


RESEARCH ARTICLE

Lack of genome elimination in adult hybrid males of the European water frog complex

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Abstract

Sexual reproduction is a hallmark of most eukaryotes, yet hybridization can lead to alternative reproductive strategies, such as hybridogenesis. During hybridogenesis, found in the European water frog (*Pelophylax esculentus*) complex, one of the parental genomes is eliminated during gametogenesis, and the other one propagates clonally to gametes. We analyzed diploid hybrid males from populations common for hybrids and *Pelophylax lessonae* (L-E systems) from the eastern part of their distribution range. All but one hybrid produced spermatocytes and spermatids with *Pelophylax ridibundus* chromosomes, suggesting premeiotic elimination of *P. lessonae* genome and endoreplication of the *P. ridibundus* genome. We also observed spermatocytes with 13 or 26 univalents, indicating alterations in genome elimination and endoreplication. By comparing spermatocytes with germ cell genome composition, we suggest that genome elimination and endoreplication do not occur in adult males. Further, we examined introgressions using mitochondrial and nuclear genetic markers and comparative genomic hybridization. Most hybrids carried *P. ridibundus* and *P. lessonae* haplogroups, but five had *Pelophylax* cf. *bedriagae* mitochondrial DNA. In one hybrid, we revealed introgression of *P. ridibundus* chromosomal segments on *P. lessonae* chromosome. Although L-E systems are generally considered as stable, our results indicate variability in hybrid male gametogenesis, which may be related to introgressive hybridization.

KEYWORDS

amphibian karyotype, chromosomal rearrangements, comparative genomic hybridization, gametogenesis, genome elimination, genome introgressions, hybridogenesis, spermatocytes

INTRODUCTION

Sexual reproduction is a universal trait of all eukaryotes. It involves the formation of reduced and recombined gametes, followed by their fusion to restore the parental genome constitution (Lenormand et al., 2016). While sexual reproduction can take different forms in various organisms, in vertebrates, it typically requires two individuals of different sexes. However, hybridization can alter the gametogenic pathways leading to various outcomes, among which hybrid sterility and genome introgression are the most common (Abbott

et al., 2013; Coyne & Orr, 2004). Another outcome of hybridization, asexual reproduction, is less understood (Stöck et al., 2021). Asexual reproduction includes clonal or hemiclinal genome transmission to new generations (Dawley & Bogart, 1989; Schön et al., 2009; Stöck et al., 2021). In clonal reproduction (parthenogenesis and gynogenesis), the maternal genome is copied to offspring without paternal contribution (Kearney et al., 2009; Lamatsch & Stöck, 2009; Neaves & Baumann, 2011). In contrast, hemiclinal reproduction (hybridogenesis) requires fertilization (Graf & Polls-Pelaz, 1989; Lamatsch & Stöck, 2009; Stöck et al., 2021). During

hemiclinal reproduction, the genome of one of the parental species is transmitted clonally across generations of hybrids (Lavanchy & Schwander, 2019). This occurs through programmed genome elimination of chromosomes from one parental species, while the genome of the other one undergoes endoreplication (Dedukh & Krasikova, 2022). Subsequent fusion with gametes containing chromosomes of the complementary parental species restores the hybrid chromosomal set (Dedukh & Krasikova, 2022; Lavanchy & Schwander, 2019). Despite progress in understanding these processes, the mechanisms of genome elimination remain to be elucidated.

Hybridogenesis has been discovered in a number of hybrids across different taxonomic groups: fishes from the genera *Poeciliopsis* (Schultz, 1969), *Squalius* (Carmona et al., 1997), *Hexagrammos* (Kimura-Kawaguchi et al., 2014), and *Hypseleotris* (Schmidt et al., 2011), frogs from the genus *Pelophylax* (Berger, 1968; Tunner, 1973), and stick insects in the genus *Bacillus* (Mantovani & Scali, 1992). In addition, hybridogenesis has been observed in triploid hybrids in *Cobitis hankugensis (siniensis)*-*Iksookimia longicorpa* (Kim & Lee, 1990), *Phoxinus eos-neogaeos* (Goddard et al., 1998), *Misgurnus anguillicaudatus* (Zhang et al., 1998), *Squalius alburnoides* (Alves et al., 2001), and *Bufo taurae* (Stöck et al., 2012). In the western Palearctic water frog group, hybridogenetic reproduction has been found pretty broadly in hybrids from at least three species complexes: *Pelophylax esculentus*, *Pelophylax hispanicus*, and *Pelophylax grafi* (Dufresnes & Mazepa, 2020; Graf & Polls-Pelaz, 1989; Plötner, 2005; Tunner, 1973). Among these hybrid complexes, *Pelophylax esculentus* is the most widespread and also includes polyploid hybrids (Dufresnes & Mazepa, 2020; Plötner, 2005). This complex includes two parental species, the pool frog *Pelophylax lessonae* (Camerano, 1882), and the marsh frog *Pelophylax ridibundus* (Pallas, 1771), as well as their natural hybrid, the edible frog *P. esculentus* (Linnaeus, 1758) (Berger, 1968). Parental species have diploid chromosome sets, whereas hybrids can be diploid, triploid, or tetraploid (Arioli, 2007; Dufresnes & Mazepa, 2020; Graf & Polls-Pelaz, 1989; Plötner, 2005). During gametogenesis in hybrids, one of the parental genomes (usually that of *P. lessonae*) is eliminated, and the remaining genome undergoes endoreplication (Heppich et al., 1982; Tunner, 1973; Tunner & Heppich, 1981). Based on the analysis of meiotic chromosome sets from testes and ovaries, basic histology, and flow cytometry, genome elimination was suggested to occur before meiosis (Bucci et al., 1990; Dedukh et al., 2015; Heppich et al., 1982; Tunner & Heppich, 1981; Vinogradov et al., 1990). Furthermore, studies of tadpole gonads revealed the presence of micronuclei involved in the selective elimination of one parental genome (Chmielewska et al., 2018; Dedukh et al., 2019, 2020; Ogielska, 1994). However, it remains unclear whether genome elimination occurs exclusively during early gametogenesis or still persists in

adult hybrids. We, therefore, aimed to determine whether natural adult hybrid males do not undergo genome elimination and endoreplication, and whether the formation of their gametes is already determined by processes that occurred during early tadpole development.

To persist, *P. esculentus* requires crosses with one of the parental species forming so-called population systems (Dufresnes & Mazepa, 2020; Graf & Polls-Pelaz, 1989; Plötner, 2005). In L-E population systems, hybrids coexist with *P. lessonae*, while in R-E systems hybrids coexist with *P. ridibundus* (Graf & Polls-Pelaz, 1989; Hoffmann et al., 2015; Plötner, 2005; Svinin et al., 2021). Some L-E and R-E population systems also include triploid or tetraploid hybrids, which affects the formation of the gametes and thus the maintenance of hybrids in such systems (Borkin et al., 2004; Hoffmann et al., 2015; Mikulíček & Kotlík, 2001; Rybacki & Berger, 2001). The majority of known R-E systems are generally considered to be unstable, with hybrids producing variable types of gametes (Biriuk et al., 2016; Doležalková-Kaštánková et al., 2018; Pustovalova et al., 2022; Uzzell et al., 1976). In contrast, L-E systems tend to be more stable, exhibiting fewer abnormalities during gametogenesis in hybrids (Plötner, 2005). Moreover, the ability of *P. esculentus* to reproduce via hybridogenesis varies considerably across its range, potentially influenced by genomic introgressions between *P. lessonae* and *P. ridibundus* and from other *Pelophylax* species into the *P. ridibundus* genome (Holsbeek & Jooris, 2010; Hotz et al., 1985; Mikulíček et al., 2014; Pustovalova et al., 2025). Therefore, we aimed to study gamete formation in diploid hybrid males from the L-E systems and to assess the potential role of genomic introgressions in their reproduction.

MATERIALS AND METHODS

Samples studied

P. esculentus ($n=16$), and *P. lessonae* ($n=1$) males were collected from four L-E systems located in the Middle Volga River drainage: the Mari El Republic (Kuguvan village, 56.7875 N, 47.7730 E, Myamikeevo village, 56.1984 N, 46.5348 E) and Mordovia Republic (Smolny settlement, 54.7211 N, 45.2839 E, and Obrezki village, 54.8337 N, 45.3746 E) of Russia (Table S1). These localities inhabited by water frog population systems contained diploid hybrids and *P. lessonae* individuals of both sexes (Svinin et al., 2021). Additionally, tissues from two *P. ridibundus* individuals from waterbodies located in the city of Yoshkar-Ola (56.6069 N, 47.9280 E), which were involved in crossing experiments in previous studies (Dedukh et al., 2019), were used for the comparative analysis of the gametogenesis. Water frogs were captured using a hydrobiological net in four water bodies during May–June 2018. All manipulations with animals were carried out following national and international guidelines.

No endangered or protected species were involved in the field studies. Techniques used for animal capture, tissue sampling, and euthanasia were designed to minimize animal suffering. Each individual was anesthetized by immersion in a 0.5% solution of 3-aminobenzoic acid ethyl ester (MS 222). All procedures were approved by the Ethics Committee for Animal Research of Saint-Petersburg State University (#131-03-3 dated 14.03.2016 and #131-04-7 dated 25.03.2019).

Molecular identification of species

A tissue sample comprising two distal phalanges of the fourth digit on the hind limb was collected from each individual. DNA was extracted using a standard salt-extraction method supplemented with proteinase K (Aljanabi & Martinez, 1997). For species identification, we used a multiplex PCR method (Ermakov et al., 2019), which is based on the separation of gene fragments of different lengths for *P. ridibundus*, *Pelophylax* cf. *bedriagae*, and *P. lessonae*. Separate PCR mixtures were prepared for mitochondrial (cytochrome c oxidase subunit I gene, COI) and nuclear (serum albumin intron-1, SAI-1) markers, ensuring equal primer concentrations in each mixture (primers and method described previously; (Ermakov et al., 2019)). PCR amplification was performed at 94°C for 30s, followed by annealing at 60°C and 62°C for SAI-1 and COI, respectively, for 30s, and an extension step at 72°C for 30s, repeated for a total of 30 cycles. Each PCR reaction mixture (12 µL) contained 30–50 ng of DNA, 0.25 µM of each primer, 0.2 mM of dNTPs, 1.5 mM of MgCl₂, 1.2 µL of 10× PCR buffer (10 mM Tris–HCl, pH 8.3, and 50 mM KCl), and 1 unit of Taq polymerase (Thermo Scientific). The PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel, followed by staining with ethidium bromide and visualization under UV light. Previous studies involving target gene sequencing have demonstrated this method to be effective for species delimitation. Although it is able to discriminate between three water frog species, this method is not sufficient to detect *Pelophylax kurtmuelleri*, whose haplotypes and alleles are rarely found in the water frogs from the Volga River basin (Miura et al., 2021; Svinin et al., 2021).

Preparation of metaphase chromosomes

Adult individuals received an intraperitoneal injection of 1 mL of a 0.3% colchicine solution (Sigma-Aldrich) 24 h before dissection. Following euthanasia with MS-222 (Sigma-Aldrich), intestinal and testes tissues were dissected. The testes were sectioned, and one part was fixed in 2% PFA in 1× PBS (phosphate-buffered saline) for 1 h and used for subsequent analysis of gonadal microanatomy. Another part, along with the intestines,

was used for cytogenetic analysis. To achieve this, the tissues were immersed in a hypotonic solution of 0.075 M KCl for 25 min and then transferred to a 3:1 fixative (3 volumes of ethanol: 1 volume of glacial acetic acid) for 30 min. After two changes of the fixative, the tissues were stored at +4°C for the preparation of metaphase chromosome spreads. A fragment of the fixed gonad tissue was suspended in 100 µL of a 70% acetic acid solution and disintegrated using two forceps. The resulting cell suspension was applied to slides preheated to 60°C. After evaporation of the solution, metaphase spreads and interphase nuclei remained on the slide.

Probe labeling and fluorescence in situ hybridization

To identify genomes of both parental species on meiotic chromosome spreads, we used fluorescent in situ hybridization (FISH) with probes to *P. ridibundus* (RrSl), (Ragghianti et al., 1995) and *P. lessonae* (PlesSat01-48) (Choleva et al., 2023) tandem repeats. Probes to RrSl and PlesSat01-48, were amplified from *P. ridibundus* (1_2017_M) and *P. lessonae* (ML_18) genomic DNA, respectively. Genomic DNA was extracted from tissue samples using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer protocol. Both probes were labeled by biotin-16-2'-deoxyuridine 5'-triphosphate (dUTP) or digoxigenin-11-dUTP by PCR (with an annealing temperature of 61°C). The following primers were used for RrSl probe labeling:

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

The following primers were used for PlesSat01-48 probe labeling:

Forward: 5'-TTTGGCTTCCAAGGGCCGGG-3'
Reverse: 5'-TGACCAAAAACGACACTCCC-3'

FISH was conducted on mitotic and meiotic metaphase chromosomes according to (Liehr et al., 2017). Prior to FISH, slides were treated with 200 ng/mL RNase A solution in 2× sodium chloride–sodium citrate buffer (SSC; 20× SSC: 3 M NaCl, 300 mM Na₃C₆H₅O₇) for 1 h at +37°C and 0.1 mg/mL pepsin solution in 0.1 M HCl for 7 min at +37°C. Chromosomal DNA was denatured in a 75°C solution of 70% formamide in 2× SSC. The slides were then treated with a series of ice-cold (–20°C) ethanol (50%, 70%, and 96%). PCR-labeled probes in a hybridization mixture (50% formamide, 10% dextran sulfate, 2× SSC, 5 ng/µL labeled probe, and a 10–50-fold excess of salmon sperm DNA) were denatured individually by heating at 86°C for 10 min in a heating block (Grant Bio PCH1, UK) or thermal cycler (C1000, BioRad, USA). After denaturation, the probe was placed on ice.

The hybridization mixture was then applied to the slides, covered with coverslips, and sealed with a rubber glue.

The slides were incubated at 37°C for 12–24 h. After hybridization, slides were washed three times in 0.2× SSC at 60°C and once in 2× SSC at 42°C with shaking. Biotin was detected by Alexa-488-conjugated avidin (# S32354, Thermo Fisher Scientific), and digoxigenin was detected with rhodamine-conjugated antidigoxigenin (# 11207750910, Merck). After washing in 4× SSC at 42°C, slides were dehydrated in an ethanol series (50%, 70%, and 96%), dried, and mounted in DABCO antifade solution (Merck) containing 1 µg/mL DAPI.

Comparative genomic hybridization

Total genomic DNA was extracted from the skeletal muscle of *P. lessonae* and *P. ridibundus* tissues using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA samples were then labeled with dUTP coupled with digoxigenin (*P. lessonae* genome, 1 µg per slide) or biotin (*P. ridibundus* genome, 1 µg per slide) by nick translation kit according to the manufacturer's protocol (07J00-001, Abbott). Nick translation was carried out at 17°C for 2.5 h. *P. ridibundus* and *P. lessonae* probes were then combined and mixed with 3.5 µg salmon sperm DNA before precipitation. The mixture was then dissolved in a hybridization solution (50% formamide, 10% dextran sulfate, 2× SSC, 0.04 M NaPO₄ buffer, 0.1% sodium dodecyl sulfate (CH₃(CH₂)₁₁OSO₃Na), Denhardt's reagent (Merck, D9905). Chromosome treatment with RNase and pepsin as well as probe and chromosome denaturation were performed similarly to the FISH section. For the hybridization of the probe and chromosomal DNA, slides were incubated at 37°C for 3 days. The slides were then washed in 0.2× SSC at 60°C with shaking. Biotin and digoxigenin detection, along with subsequent washes were performed as described for FISH procedure.

Whole-mount FISH

Whole-mount FISH was conducted on testis fragments from adult individuals using the probe for the pericentromeric repeat RrS1 of *P. ridibundus* according to (Dedukh et al., 2020). Gonadal fragments from individuals used for chromosomal spreading were also fixed in a 2% paraformaldehyde solution in 1× PBS, and stored at +4°C in 1× PBS with 0.02% sodium azide. Before the whole-mount FISH, tissues were incubated in a 0.5% solution of Triton X100 in 1× PBS for 4–5 h at room temperature (RT), followed by a 15-min wash in 1× PBS. Tissues were then impregnated with a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC for 3–4 h at 37°C. Afterward, tissues were transferred to the hybridization mixture (50% formamide, 2× SSC, 10% dextran sulfate, 20 ng/µL probe, and a 10- to 50-fold excess of salmon

sperm DNA/yeast DNA) and heated at 82°C for 15 min for DNA and probe denaturation. After hybridization for 24 h at RT the tissues were washed in three changes of 0.2× SSC at 42°C for 15 min each and blocked in 4× SSC containing 1% blocking reagent (Roche) in 4× SSC for 1 h at RT. Biotin was detected by incubation with streptavidin-AlexaFluor 488 (Invitrogen). Afterwards, the tissues were stained with DAPI (1 µg/µL) (Sigma), prepared in 1× PBS at RT overnight. Finally, tissues were transferred into a 10-µL drop of antifade solution on a slide, covered with coverslips and subjected to confocal microscopy.

Confocal laser scanning microscopy and fluorescence microscopy

Slides with chromosome spreads were analyzed by Leica DM 4000B (Leica Wetzlar GmbH, Germany) and Axio Imager Z2 microscope equipped with a CoolCube 1 black/white digital camera (MetaSystems, Altlussheim, Germany). MetaSystems platform was utilized for automated search, acquisition and image processing. IKAROS and ISIS imaging software (MetaSystems, Altlussheim, Germany) were also applied for capturing and initial image processing. Final image processing was performed in Adobe Photoshop CS6.

Analysis of the 3D morphology of the gonads was performed by laser scanning confocal microscopy using a Leica TCS SP5 based on the Leica DMI 6000 CS inverted microscope (Leica-Microsystems). Tissues were placed in a drop of DABCO antifade solution containing 1 mg/mL DAPI. Specimens were analyzed in XYZ planes using an HC PL APO 20×, 40× objectives. Diode, argon, and helium–neon lasers were used to excite the DAPI fluorescent dye, and the Alexa488 and rhodamine fluorochromes, respectively. Images were acquired using LAS AF software (Leica Microsystems, Germany). Processing and counting of the number of micronuclei and the fluorescent signals in the cells were performed using LAS AF software (Leica-Microsystems).

RESULTS

We analyzed gametogenesis in 17 adult hybrid males from European water frog L-E population systems along with two *P. lessonae* and one *P. ridibundus* males (Table S1). To do this, we applied FISH with available species-specific probes on mitotic and meiotic chromosomal spreads from the testes, followed by a detailed analysis of gonadal microanatomy and identification of genome composition and ploidy of germ cells by whole-mount FISH. Furthermore, to unravel genome rearrangements and introgressions from the genomes of other closely related species, we utilized genetic markers and comparative genomic hybridization (CGH) on the somatic tissues of nine hybrid males.

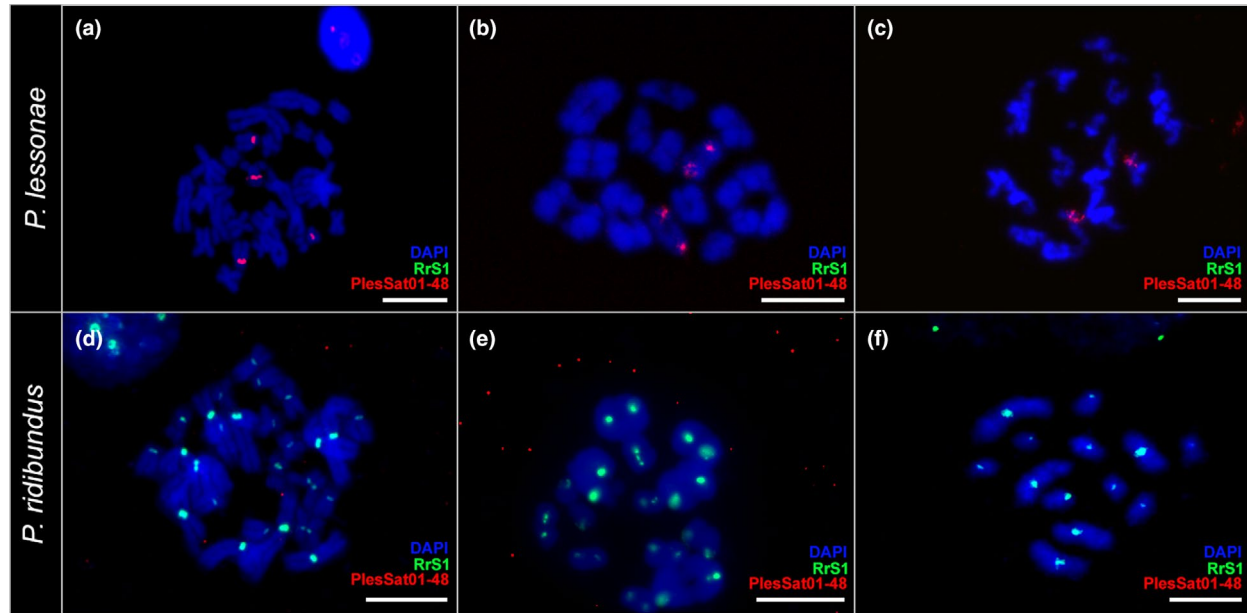


FIGURE 1 Fluorescent in situ hybridization mapping of species-specific probes to somatic (a, d) and meiotic (b, c, e, f) chromosomal spreads of *Pelophylax lessonae* (a–c) and *Pelophylax ridibundus* (d–f) individuals. Probe to *P. lessonae* species repeat, PlesSat01-48 (red), localizes in centromeric regions of two pairs of chromosomes on somatic chromosomal spreads (a), two bivalents on spermatocyte I chromosome spread with 13 bivalents (b) and two chromosomes on spermatocyte II chromosome spread with 13 univalents (c). Probe to *P. ridibundus* tandem repeat, RrS1 (green), visualizes centromeric regions of all *P. ridibundus* chromosomes in somatic chromosomal spreads (d), spermatocyte I chromosome spreads with 13 bivalents (e) and spermatocyte II chromosome spreads with 13 univalents (f). Chromosomes are counterstained with DAPI (blue). Scale bar = 10 μm .

The application of RrS1 and PlesSat01-48 probes enabled discrimination of parental species genomes in mitotic and meiotic metaphases, along with spermatids following the previous studies (Choleva et al., 2023; Raghianti et al., 1995). In mitotic metaphase *P. lessonae* chromosomes from somatic cells, we observed weak or no signals from the RrS1 probe, while signals from the PlesSat01-48 probe were clearly visible on two pairs of small chromosomes, as expected (Figure 1a). In mitotic metaphase *P. ridibundus* chromosomes, RrS1 repeat localized in centromere regions of all chromosomes, while PlesSat01-48 repeat was not detectable (Figure 1d).

Genome composition of spermatogonia, spermatocytes, and spermatids in diploid adult hybrid males from the L-E population systems

Analysis of 264 meiotic cells in *P. lessonae* and 218 meiotic cells in *P. ridibundus* males revealed only spermatocytes with 13 bivalents ($n=440$, 91%) or with 13 univalents ($n=42$, 9%; Figure 1b,c,e,f; Table S2). We assume that cells with 13 bivalents correspond to primary spermatocytes (meiosis I) and cells with 13 univalents correspond to secondary spermatocytes (meiosis II). Additional analysis of mitotic metaphase plates revealed only cells with 26 chromosomes (Table S2).

In hybrids, mitotic and meiotic chromosomal sets were first examined by FISH with the probe to RrS1 repeat and then with a combination of probes to RrS1

and PlesSat01-48 repeats to confirm the initial analysis. Somatic chromosomal sets of hybrids included 13 *P. ridibundus* and 13 *P. lessonae* chromosomes, distinguished by applying the probe to RrS1 repeat only or by combining the probes to RrS1 and PlesSat01-48 repeats (Figure 2a,j). Next, we analyzed 1556 meiotic and 796 mitotic cells from the gonads of 17 hybrid males from the studied localities (Table S2). The number of spermatocytes and their genome composition varied between hybrid individuals. In the gonads of one hybrid male (ML_8_2019), we did not find any meiotic or mitotic chromosomal spreads (Table S2). In the other 16 individuals, we found primary spermatocytes with 13 bivalents represented by *P. ridibundus* chromosomes (Figure 2d, Table S2). The presence of spermatocytes with 13 bivalents of *P. ridibundus* suggests the elimination of *P. lessonae* genome and endoreplication of the remaining *P. ridibundus* genome. Nevertheless, in five individuals, the number of spermatocytes with 13 bivalents of *P. ridibundus* was low suggesting problems in selective genome elimination and endoreplication (Table S2). We also found mitotic cells with 26 chromosomes represented exclusively by *P. ridibundus* chromosomes in all but one hybrid male (Figure 2b, Table S2). Although we cannot distinguish between somatic and germline mitotic cells, we speculate that these cells represent germline cells, as genome elimination and endoreplication do not occur in somatic cells (Bucci et al., 1990; Heppich et al., 1982; Tunner & Heppich, 1981).

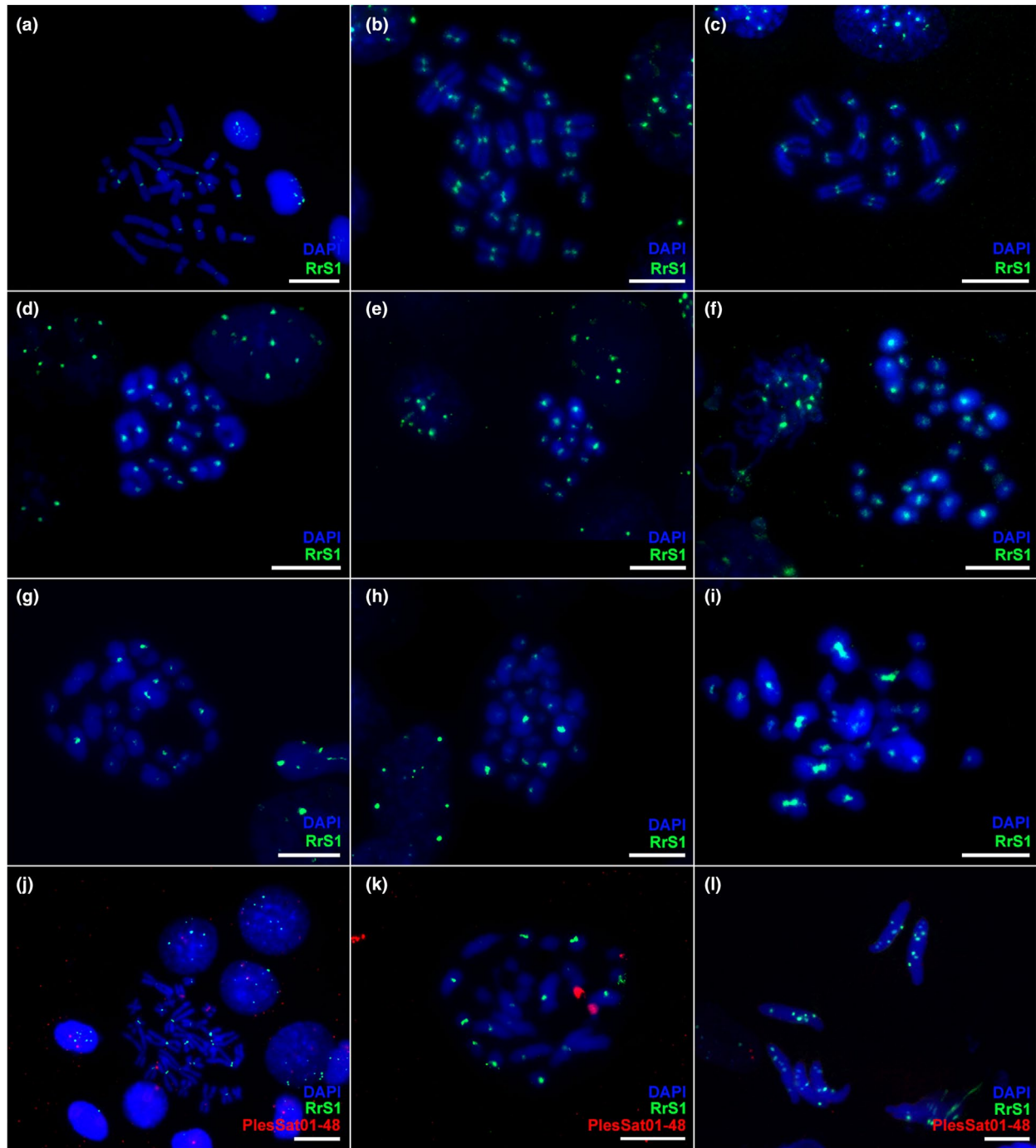


FIGURE 2 Identification of genotypes and ploidy in mitotic and meiotic spreads from hybrids by fluorescent in situ hybridization with a probe to RrS1 repeat (a–i) and a combination of probes to RrS1 and PlesSat01-48 repeats (j–l). Somatic chromosomal plate of hybrid with 13 *Pelophylax ridibundus* chromosomes having bright RrS1 signal (green) and 13 *Pelophylax lessonae* chromosomes without RrS1 signals (a). Mitotic metaphases with 26 *P. ridibundus* chromosomes (b) and 13 *P. ridibundus* (c) chromosomes from testes spreads. Spermatocyte chromosomal spreads with 13 bivalents of *P. ridibundus* chromosomes (d), 13 univalents corresponding to *P. ridibundus* chromosomes (e), 26 univalents corresponding to *P. ridibundus* chromosomes (f). Spermatocyte spreads with 26 univalents, with 13 univalents corresponding to *P. lessonae* chromosomes and 13 univalents corresponding to *P. ridibundus* chromosomes (g, h) and spread with a mixture of bivalents and univalents corresponding to *P. ridibundus* chromosomes (i). Application of two species-specific probes (PlesSat01-48: red, RrS1: green) on somatic metaphase of hybrid revealed 13 chromosomes of *P. ridibundus* and two chromosomes of *P. lessonae* (j). Meiotic chromosome spread with 26 univalents consisting of 13 univalents corresponding to *P. ridibundus* chromosomes and 13 univalents corresponding to *P. lessonae* chromosomes (k). Spermatids with exclusively *P. ridibundus* chromosomes (l). Chromosomes are counterstained with DAPI (blue). Scale bar = 10 μm .

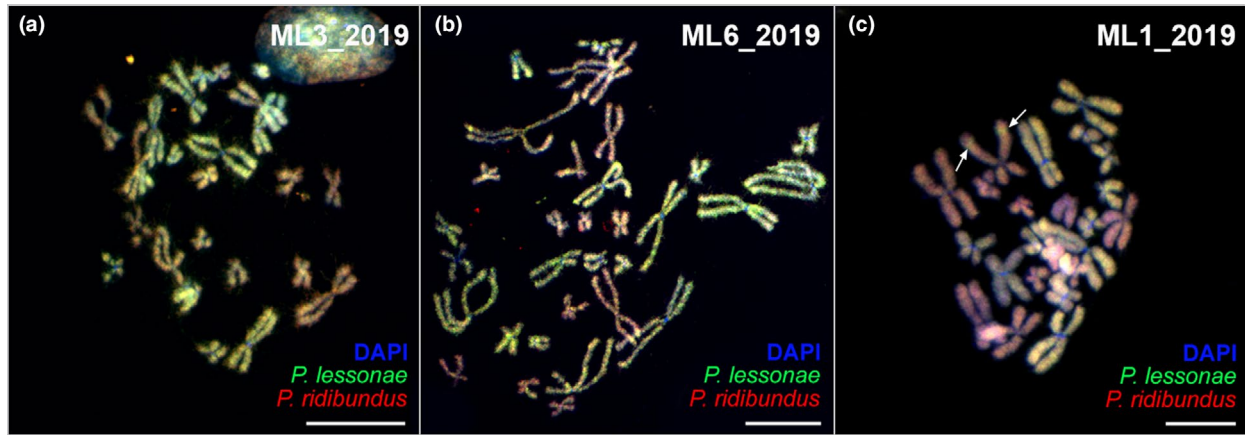


FIGURE 3 Comparative genomic hybridization on somatic chromosomal plates of three hybrid individuals with probes to *Pelophylax lessonae* (green) and *Pelophylax ridibundus* (red) genomes. After comparative genomic hybridization on somatic chromosomal spreads of hybrid individuals, 13 *P. ridibundus* and 13 *P. lessonae* chromosomes were visualized. In two individuals, no chromosomal rearrangements were observed (a, b) while in one individual (c), *P. lessonae* chromosomal segment was detected on the long arm of chromosome 3 of *P. ridibundus* (arrows). Chromosomes are counterstained with DAPI (blue). Scale bar = 10 μ m.

Spermatocytes with 13 univalents corresponding to *P. ridibundus* chromosomes were observed in 16 hybrid males (Figure 2e, Table S2). Such cells may arise during the metaphase of the second meiotic division after the normal chromosome segregation in spermatocyte I with 13 bivalents. Alternatively, such cells may represent spermatocytes I in case of the elimination of *P. lessonae* genome and the absence of genome endoreplication. We noticed that the number of spermatocytes with 13 univalents was higher in hybrids compared to parental species (Table S2). Moreover, in almost all diploid hybrids, we observed cells with 13 *P. ridibundus* chromosomes in gonadal spreads (Figure 2c, Table S2). Thus, we suggest, that in gonadal spreads of hybrid males, at least some spermatocytes originated from cells with 13 chromosomes, indicating alterations in genome endoreplication.

In eight hybrids, we observed spermatocytes with 26 univalents represented only by *P. ridibundus* chromosomes (Figure 2f, Table S2). To form such spermatocytes, both genome elimination and endoreplication occurred, but *P. ridibundus* chromosomes could not form bivalents during meiosis. In 15 hybrid males, we also observed spermatocytes with 26 univalents, including 13 univalents of *P. ridibundus* chromosomes and 13 univalents of *P. lessonae* chromosomes (Figure 2g,h,k, Table S2). The presence of such cells indicates the absence of genome elimination and endoreplication. Alternatively, such cells may originate from spermatocytes I with 26 bivalents. However, we do not consider this possibility as we did not observe any spermatocytes I with 26 bivalents. Additionally, in five hybrid males, we found 10 spermatocytes with a mixture of bivalents and univalents indicating aberrant pairing (Figure 2i, Table S2). Analysis of 551 spermatids on testis spreads performed from hybrid males revealed that they bear *P. ridibundus* chromosomes (Figure 2l).

CGH results and genome introgressions

To assess whether gametogenic alterations may be connected with genome introgressions and rearrangements between *P. lessonae* and *P. ridibundus* chromosomes, we first performed the multiplex PCR analysis for mitochondrial (COI) and nuclear (SAI-1) markers (Figure S1, Table S1). COI analysis revealed *P. ridibundus* mitochondrial DNA in two hybrids, *P. lessonae* haplotypes in nine hybrids, and *P. cf. bedriagae* haplotypes in five hybrids (Figure S1A, Table S1). SAI-1 analysis revealed *P. ridibundus* and *P. lessonae* alleles in all examined samples (Figure S1, Table S1B).

CGH with *P. lessonae* and *P. ridibundus* whole genomic probes was performed on somatic cells of nine hybrids by visualization of 13 chromosomes of *P. lessonae* and 13 chromosomes of *P. ridibundus* (Figure 3, Table S2). By analyzing at least five full metaphase plates per individual, we did not observe any detectable large-scale chromosomal rearrangements in eight out of nine individuals (Figure 3a,b; Table S2). In one hybrid individual (ML1), we detected introgression of *P. lessonae* chromosomal segments on the long arm of chromosome 3 of *P. ridibundus* (Figure 3c; Table S2).

Analysis of gonadal microanatomy revealed diploid and haploid gonial cells

Analysis of the gonadal microanatomy of nine adult males revealed distinct germ cell populations. By morphology, we distinguished gonocytes, meiotic cells, and spermatids in one *P. ridibundus* individual (Figure 4a) and seven out of eight hybrid males (Figure 4b–d). According to the nuclear morphology after DAPI staining, spermatogonia were distinguished as large cells with multiple nucleoli and less intensive chromatin staining compared to

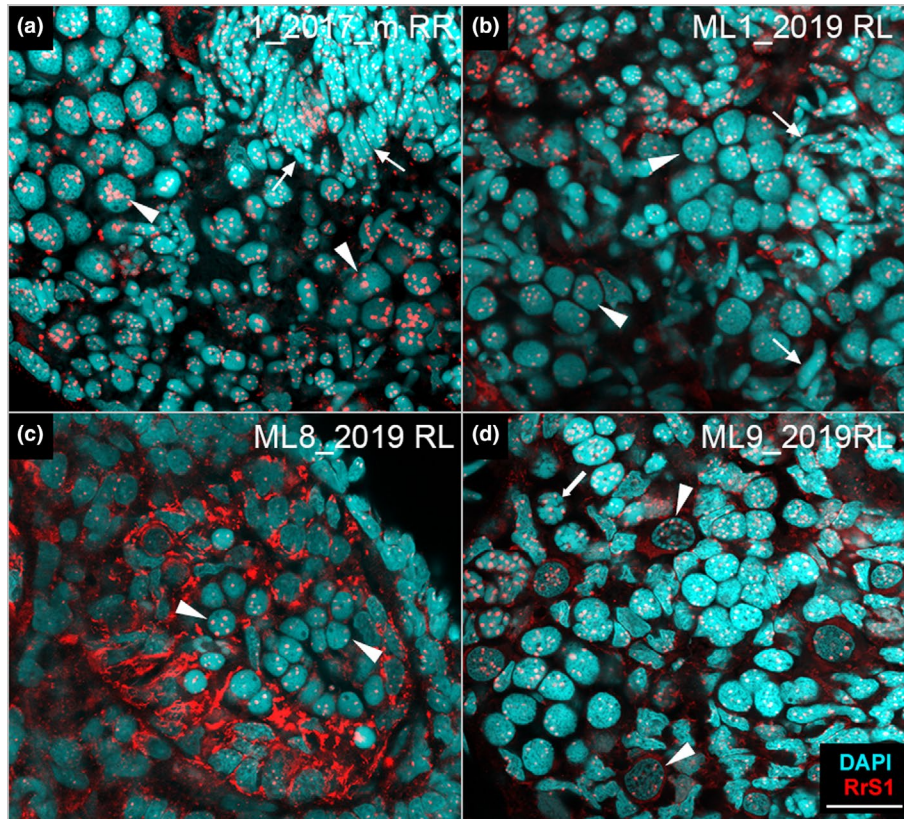


FIGURE 4 Whole mount fluorescent in situ hybridization with RrS1 probe (red) on intact gonadal fragments of *Pelophylax ridibundus* (a) and three hybrid individuals (b–d). Gonocytes (indicated by arrowheads) and spermatids (indicated by thin arrows) with *P. ridibundus* chromosomes were highly numerous in gonadal fragments from *P. ridibundus* (a) and some *Pelophylax esculentus* (b). Other hybrids have gonocytes and spermatocytes (indicated by thick arrows) but do not show any spermatids (c, d). Chromosomes are counterstained with DAPI (cyan). Scale bar = 20 μ m.

somatic cells, spermatocytes were distinguished as cells during divisions with bivalents and univalents, while spermatids were distinguished as small elongated cells with intense DAPI staining (Chmielewska et al., 2018, 2022; Dedukh et al., 2020; Ogielska et al., 2024). In two individuals (ML_8 and ML_9), no spermatids were observed, whereas germ cells and spermatocytes were present (Figure 4c,d). These results are consistent with the analysis of gonial chromosomal spreads in hybrid males studied.

Next, by applying a probe specific to the RrS1 repeat found in *P. ridibundus*, we identified the genome composition of gonocytes and meocytes in the testes of eight hybrid males (Figure 4b–d) and one *P. ridibundus* male (Figure 4a). In five hybrid males, most germ cells ($n=4517$, 60%) contained around 13 chromosomes corresponding to *P. ridibundus* (Figure 4c). *P. lessonae* chromosomes were distinguished as those that do not bear signals from the RrS1 probe. Nevertheless, we infer that at least some of those cells are haploid, given the presence of mitotic figures with 13 chromosomes and spermatocytes in meiosis I. In the other three hybrid males, gonial cells with 13 *P. ridibundus* chromosomes were also present although in smaller numbers ($n=3183$, 39%). Other gonial cells contained 26 *P. ridibundus* chromosomes suggesting

regular processes of *P. lessonae* genome elimination and *P. ridibundus* genome endoreplication.

DISCUSSION

Gametogenesis in diploid hybrid males from L-E water frog population systems

Here, we analyzed genome composition in spermatogonia, spermatocytes, and spermatids, investigated gonadal microanatomy, and examined genome introgressions by molecular and cytogenetic methods in *P. esculentus* males from the Eastern locality of their range located in the Mari El and Mordovia regions of Russia.

To address our primary aim, we analyzed gametogenesis in adult hybrids and demonstrated that the genome elimination does not occur in adult hybrid males. In spreads from gonads, we found both gonial cells and spermatogonia with similar genome composition. Furthermore, based on the analysis of gonadal microanatomy of adult hybrid males, we did not find any micronuclei, which serve as a marker for genome elimination in hybrid tadpoles (Chmielewska et al., 2018, 2022; Dedukh et al., 2019, 2020; Dudzik et al., 2025;

Ogielska, 1994) as well as other organisms with programmed DNA elimination (Dedukh et al., 2025; Dedukh, Majtánová, et al., 2024; Gernand et al., 2005; Goday & Pigozzi, 2010; Sanei et al., 2011). Similarly, in juveniles and adult diploid and triploid hybrids from laboratory crosses, genome elimination was not observed and restricted only to primordial germ cells present during early tadpole development (Chmielewska et al., 2022). Thus, we suggest that genome composition of germ cells is established only during tadpoles' development. Similarly, in hybridogenetic complexes from *Hypseleotris*, and probably *Hexagrammos*, and triploid hybrids from *Iksookimia longicorpa*–*Cobitis hankugensis* complex, genome elimination is also restricted to early developmental stages (Dedukh et al., 2025; Dedukh, Majtánová, et al., 2024; Dedukh, Marta, et al., 2024). These data fit recent suggestion that primordial germ cells exist only in early developmental stages while later replaced by spermatogonial stem cells in adults (Chmielewska et al., 2022; Ogielska et al., 2024).

Further, we analyzed gametogenesis in hybrid males and found that the majority of diploid hybrids have normal hybridogenetic reproduction. Most hybrid males produced spermatocytes with only *P. ridibundus* chromosomes, but not spermatocytes with *P. lessonae* chromosomes (Figure 5). Similarly, the analysis of spermatids revealed spermatozoa with *P. ridibundus* chromosomes only. These findings align with previous studies on hybrid males and females from the studied localities. Earlier, it was demonstrated that hybrid females and males from L-E population systems predominantly produced gametes with the *P. ridibundus* genome (Dedukh et al., 2019; Svinin et al., 2021). The formation of such gametes with the *P. ridibundus* genome enables hybrids to reproduce with the *P. lessonae* individuals, which are predominant in such localities, and thereby produce hybrid offspring. Gametes with *P. ridibundus* genomes have also been documented in hybrids from L-E systems mostly by the analysis of laboratory crosses and occasionally by cytogenetic and population genomic studies (Bucci et al., 1990; Chmielewska et al., 2022; Dubey et al., 2019; Heppich et al., 1982; Pruvost et al., 2013; Tunner & Heppich, 1981; Uzzell et al., 1980).

Surprisingly, we also observed quite high numbers of spermatocytes with different ploidy and genome composition indicating abnormalities in genome elimination and endoreplication in some hybrid males. We found gonial cells with haploid *P. ridibundus* genome in some hybrids. Since we observed no evidence of genome elimination and micronuclei formation, we suggest that at least some gonial cells remain haploid in adult hybrid males. This finding may indicate genome elimination without a subsequent round of endoreplication to restore the diploid chromosomal set (Figure 5). Interestingly, such haploid gonial cells may be likely to enter meiosis and form spermatocytes with univalents. Similarly, spermatocytes with 13 univalents were earlier observed

in hybrid water frogs from the R-E systems (Biriuk et al., 2016; Pustovalova et al., 2022, 2025), although the haploid composition of germ cells has not been established so far.

Further, we found diploid spermatocytes with genomes of both parental species that fail to conjugate and remain as univalents. Such spermatocytes suggest the absence of selective genome elimination and subsequent endoreplication of the remaining genome (Figure 5). Similarly, we detected oocytes with 26 univalents with both parental genomes in the oocytes of some hybrid females from the studied localities (Dedukh et al., 2019). Furthermore, some males exhibited a low number of spermatocytes, indicating a potential decrease in fertility and spermatozoa production. These males produced a relatively high number of spermatocytes with univalents, suggesting aberration in genome elimination and endoreplication. In one hybrid male with reduced spermatocyte counts, analysis of gonad morphology revealed a complete absence of spermatids. These findings suggest that gametogenic alterations during reproduction affect hybrid fertility. Similar disruptions, accompanied by reduced fertility, have been reported in R-E systems in Eastern Ukraine and Central Europe (Biriuk et al., 2016; Doležalková et al., 2016; Doležalková-Kaštánková et al., 2024; Pustovalova et al., 2025) and were recently observed in the hybrid male offspring emerged from laboratory crosses of hybrids from E and L-E systems (Chmielewska et al., 2022). Interestingly, aneuploidy and spermatocytes with aberrant pairing were more numerous in juveniles than in adult hybrids from the same clutch, suggesting some correction mechanisms (Chmielewska et al., 2022).

Although gametogenesis in diploid hybrids from L-E systems is generally considered more stable compared to hybrids from R-E systems, we identified some irregularities in hybrids from L-E systems that impacted their fertility. Nevertheless, studied hybrid males produced haploid gametes with *P. ridibundus* genome required for hybrid maintenance.

Genome introgressions as a potential cause of deviations in hybrid gametogenesis

To further investigate the potential causes affecting alterations in hybrid gametogenesis, we analyzed mitochondrial and nuclear genes and checked the presence of large-scale genome rearrangements and introgressions by CGH. We identified a high number of frogs with *P. cf. bedriagae* COI suggesting the mitogenomes admixture, and, possibly, admixture of nuclear genomes also. We did not reveal nuclear alleles of SAI-1 gene in the hybrids. However, previous studies have shown the introgression of *P. cf. bedriagae* SAI-1 alleles into the genetic pool of *P. ridibundus* in these localities (Fayzulín et al., 2018;

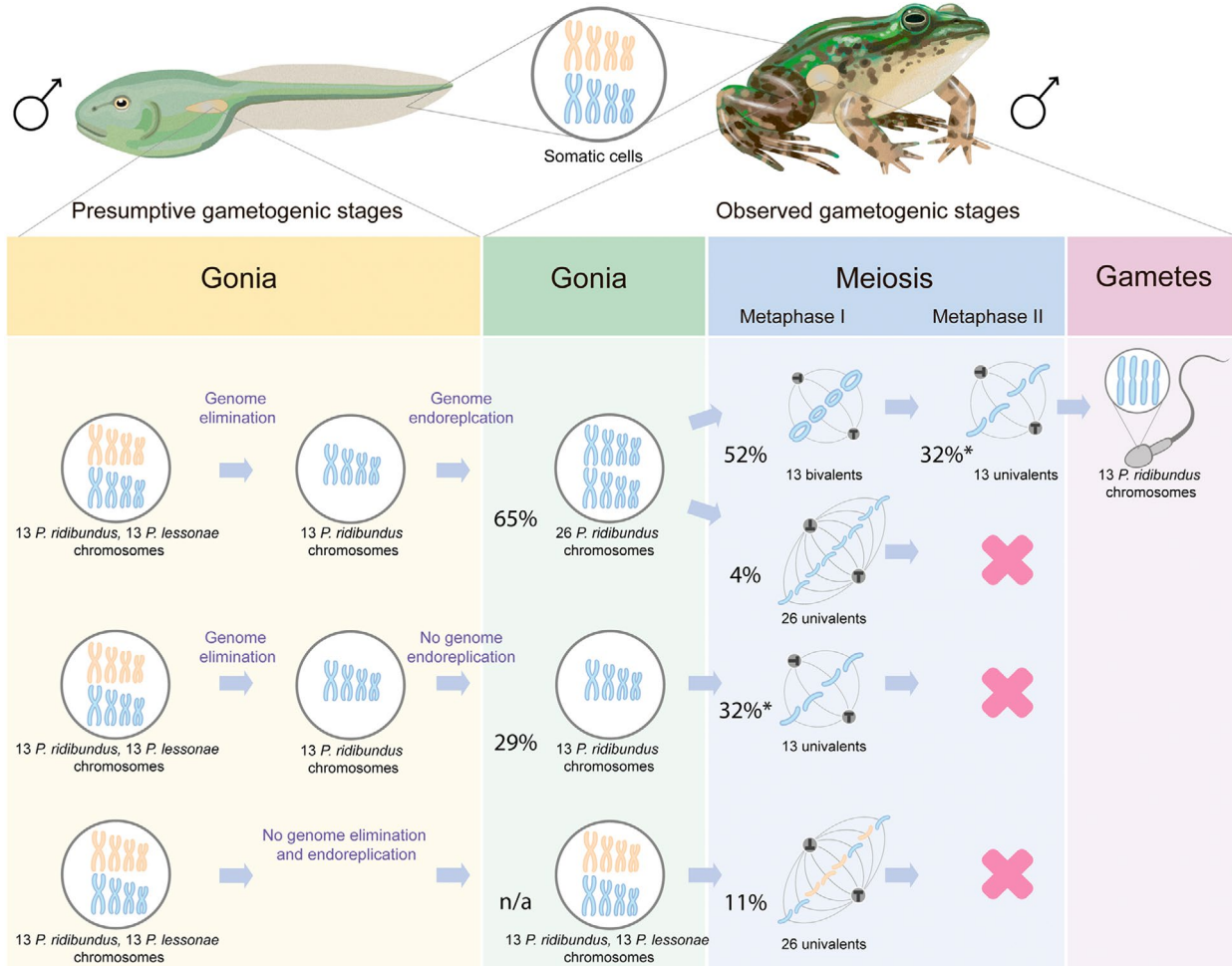


FIGURE 5 Schematic representation of observed mitotic and meiotic spreads and presumptive gametogenic stages. Germ cells retain a hybrid genome composition containing 13 *Pelophylax ridibundus* (blue) and 13 *Pelophylax lessonae* (orange) chromosomes. During tadpole development, *P. lessonae* genome is eliminated while *P. ridibundus* genome is endoreuplicated, resulting in gonial cells with 26 *P. ridibundus* chromosomes. These cells enter meiosis, forming 13 bivalents during meiosis I, followed by 13 univalents in meiosis II. After meiotic completion, such cells lead to haploid sperm carrying *P. ridibundus* genome. Nevertheless, gonial cells with 26 *P. ridibundus* chromosomes may also form 26 univalents in meiosis I. Additionally, some germ cells do not undergo premeiotic genome endoreplication after the elimination of *P. lessonae* genome resulting in cells with only 13 chromosomes, which probably form spermatocytes with 13 univalents in meiosis I. Asterisks indicate cases where meiosis I and meiosis II cannot be distinguished. In the absence of genome elimination and endoreplication, spermatocytes I containing both *P. ridibundus* and *P. lessonae* genomes were observed. We suggest that spermatocytes I with univalents and a mixture of bivalents and univalents are unable to form spermatozoa. Percentages are given according to the analysis of karyotype composition on meiotic and mitotic cell spreads (Table S2). Extremely rare cases of meiotic cells with bi- and univalents are not depicted.

Svinin et al., 2016). Additionally, SAI-1 analysis revealed intergenomic introgressions within the *P. ridibundus* clade, including *P. kurtmuelleri*, *P. cf. bedriagae*, and *P. ridibundus* species (Dubey et al., 2014; Kolenda et al., 2017; Litvinchuk et al., 2020; Plötner et al., 2015).

Such introgressions may explain the lower occurrence of hybrids as well as their decreased fertility along the Volga River basin (Dedukh et al., 2019; Litvinchuk, 2018; Litvinchuk et al., 2016; Svinin et al., 2021). Although the role of *P. cf. bedriagae* or genetic material from another species in selective genome elimination in *P. esculentus* is not well understood, intense genome introgressions and rearrangements between *P. lessonae* and *P. ridibundus*, along with unidentified water frog species, have

recently been detected in the genomes of some diploid and triploid hybrids from localities in Eastern Ukraine (Pustovalova et al., 2025). Moreover, hybrid frogs with rearranged chromosomes have demonstrated pairing between *P. lessonae* and *P. ridibundus* chromosomes but reduced fertility (Pustovalova et al., 2025). However, after CGH, we did not reveal a similar pattern of introgression of the additional genome into the genetic pool of the hybrid males studied and detectable chromosomal rearrangements. Similarly, even in cases where both genomes were present in primary spermatocytes, they remained as 13 *P. ridibundus* and 13 *P. lessonae* univalents with no evidence of pairing.

In one individual, we observed introgression of *P. lessonae* chromosomal fragments into one chromosome

of *P. ridibundus*. Such introgression suggests at least partial recombination between *P. lessonae* and *P. ridibundus* chromosomes in hybrids that have not undergone premeiotic elimination of *P. lessonae* genome. Since we observed introgression into the *P. ridibundus* genetic pool, which is normally eliminated from hybrids, this may suggest that introgressions into the *P. lessonae* genetic pool occur through hybrids. Nevertheless, such animals with genome introgressions were found rarely (current data and (Mikuliček et al., 2014; Pustovalova et al., 2025; Zalešna et al., 2011), so we might expect that clonal and hemiclinal genome transmission maintains the integrity of *P. ridibundus* and *P. lessonae* genetic material (Dedukh et al., 2023; Zalešna et al., 2011). Nevertheless, in this individual with introgression of chromosomal fragments we did not find the increase of gametogenic alterations compared to other hybrids suggesting that we did not find correlation of chromosomal rearrangements and mitochondrial genome introgressions with aberrant gametogenesis.

CONCLUSION

Our study investigates gametogenesis of diploid hybrid males from L-E population systems of the European water frog complex from most Eastern distribution range. We demonstrated that selective genome elimination and endoreplication did not occur during gametogenesis in adult hybrids. Adult hybrids maintain various genome combinations and ploidy level in gonial cells that were established during early gametogenesis. Genome elimination and endoreplication play a crucial role in maintaining hybrid fertility by ensuring the transmission of the *P. ridibundus* genome. However, irregularities in these processes observed in some hybrids may lead to spermatogenic abnormalities and reduced fertility. Hybridogenic reproduction maintains stability of parental species genomes, although some large-scale rearrangements can occasionally occur in some hybrids.

AUTHOR CONTRIBUTIONS

Conceptualization: D.D., A.K.; Data curation: A.K., D.D.; Sampling: A.S.; Investigation: S.B., Y.N., A.R., O.E.; Methodology: S.B., Y.N., A.R., O.E.; Resources: A.K., D.D.; Supervision: A.K., D.D.; Writing: original draft: D.D., S.B.; Writing: review and editing: all co-authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, Tables S1, S2, and Figure S1.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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