartmańska, 1999). However, a notable proportion of meiotic metaphases lacked proper chromosomal conjugation, likely indicating failed synapsis or impaired recombination. Such disruptions may reflect a regulatory mechanism limiting recombination, which could help preserve genome stability by preventing deleterious rearrangements. This interpretation aligns with recent observations in *P. esculentus* hybrids, where univalent formation is associated with disrupted meiosis and abnormal gametes (Pustovalova et al., 2024).

Introgression from *P. kurtmuelleri* or *P.* cf. *bedriagae* has been implicated in disrupted gametogenesis in *P. esculentus* hybrids (Svinin et al., 2021; Dufresnes et al., 2024; Pustovalova et al., 2024). Similarly, mitochondrial introgression has been associated with increased embryo mortality not only in hybrid × hybrid but also in parental × hybrid and even parental × parental crosses (Doležálková-Kaštánková et al., 2022, 2024). While these effects are well documented in hybrids, their consequences for gametogenesis in parental species remain largely unexplored. In our study, the most frequent cytogenetic abnormality observed in *P. ridibundus* males was chiasma disruption between homologous chromosomes in bivalents (Fig. 4, G), accounting for approximately 90 % of meiotic irregularities per individual. These pairing defects may reflect recombination failure linked to hidden genomic incompatibilities, potentially arising from ancient or ongoing introgression. Given the high prevalence of cryptic nuclear and mitochondrial introgression reported in eastern European *Pelophylax* populations (Dufresnes et al., 2024), further cytogenomic investigation is needed to determine whether such processes compromise gamete quality even in phenotypically pure parental individuals. Unfortunately, due to the limited number of informative metaphases, aneuploidy rates could not be reliably assessed in *P. lessonae* males, although their declining population status already suggests potential reproductive vulnerability.

Conclusion

We investigated spermatogenesis in 41 males representing both parental species of the *Pelophylax esculentus* complex. Most spermatogonia and spermatocytes exhibited chromosome numbers consistent with the production of viable gametes. Yet, despite this apparent stability, we detected a striking prevalence of meiotic cells with disrupted chromosomal conjugation in bivalents, suggesting irregular synapsis potentially driven by divergence in repetitive DNA content. These structural mismatches may reflect the underlying genomic introgression from cryptic lineages. Compared to hybrid *P. esculentus*, parental *P. ridibundus,* and likely *P. lessonae*, displayed a significantly lower incidence of aneuploid metaphases, supporting their relative meiotic stability. However, the presence of pairing anomalies in otherwise karyotypically intact individuals raises the possibility that even parental populations are not entirely insulated from introgressive influence. These findings underscore the dynamic interplay between genome integrity and gene flow in *Pelophylax* species and point to cytogenetic resilience as a key factor in maintaining reproductive competence amid natural and human-altered habitats.

Acknowledgments and funding sources. The authors would like to thank the Armed Forces of Ukraine who fight every day during the large-scale russian invasion

for free and independent Ukraine. Eleonora Pustovalova would also like to thank Dr Volodymyr Strashnuk for the inspiration to write this manuscript and Dr Dmytro Didukh for the discussion of the results obtained. Additionally, the authors would like to express their sincere gratitude to Dr Halyna Mykytynets for joint field expeditions and Dr Anna Fedorova for comprehensive assistance. The work of Eleonora Pustovalova was supported by the Academy of Sciences of the Czech Republic (grant #RRFU-22-21) and EMBO (grant #SLG-5411).

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Received 6 February 2025
Accepted 21 October 2025