The high diversity of gametogenic pathways in amphispermic water frog hybrids from Eastern Ukraine

Eleonora Pustovalova[1,2,3], Lukáš Choleva[2,3], Dmytro Shabanov[1], Dmitrij Dedukh[2]

[1] Laboratory of Amphibian Population Ecology, School of Biology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine

[2] Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics of the CAS, v.v.i., Libechov, Czech Republic

[3] Department of Biology and Ecology, Faculty of Science, University of Ostrava, Ostrava, Czech Republic

Corresponding Author:
Dmitrij Dedukh[2]
89 Rumburská, Liběchov, 277 21, Czech Republic
Email address: dmitrijdedukh@gmail.com

Introduction

Meiosis is a conserved process for all eukaryotic organisms and represents a hallmark of sexual reproduction (Lenormand et al., 2016). Chromosome conjugation during meiosis relies on sufficient homology between chromosomes (McKee, 2004), whereas insufficient pairing may lead to meiotic abruption and formation of aneuploid gametes. These mechanisms keep taxa prezygotically reproductively isolated (Zong & Fan, 1989; Borodin et al., 1998; Ishishita et al., 2015; Torgasheva et al., 2016; Dedukh et al., 2020). Interspecific hybridization has both positive (Mallet, 2010; Abbott et al., 2013) and negative impacts (Arnold & Hodges, 1995; Rieseberg, 2001; Coyne et al., 2004) and plays a key role in evolution. One of the outcomes of hybridization is the creation of individuals with clonal and hemiclonal reproductive modes (Dawley & Bogart, 1989; Schön et al., 2009; Neaves & Baumann, 2011; Stöck et al., 2021). Hybrid clonal animals form gametes with a chromosomal composition identical to that of their somatic cells (Dawley & Bogart, 1989; Schön et al., 2009; Neaves & Baumann, 2011; Stöck et al., 2021). Hybrid hemiclonal animals produce unrecombined haploid gametes that require fertilization to restore diploid chromosomal sets in their offspring (Dawley & Bogart, 1989; Schön et al., 2009; Stöck et al., 2021; Dedukh & Krasikova, 2021). A switch to asexual reproduction requires significant modifications to gametogenesis, rescuing hybrids from sterility, and the creation of alternative pathways for successful reproduction. Thus, our understanding of reproductive ability and evolutionary potential of hybridization lies in our understanding of hybrid gametogenesis. Hemiclonal reproduction, also known as hybridogenesis, has been found in European water frogs of the genus *Pelophylax* (Tunner, 1974). This animal system includes two parental species: *P. lessonae* (Camerano, 1882) (LL genotype) and *P. ridibundus* (Pallas, 1771) (RR genotype), and their hybrid *P. esculentus* (Linnaeus, 1758). Hybrids can be represented in diploid (RL) and triploid (LLR, LRR) forms (Günter et al., 1979; Berger, 1983). The classical model of hybridogenetic reproduction states that one parental genome is eliminated during gametogenesis while the other is duplicated and transmitted to gametes, which appear to be clonal (Tunner, 1973; Tunner & Heppich, 1981; Tunner & Heppich-Tunner, 1991; Chmielewska et al., 2018; Doležálková-Kaštánková & Mazepa et al., 2021). Triploid hybrids usually eliminate a genome present in one copy, whereas the genome present in two copies enters meiosis and forms recombinant gametes (Günther et al., 1979; Graf & Polls-Pelaz, 1989; Ogielska, 1994; Plötner, 2005; Christiansen & Reyer, 2009; Dedukh et al., 2015; Dufresnes & Mazepa, 2020; Dedukh et al., 2020; Dedukh & Krasikova, 2021). However, the detailed principles of genome elimination and duplication during hybrid gametogenesis remain unknown.

Hybridogenetic gametogenesis makes hybrids dependent on parental species and leads to the formation of population systems where hybrids coexist with one or both parental species, or for all-hybrid populations with various ploidy and genomic compositions (Graf & Polls-Pelaz, 1989; Plötner, 2005; Christiansen & Reyer, 2009). In most of the distribution range, *P. esculentus* coexists with *P. lessonae*, creating the L-E system (Graf & Polls-Pelaz, 1989; Plötner, 2005; Pruvost et al., 2013; Svinin et al., 2013; 2021; Hoffman et al., 2015; Dufresnes & Mazepa, 2020). Here, hybrids have a typical hemiclonal gametogenesis with preferential elimination of

57 the *P. lessonae* genome, followed by the transmission of *P. ridibundus* genome to gametes
58 (Günther, 1983; Bucci et al., 1990; Pruvost et al., 2013; Dedukh et al., 2019; Svinin et al., 2021).
59 The R-E system forms hybrids mixed in populations with *P. ridibundus*. *P. esculentus* from this
60 system is specific to significant alterations in gametogenic pathways, resulting in decreased
61 fertility and increased numbers of aneuploid gametes (Uzzell et al., 1976; Günther, 1983;
62 Vinogradov et al., 1991; Borkin et al., 2004; Ragghianti et al., 2007; Doležálková et al., 2016;
63 Dedukh et al., 2015, 2017; Biriuk et al., 2016). Studies of geographic variation showed that in
64 Central Europe (Doležálková et al., 2016; Doležálková-Kaštánková et al., 2018; 2021), *P.*
65 *esculentus* is present only in a male sex, and both sexes of *P. ridibundus* coexist in Eastern
66 Europe. *P. esculentus* syntopic with *P. ridibundus* is present in both sexes and at two ploidy
67 levels (RL, RRL, and LLR) (Borkin et al., 2004; Shabanov et al., 2020).
68 Previous studies from Eastern Ukraine have shown that hybrid females frequently produce
69 haploid gametes with the R genome and diploid gametes with the RL genome, whereas gametes
70 with L genomes have never been detected (Dedukh et al., 2015, 2017). Additionally, diploid
71 hybrid males usually simultaneously produce a mixture of gametes with the L and R genomes.
72 This phenomenon, called hybrid amphispermy (Vinogradov et al., 1991), includes the
73 simultaneous formation of L and R sperms, and was first observed in Central Europe
74 (Vinogradov et al., 1991; Doležálková et al., 2016). Vinogradov and colleagues (1991) suggested
75 the existence of at least two germ cell populations that can eliminate either *P. ridibundus* or *P.*
76 *lessonae* genome during amphispermic reproduction. An alternative hypothesis proposed the
77 absence of premeiotic genome elimination and a different separation of the L and R genomes in
78 the first meiotic division (Doležálková et al., 2016).
79 In the current study, we analyzed hybridogenetic gametogenesis in Eastern Europe. Using
80 fluorescent *in situ* hybridization (FISH) with probe RrS1 specific to centromeric regions of *P.*
81 *ridibundus* chromosomes, we identified the genomes of *P. ridibundus* during metaphase of
82 meiosis I, spermatids, and mitotic spreads on chromosomal spreads from hybrid male gonads.
83 Combining these data, we tested (i) whether amphispermy is widespread gametogenesis in hybrid
84 males over R-E systems from Eastern Ukraine. Further, we tested (ii) whether premeiotic
85 genome elimination of both L and R genomes occurs in different gonial cells of amphispermic
86 males, or not.

Materials and methods

Samples

89 Sampling was conducted in Kharkiv Oblast, Eastern Ukraine, during 2016–2019. We collected
90 six adult *P. esculentus* males from the Mozh River (49.749167; 36.162778), five males from the
91 Iskiv water body (49.627778; 36.282778), and one male from the Udy River (49.968333;
92 36.136944) (Fig. S1). These geographically isolated population systems are characterized by the
93 coexistence of di- and triploid hybrids of both sexes, represented by LR, LLR, and LRR
94 genotypes, and *P. ridibundus* of both sexes. Animals were caught at night using a torch. All
95 specimens were collected outside of the protected areas within Eastern Ukraine and therefore, no
96 specific permissions were required. All animal manipulations were performed according to

97   national and international guidelines. Standard techniques for capture, tissue sampling, and
98   euthanasia were used to minimize animal suffering. Before euthanasia, each individual was
99   anesthetized by submersion in ethyl ethanoate (ETAC). All procedures were approved by the
100   Committee on Bioethics of the V. N. Karazin Kharkiv National University (minutes №4,
101   21.04.2016). The previous species and ploidy identification were determined by a complex of
102   morphological features and Ag-staining (Birstain, 1984) with some modification and further
103   confirmed within the preparation of somatic tissue chromosomes followed by fluorescent in *situ*
104   hybridization (FISH) with species-specificity (Ragghianti et al., 1995; Dedukh et al. 2015, 2017).
105   **Preparation of mitotic and meiotic chromosomes**
106   Before euthanasia in ETAC, each frog was injected with ☐0.05% colchicine for 12 h. The
107   intestines and testes were dissected, cleaned, and treated hypothonically (0.07M KCl) for 20 min.
108   The tissues were transferred to Carnoy's fixative (3:1 methanol: glacial acetic acid), and the
109   solution was changed thrice. To prepare chromosomal spreads, the tissue fragments were
110   transferred to 70% acetic acid solution for maceration in a suspension of cells and dropped onto
111   slides pre-heated to 60 °C (Biriuk et al., 2016). The chromosomal and cell nuclei spreads were
112   dried on a heating table at 60 °C for 1 h.
113   **Fluorescent in *situ* hybridization**
114   Male gametogenesis was further analyzed using the FISH method on mitotic and meiotic
115   chromosomes, following Dedukh et al. (2015, 2017). The slides were treated with RNAse (100–
116   200 µg/ml) for 1 h and pepsin D (0.005%, diluted in 0.01 N HCl) for 3 min. The probe was
117   labelled with biotin l from the genomic DNA of *P. ridibundus* by PCR using the following
118   primers to RrS1 centromeric repeat:5`-AAGCCGATTTTAGACAAGATTGC-3`; 5`-
119   GGCCTTTGGTTACCAAATGC-3`. The probe was added to the hybridization mixture (50%
120   formamide, 1 µl 2xSSC and tRNA, 10% dextran sulphate, 1.5 µl labelled probe). Slides
121   containing mitotic and meiotic chromosomes were denatured at 77 °C for 3 min and incubated at
122   room temperature for 12–18 h. The slides were then washed thrice in 0.2xSSC at 60 °C. Biotin
123   was detected using avidin conjugated with the fluorochrome Alexa 488 or Cy3. After washing in
124   4xSSC slides, they were dehydrated in an ethanol series, air-dried, and mounted in DABCO
125   antifade solution containing 1 µg/ml DAPI.
126   **Image Processing**
127   Mitotic and meiotic chromosomes were inspected after FISH using Provis AX70 Olympus
128   microscopes and Leica DM 2000 equipped with standard fluorescence filter sets.
129   Microphotographs of chromosomes were captured with a CCD camera (DP30W Olympus) using
130   Olympus Acquisition Software and a Leica DFC3000 G camera using Leica LASX Software.
131   Microphotographs were adjusted and arranged in the Adobe Photoshop CS6 software. FISH-
132   based mapping of RrS1 pericentromeric repeats visualizes the centromeric regions of *P.*
133   *ridibundus* chromosomes (Ragghianti et al., 1995), but cannot identify *P. lessonae* genome
134   during interphase. The analysis allowed us to discriminate different gametogenic stages, as we
135   identified the presence of *P. ridibundus* genome in mitotic (from both somatic and germ cells)
136   and meiotic chromosome plates as well as in the nuclei of somatic and germ cells and spermatids

137   (Table S1). Interphase cells and spermatids with 5–13 signals were discriminated as cells with *P.*
138   *ridibundus* genome. Among these signals five were usually bright and clearly distinguishable
139   while remaining eight signals were either weak or absent. Cells with 1-4 signals were not taken
140   into account. Five signals observed in interphase cells and spermatids corresponded to the
141   haploid *P. ridibundus* chromosomal set, where we observed five bright signals on all large
142   chromosomes and one small chromosome while signals on the other chromosomes were either
143   weak or absent. Ragghianti and co-authors (1995, 2007) observed six signals in interphase cells
144   of diploid hybrids. Interestingly, Dedukh and co-authors (2019, 2020) detected 13 signals in a
145   haploid set of *P. ridibundus* chromosomes, while they also found a difference in the signal
146   intensity. A signal variation and polymorphism of the studied pericentromeric repeat may explain
147   technical differences in laboratory protocol used, the source of genomic DNA used for probe
148   preparation or the interpopulation polymorphism.

149   **Results**

150   The two geographically isolated populations of *P. esculentus* were characterized by the
151   coexistence of diploid and polyploid hybrids. Here, we used FISH with the RrS1 probe to
152   identify the genome composition of interphase nuclei, spermatids, and meiotic and mitotic
153   chromosomal plates obtained from the testes of 11 diploid *P. esculentus* males. The hybrid testes
154   were round in shape without any visible anomalies. In nine males, the left testis was larger than
155   the right (left mean 5.8 mm; right mean 4.1 mm) and two males had testes of equal sizes (frogs'
156   ID: 19I-60, 19I-62) (Table S2). Testes size difference is common in *P. esculentus* and might be
157   accompanied by decreased fertility (Berger, 1970; Ogielska & Bartmańska, 1999). Data from a
158   single male from the Udy River (17U-4.2) were insufficient to evaluate hybrid gametogenesis in
159   this locality. Raw data on the number of each type of gametes produced by this male are
160   presented in Supplementary Table S1.

161   **Gametogenesis in diploid hybrid males in Mozh River**

162   Analysis of 436 interphase nuclei from four diploid hybrid males (17T-5, 17T-10, 18T-8, 18T-7)
163   showed the presence of interphase nuclei with 3–18 signals (Fig. 1D, E, G, H, J) along with
164   interphase nuclei without signals (Fig. 1H). Interphase nuclei without signals were those with
165   exclusive content of *P. lessonae* chromosomes. Nuclei with 5–13 signals contained at least a
166   haploid set of *P. ridibundus* chromosomes, whereas nuclei with more than 13 signals contained
167   an aneuploid or diploid chromosomal set of *P. ridibundus*. The analysis of 79 metaphase plates
168   during mitosis showed 0–24 signals, among which most metaphase plates had 12–13 signals
169   (Fig. 1E). These results fit well with the interphase nuclei analysis, suggesting at least three cell
170   populations: cells with 26 *P. lessonae* chromosomes, cells with 13 *P. ridibundus* and 13 *P.*
171   *lessonae* chromosomes, and cells with 26 *P. ridibundus* chromosomes. Distinguishing germ cells
172   from somatic cells is difficult. However, as genome elimination and endoreplication occur only
173   in germ cells, we considered cells with *P. lessonae* chromosomes as germ cells. During meiosis
174   I, we observed spermatocytes with 13 bivalents of *P. ridibundus* and spermatocytes with 13
175   bivalents of *P. lessonae* in all four males analyzed (Fig. 1F, G). In two of these males (18T-7,
176   17T-10), bivalents with *P. ridibundus* chromosomes dominated (87% and 77%). During meiosis

177   II, we detected spermatocytes with 13 univalents of *P. ridibundus* chromosomes (Fig. 1H) and

178   13 univalents of *P. lessonae* chromosomes (Fig. 1I). Additionally, we observed many cells with

179   aberrant pairing in all analyzed males. The observed hybrids potentially eliminated different

180   genomes in different cells premeiotically, or had some problems with selective elimination. We

181   detected spermatids in which the signal of *P. ridibundus* probe varied from 0 to 12, suggesting

182   the presence of spermatids in *P. lessonae* and *P. ridibundus* genomes (Fig. 1D, J). These males

183   transmitted two parental genomes in their cells simultaneously, i.e., they were amphigametic.

184   Fifty-four examined interphase cells of one male (18-T6) had at least five signals, indicating the

185   presence of the haploid *P. ridibundus* genome (Fig. 1C). The analysis of 14 mitotic chromosomal

186   plates showed 8 plates with 26 chromosomes, of which 13 belonged to *P. ridibundus* and 13 to

187   *P. lessonae*, the other 6 mitotic chromosomal plates were aneuploid. During the analysis of 32

188   metaphases of meiosis I, we detected 13 bivalents of *P. ridibundus* (Fig. 1A). We also detected

189   five metaphases of meiosis II with 13 univalents of *P. ridibundus* (Fig. 1B). In addition, 24

190   aneuploid chromosomal plates (Fig. 1C) were observed. The analyzed spermatids (n=48)

191   exclusively exhibited the presence of *P. ridibundus* chromosomes. We suggest that during

192   gametogenesis in this male, the genome of *P. lessonae* was premeiotically eliminated, followed

193   by endoreplication of the *P. ridibundus* genome.

194   In one individual (17T-8), we observed interphase nuclei with 3–26 signals (Fig. 2A, B, D).

195   Haploid *P. ridibundus* genome was suggested in cells with 5-13 signals; diploid *P. ridibundus*

196   genome was suggested in cells with 15-26 signals. The analysis of 14 mitotic chromosomal

197   plates from this individual showed 3 mitotic chromosomal plates with approximately 52

198   chromosomes, including chromosomes exclusive to *P. ridibundus* (Fig. 2B) and chromosomes

199   exclusive to *P. lessonae* (Fig. 2C). In 8 metaphase plates, we observed 26 chromosomes

200   exclusive to *P. ridibundus* (Fig. 2D) as well as both *P. ridibundus* and *P. lessonae* chromosomes

201   (Fig. 2I). In meiosis I, we detected chromosomal plates with 13 tetravalents of *P. ridibundus* and

202   metaphase plates with 13 tetravalents of *P. lessonae* (Fig. 2G) (23% of the total amount). One of

203   the genomes was eliminated to form spermatocytes with genome-specific tetravalents, whereas

204   the other underwent two rounds of genome endoreplication. We also found metaphase plates of

205   meiosis I with approximately 13 tetravalents, including 26 chromosomes of *P. ridibundus* and 26

206   chromosomes of *P. lessonae* (Fig. 2C, F). Spermatids of this male had 3–19 signals, suggesting

207   the presence of two *P. ridibundus* genomes at least in some spermatids (Fig. 2F-H). This pattern

208   also supports the amphigametic production.

209   **Gametogenesis in diploid hybrid males in Iskiv pond**

210   Analysis of interphase nuclei of one male (19I-60) revealed both interphase cells without signals

211   and those with RrS1 signals (Fig. S2J). Some cells had, therefore, chromosomes exclusive to *P.*

212   *lessonae*, and some cells had at least one haploid genome of *P. ridibundus*. Mitotic metaphase

213   plates of this individual were represented by 26 chromosomes, with 13 *P. ridibundus*

214   chromosomes, 13 *P. lessonae* chromosomes, and 26 chromosomes exclusive to *P. ridibundus*

215   (Fig. S2J). Our metaphase inspection of meiosis I clearly distinguished 13 *P. ridibundus*

216   bivalents (Fig. S2K-L). To form such spermatocytes, *P. lessonae* genome must have been

217 premeiotically eliminated, whereas *P. ridibundus* genome was endoreplicated. Additional
218 aneuploid cells (n=30) suggest aberrant genome elimination and endoreplication. The analysis of
219 spermatids (n=29) revealed that most spermatids had *P. lessonae* genome, and only a few
220 spermatids had *P. ridibundus* genome (Fig. S2L). Though we observed both interphase nuclei
221 and spermatids exclusively in the *P. lessonae* genome, we did not detect meiotic plates with *P.*
222 *lessonae* bivalents. Therefore, we suggest that spermatocytes with *P. lessonae* must be present in
223 this individual, i. e. the individual was amphispermic with the prevalence of L-gametes.
224 The analysis of interphase nuclei (n=307) from two males (19I-62 and 18I-90) showed some
225 interphase nuclei only in *P. lessonae* chromosomes and others in *P. ridibundus* chromosomes
226 (Fig. S2A-C). During the analysis of mitotic metaphases (n=44), we detected metaphase plates
227 with 26 chromosomes, including 13 *P. ridibundus* and 13 *P. lessonae* chromosomes (Fig. S2B).
228 Most spermatocytes had 13 bivalents of *P. ridibundus* (Fig. S2C) while only a few spermatocytes
229 had 13 *P. lessonae* bivalents. We detected 58 aneuploid chromosome plates in both males (Fig.
230 S2D). In meiosis II, we observed spermatocytes with 13 univalent *P. ridibundus* and 13
231 univalent *P. lessonae* (Fig. S2A). In spermatids (n=114), we found those with *P. ridibundus*
232 chromosomes and exclusive *P. lessonae* chromosomes (Fig. S2B), supporting the pattern of
233 amphigametic production.
234 Analysis of interphase nuclei (n=110) in two other males (18I-91 and 19I-61) revealed nuclei
235 exclusively with *P. lessonae* chromosomes and nuclei with *P. ridibundus* chromosomes (Fig.
236 S2E-G, I). During the analysis of mitotic metaphases (n=13) obtained from the other male (19I-
237 61), we found metaphase plates with 26 chromosomes, among which 13 chromosomes were
238 from *P. lessonae* and 13 were from *P. ridibundus* (Fig. S2E), while mitotic chromosomal plates
239 were not detected in one of the males (18I-91). Both males simultaneously produced
240 spermatocytes with 13 *P. ridibundus* bivalents (Fig. S2F) and 13 *P. lessonae* bivalents. During
241 meiosis II, we detected spermatocytes with 13 *P. lessonae* univalents (Fig. S2H, I) and with 13
242 *P. ridibundus* univalents (Fig. S2G). In spermatids, the number of signals was varied from 0 to
243 13. Spermatids with no signal were considered as bearing *P. lessonae* genome (Fig. S2I);
244 spermatids with 5-13 were considered as bearing *P. ridibundus* genome (Fig. S2F-H). These two
245 males (18I-91, 19I-61) potentially eliminated different genomes in different cells premeiotically
246 and transmitted the two genomes in their cells, thus being amphigametic.

247 **Discussion**

248 **Diverse spermatogenesis in diploid hybrids**

249 Our study of hybrid *P. esculentus* males from Eastern Ukrainian populations revealed diverse
250 gamete formation (Fig. 3, Fig. S3, Table S1). Nine out of eleven males simultaneously produced
251 two types of haploid gametes with parental chromosomes (amphispermic male, Fig. 4, Pathway
252 III), one with *P. lessonae* genome and one with *P. ridibundus* genome, free of recombination and
253 crossover between the genomes of parental species. A single male represented the second type of
254 spermatogenesis-producing spermatid with *P. ridibundus* genome only (Fig. 3B, Table S1). We
255 also found a male suspected to form diploid sperm based on sperm analysis and tetravalent
256 observations during meiosis, which corresponded to the third type of spermatogenesis (Fig. 3B,

257   D). The simultaneous production of fertile gametes with *P. lessonae* and *P. ridibundus* genomes
258   (amphispermy) was determined using DNA flow cytometry in the Iskiv pond population (Biriuk
259   et al., 2016) and from artificial crosses in the Mozh River (Mazepa et al., 2018). By analyzing
260   the process of gametogenesis in detail, we provide clear pathways on the mechanisms of the
261   origins of diverse gametes in these tetrapod animals.
262   Inspecting meiosis, we revealed spermatocytes with 13 univalents or bivalents of *P. ridibundus*
263   (39% for Mozh, 47% for Iskiv, 43% for both) as well as 13 univalents or bivalents of *P. lessonae*
264   (32% for Mozh, 20% for Iskiv, 26% for both) (Fig. S3A). Interphase nuclei and mitotic
265   chromosomes from testis cell suspensions often bear either *P. ridibundus* or *P. lessonae*
266   chromosomes (Fig. 3A, C). The methodology used cannot distinguish whether interphase nuclei
267   and metaphase chromosomes belong to germ cells or somatic cells. However, as genome
268   elimination and endoreplication occur only in the germ cells, we considered the observed cells as
269   germ cells. As we detected germ cells and spermatocytes bearing only *P. ridibundus* or *P.*
270   *lessonae* chromosomes, we suggest that genome elimination and endoreplication occurred in
271   germ cells before meiosis (Fig. 4, Way III). A phenomenon of premeiotic genome elimination
272   has been described earlier in water frog hybrids during tadpole development and causes the
273   classical formation of a single gamete type (Tunner & Heppich-Tunner, 1991; Ogielska, 1994;
274   Dedukh et al., 2017; 2019; 2020; Chmielewska et al., 2018). The presence of cells with only *P.*
275   *ridibundus* and *P. lessonae* genomes indicated the existence of at least two cell population types
276   eliminating different parental genomes, even in a single individual, as proposed by Vinogradov
277   et al. (1991). Comparative genomic hybridization on Central-European amphispermic males has
278   revealed meiotic metaphase I with univalent and bivalent-like configurations, including bivalent-
279   like configurations between the two parental genomes (Doležálková et al., 2016). Based on these
280   observations, Doležálková et al. proposed a hypothesis in which premeiotic elimination would be
281   absent in these cases, followed by segregation of *P. ridibundus* and *P. lessonae* chromosomes
282   during meiosis I. Diploid hybrid males from Eastern Europe likely do not use this hypothetical
283   strategy, as evidenced by our observation of premeiotic genome elimination followed by genome
284   duplication in different germ cell populations (Fig. 4). However, it should be noted that bivalent-
285   like configurations between the two different parental genomes were not observed in our males.
286   The presence of aneuploid cells during meiosis (on average 25% for Mozh, 33% for Iskiv, 29%
287   for both) indicates problems with genome elimination and/or endoreplication (Fig. 4, Way V).
288   Aneuploid meiocytes and meiocytes with unusual pairings were detected earlier in both hybrid
289   females (Dedukh et al., 2015, 2017) and males (Biriuk et al., 2016) from the same locality and
290   generally in various population types (Heppich et al., 1982; Bucci et al., 1990; Christiansen et
291   al., 2005; Christiansen, 2009; Christiansen & Reyer, 2009; Dedukh et al., 2019). It should be
292   noted that aberrations were highly numerous in hybrid frogs from a mixed population of *P.*
293   *ridibundus*, suggesting difficulties in genome elimination and duplication during hybrid
294   gametogenesis (Uzzell et al., 1976; Ragghianti et al., 2007; Doležálková et al., 2016; Dedukh et
295   al., 2015; 2017; Biriuk et al., 2016).
296   A single hybrid male produced spermatocytes with 13 tetravalents of *P. ridibundus* and 13

297   tetravalents of *P. lessonae*, indicating that it underwent an additional round of genome
298   duplication (Fig. 3B). To form spermatocytes with 13 tetravalents of *P. ridibundus*, the cells
299   must first eliminate *P. lessonae* chromosomes, followed by two rounds of duplication of *P.*
300   *ridibundus* chromosomes, and vice versa for *P. lessonae* tetravalents (Fig. 4, Way IV).
301   Additional detection of spermatocytes with 13 tetravalents during meiosis I with both genomes
302   of the parental species suggests the absence of genome elimination and two rounds of genome
303   endoreplication. Interphase cells with 26 *P. ridibundus* chromosomes (Fig. 2A) resembled the
304   results obtained for the diploid hybrid males with metaphase plates and tetravalents (Ragghianti
305   et al., 2007). Similar observations were made by Dedukh et al. (2015) during lampbrush
306   chromosome analysis, where the authors found one hybrid female with 26 *P. ridibundus*
307   bivalents. In addition, such a pattern supports the presence of two rounds of genome
308   endoreplication preceding meiosis after the elimination of one of the parental genomes.
309   Chromosomal plates with tetravalents are typically formed in autopolyploid frogs of the
310   *Pleuroderma* genus (Salas et al., 2014). Nevertheless, in these species, bi-, tetra-, and octavalents
311   were also detected among metaphase plates, suggesting some pairing inaccuracies (Salas et al.,
312   2014). Bi and Bogart (2010) showed the presence of quadrivalents (the same as tetravalents) in
313   *Ambystoma* hybrid females by investigating lampbrush chromosomes, suggesting occasional
314   synapses between homologous chromosomal regions. Nevertheless, such oocytes are a rare
315   phenomenon in *Ambystoma* (Bi & Bogart, 2010), while in water frogs, we provide frequent
316   observations with numbers of spermatocytes with tetravalents varying in their genome
317   composition. We hypothesized that these cells could proceed through meiosis and form diploid
318   sperm with the LL, RL, and RR genomes (Fig. 4, Way IV). Such gametes may lead to the
319   emergence of triploid frogs (approximately 5%) observed in the Mozh Basin (Drohvalenko et al.,
320   2022). However, the fertilization success of diploid sperms to compete with haploid sperms
321   requires further investigation.
322   As not only hybrid males but hybrid females (Dedukh et al., 2015, 2017; Christiansen et al.,
323   2009; Christiansen and Reyer, 2009; Pruvost et al., 2013) can also produce gametes of both
324   parental species, Dubey et al. (2019) called this phenomenon as 'amphigamy.' However, this
325   term has following interpretations according to Rieger et al. (1991): (1) the fusion of two sex
326   cells and the formation of conjugated pairs of nuclei (dikaryophase). If a. immediately follows
327   karyogamy, the process is referred to as amphimixis (Renner, 1916); and (2) the normal
328   fertilization process (Battaglia, 1947). Therefore, we considered correcting the term to
329   'amphigameticity' to indicate the ability of interspecific hybrid males and females to produce
330   gametes of both parental species.
331   **The gain and loss during diverse gamete formation**
332   To establish successful hemiclonal genome propagation, hybrid organisms must adapt
333   gametogenesis accordingly. The F1 hybrids of *P. ridibundus* and *P. lessonae* showed premeiotic
334   genome elimination and endoreplication, rescuing their fertility (Tunner & Heppich-Tunner,
335   1991; Dedukh et al., 2019). However, premeiotic genome elimination and endoreplication do not
336   occur in all populations of germ cells, causing unusual pairing in meiosis and abruption of

337   gamete formation, thereby decreasing fertility in otherwise vital individuals (Vorburger et al.,
338   1991; Dedukh et al., 2015, 2019, 2020; Doležálková et al., 2016). Reported cases of genome
339   elimination and/or endoreplication failure cause the formation of aneuploid cells during mitosis
340   and meiosis (Fig. 3, Fig. S3). Not all changes in genome elimination and endoreplication
341   machinery harm the reproduction of hybrid frogs. At least one hybrid male from Eastern Ukraine
342   potentially produced diploid spermatozoa with LL, RL, and RR genomes. The formation of
343   diploid gametes is crucial for the emergence of triploid hybrids in some population systems
344   (Tunner & Heppich-Tunner, 1992; Brychta & Tunner, 1994; Rybacki, 1994; Mikulíček &
345   Kotlík, 2001; Pruvost et al., 2015).
346   We stress that hybrids have an additional challenge in the selective elimination of *P. ridibundus*
347   genome. During the initial crossing of *P. ridibundus* and *P. lessonae*, hybrids usually transmit
348   the *P. ridibundus* genome and eliminate *P. lessonae* (Berger et al., 1971; Dedukh et al., 2019).
349   Subsequent backcrosses of diploid hybrids with *P. lessonae* individuals ensure the maintenance
350   of hybrids and lead to the formation of a mixed population of hybrids and *P. lessonae* (Berger,
351   1971; Günther, 1983; Christiansen & Reyer, 2009). Hybridogenetic reproduction of hybrid frogs
352   in this population type is characterized by stable propagation of *P. ridibundus* genome with
353   relatively rare aberrations in genome elimination and endoreplication (Berger, 1971; Graf &
354   Müller, 1979; Pruvost et al., 2013; Dedukh et al., 2019). Surprisingly, a growing number of
355   evidence shows that also hybrid frogs in a mixed population with *P. ridibundus* produced mostly
356   R gametes and/or L gametes (this study; Uzzell et al., 1976; Graf and Polls-Pelaz, 1989;
357   Vinogradov et al., 1991; Dedukh et al., 2015, 2017; Biriuk et al., 2016, for the exceptions see
358   Doležálková-Kaštánková & Mazepa et al. 2021), although the L gametes are the crucial cells for
359   the hybrid´s persistence (Fig. S3C).) As haploid gametes with *P. ridibundus* genome would not
360   lead to hybrid progeny when coexisting with *P. ridibundus*, it is clear that these hybrids have to
361   under absence of *P. lessonae* produce fertile *P. lessonae* gametes to perpetuate themselves.
362   Obvious difficulties in forming gametes with *P. lessonae* genome may explain why mixed
363   populations of hybrids and *P. ridibundus* are rare over continental Europe compared to mixed
364   hybrid populations with *P. lessonae* (Uzzell et al., 1976; Graf & Polls-Pelaz, 1989; Plötner,
365   2005). For example, the evolutionary origin of *P. ridibundus – P. esculentus* male populations in
366   Central Europe seems to be rare event in the past time, as clonally inherited *lessonae* genomes
367   share their ancestors (Doležálková et al., 2016; Doležálková-Kaštánková et al., 2018;
368   Doležálková-Kaštánková & Mazepa et al., 2021).
369   In this light of the evidence, diploid hybrid males persisting within the R-E system in Eastern
370   Europe in high numbers over decades of observation (Borkin et al., 2004; Shabanov et al., 2020)
371   remains unclear. As hybrid males produce mainly a mixture of R and L genomes (Fig. 3, Fig.
372   S3), while female and co-occurring triploid hybrids with the RRL genotype produce R and RL
373   gametes, the proportion of hybrids that received *P. lessonae* gametes is expected to be lower than
374   observed. Moreover, long-term clonal propagation of the genome may theoretically lead to the
375   accumulation of deleterious mutations, thus decreasing the survival of hybrids (Tunner &
376   Heppich-Tunner, 1991; Christiansen et al., 2005; Christiansen, 2009; Dubey et al., 2019). The

377 maintenance of these hybrid males may explain different competition rates between *P.*
378 *esculentus* and *P. ridibundus* tadpoles (Berger, 1977; Hotz et al., 1999), or a general selection
379 against parental genotypes (Reyer et al., 2015).

Conclusion

381 We found diverse pathways of hybridogenetic reproduction in diploid hybrid males from Eastern
382 Ukraine. Investigating gametogenesis we observed one or another parental genome elimination
383 followed by endoreplication of the remaining genome in diverse germ cell populations. These
384 pathways resulted in the simultaneous formation of gametes with *P. ridibundus* and *P. lessonae*
385 genomes in most males. We found these males crucial for the hybrid´s persistence in these
386 populations because they are the only ones able to form *P. lessonae* gametes. However, genome
387 elimination and endoreplication have not always occurred correctly, resulting in aneuploidy and
388 the abruption of meiosis in some spermatocytes. We find the gametogenic diversity as the key
389 evolutionary force producing a variety of gametes with different genome compositions and
390 ploidy levels, maintaining these populations in particular and increasing global vertebrate
391 diversity in general.

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References

399 Abbot R, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A,
400 Buerkle CA, Buggs R, Butlin RK, Dieckmann U, Eroukhmanoff F, Grill A, Cahan SH, Hermansen
401 JS, Hewitt G, Hudson AG, Jiggins C, Jones J, Keller B, Marczewski T, Mallet J, Martinez-
402 Rodriguez P, Möst M, Mullen S, Nichols R, Nolte AW, Parisod C, Pfennig K, Rice AM, Ritchie
403 MG, Seifert B, Smadja CM, Stelkens R, Szymura JM, Väinölä R, Wolf JBW, Zinner D. 2013.
404 Hybridization and speciation. *Journal of Evolutionary Biology*, 26:229–246 DOI: 10.1111/j.1420-
405 9101.2012.02599.x
406 Arnold ML, Hodges SA. 1995. Are natural hybrids fit or unfit relative to their parents? *Trends in*
407 *ecology & evolution,* 10(2):67-71 DOI: 10.1016/S0169-5347(00)88979-X
408 Battaglia E. 1974. Sulla terminologia dei processi apomittici. *Nuovo Giornale botanico italiano,*
409 54:674-696 DOI: 10.1080/11263504709440462
410 Berger L. 1968. Morphology of the F1 Generation of various crosses within *Rana esculenta*-
411 complex. *Acta Zoologica Cracoviensia,* 13:301–324.
412 Berger L. 1970. Sex ratio in the F1 progeny within forms of *Rana esculenta* complex. *Genetica*
413 *Polonica*, 12:87–101.
414 Berger L. 1971. Viability, sex and morphology of F2 generation within forms of *Rana esculenta*
415 complex. *Zoologica Poloniae*, 21(4):345–393.
416 Berger L. 1977. Systematics and hybridization in the *Rana esculenta* complex. In: Taylor DH,
417 Guttman SI, eds. *The reproductive biology of amphibians*. Springer, Boston, MA, 367-388 DOI:
418 10.1007/978-1-4757-6781-0_12

419   Berger L. 1983. Systematyka i systemy genetyczne zab zielonych Europy. *Przegrad Zoologiczny*,
420   28:47-61.
421   Bi K, Bogart JP. 2010. Probing the meiotic mechanism of intergenomic exchanges by genomic in
422   situ hybridization on lampbrush chromosomes of unisexual *Ambystoma* (Amphibia: Caudata).
423   *Chromosome Research*, 18:371-382 DOI: 10.1007/s10577-010-9121-3
424   Biriuk OV, Shabanov DA, Korshunov AV, Borkin LJ, Lada GA, Pasynkova RA, Rosanov JM,
425   Litvinchuk SN. 2016. Gamete production patterns and mating systems in water frogs of the
426   hybridogenetic *Pelophylax esculentus* Complex in northeastern Ukraine. *Journal of Zoological*
427   *Systematics and Evolutionary Research*, 54(3):215–225 DOI: 10.1111/jzs.12132
428   Birstein VJ. 1984. Localization of NORs in karyotypes of four *Rana* species. *Genetica*, 64:149–
429   154 DOI: 10.1007/BF00115338
430   Borkin LJ, Korshunov AV, Lada GA, Litvinchuk SN, Rosanov JM, Shabanov DA, Zinenko AI.
431   2004. Mass occurrence of polyploid green frogs (*Rana esculenta* Complex) in eastern Ukraine.
432   *Russian Journal of Herpetology*, 11:194–213 DOI: 10.30906/1026-2296-2004-11-3-203-222
433   Borondin PM, Rogatcheva MB, Zhelezova AI, Oda S. 1988. Chromosome pairing in inter-racial
434   hybrids of the house musk sherew (*Suncus murinus*, Insectivora, Soricidae). *Genome*, 41:79–90
435   DOI: 10.1139/g97-103
436   Brychta BH, Tunner HG. 1994. Flow cytometric analysis of spermatogenesis in triploid *Rana*
437   *esculenta*. *Zoologica Poloniae*, 39:507
438   Bucci S, Ragghianti M, Mancino G, L, Hotz H, Uzzell T. 1990. Lampbrush and mitotic
439   chromosomes of the hemiclonally reproducing hybrid *Rana esculenta* and its parental species.
440   *Journal of Experimental Zoology,* 255:37–56 DOI: 10.1002/jez.1402550107
441   Chmielewska M, Dedukh D, Haczkiewicz K, Rozenblut-Kościsty B, Kaźmierczak M, Kolenda K,
442   Serwa E, Pietras-Lebioda A, Krasikova A, Ogielska M. 2018. The programmed DNA elimination
443   and formation of micronuclei in germ line cells of the natural hybridogenetic water frog *Pelophylax*
444   *esculentus*. *Scientific reports*, 8(1):1-19 DOI: 10.1038/s41598-018-26168-z
445   Christiansen DG, Fog K, Pedersen BV, Boomsma JJ. 2005. Reproduction and hybrid load in all-
446   hybrid populations of *Rana esculenta* water frogs in Denmark. *Evolution*, 59:1348–1361 DOI:
447   10.1111/j.0014-3820.2005.tb01784.x
448   Christiansen DG, Reyer HU. 2009. From clonal to sexual hybrids: genetic recombination via
449   triploids in all-hybrid populations of water frogs. *Evolution*. 63(7):1754–1768
450   DOI: 10.1111/j.1558-5646.2009.00673.x
451   Christiansen DG. Gamete types, sex determination and stable equilibria of all-hybrid populations
452   of diploid and triploid edible frogs (*Pelophylax esculentus*). 2009. *BMC Evolutionary Biology*
453   9(1):135 DOI: 10.1186/1471-2148-9-135
454   Coyne JA, Orr HA. 2004. *Speciation*. Sunderland, MA, USA: Sinauer Associates, Inc.
455   Dawley RM, Bogart JP. 1989. *Evolution and ecology of unisexual vertebrates*. Albany, New York:
456   New York State Museum Publications.
457   Dedukh D, Litvinchuk S, Rosanov J, Mazepa G, Saifitdinova A, Shabanov D, Krasikova A. 2015.
458   Optional endoreplication and selective elimination of parental genomes during oogenesis in
459   diploid and triploid hybrid European water frogs. *PLoS One*, 10(4):e0123304 DOI:
460   10.1371/journal.pone.0123304
461   Dedukh D, Litvinchuk S, Rosanov J, Shabanov D, Krasikova A. 2017. Mutual maintenance of di-
462   and triploid *Pelophylax esculentus* hybrids in R-E systems: results from artificial crossings
463   experiments. *BMC Evolutionary Biology*, 17:220 DOI: 10.1186/s12862-017-1063-3

464   Dedukh D, Litvinchuk J, Svinin A, Litvinchuk S, Rosanov J, Krasikova A. 2019. Variation in
465   hybridogenetic hybrid emergence between populations of water frogs from the *Pelophylax*
466   *esculentus* complex. *PloS One*, 14(11):e0224759 DOI: 10.1371/journal.pone.0224759
467   Dedukh D, Riumin S, Chmielewska M, Rozenblut-Kościsty B, Kolenda K, Kaźmierczak M,
468   Dudzik A, Ogielska M, Krasikova A. 2020. Micronuclei in germ cells of hybrid frogs from
469   *Pelophylax esculentus* complex contain gradually eliminated chromosomes. *Scientific reports*
470   10(1):1-13 DOI: 10.1038/s41598-020-64977-3
471   Dedukh D, Krasikova A. 2021. Delete and survive: strategies of programmed genetic material
472   elimination in eukaryotes. *Biological Reviews* DOI: 10.1111/brv.12796
473   Doležálková M, Sember A, Marec F, Ráb P, Plötner J, Choleva L. 2016. Is premeiotic genome
474   elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus
475   *Pelophylax*? *BMC Genetics*, 17:100 DOI: 10.1186/s12863-016-0408-z
476   Doležálková-Kaštánková M, Pruvost NBM, Plötner J. Reyer HU, Janko K, Choleva L. 2018. All-
477   male hybrids of a tetrapod *Pelophylax esculentus* share its origin and genetics of maintenance.
478   *Biology of Sex Differences*, 9(13) DOI: 10.1186/s13293-018-0172-z
479   Doležálková-Kaštánková M, Mazepa G, Jeffries DL, Perrin N, Plötner M, Plötner J, Guex GD,
480   Mikulíček P, Poustka AJ, Grau J, Choleva L. 2021. Capture and return of sexual genomes by
481   hybridogenetic frogs provides clonal genome enrichment in a sexual species. *Scientific reports*,
482   11(1):1-10 DOI: 10.1038/s41598-021-81240-5
483   Drohvalenko M, Pustovalova E, Fedorova A, Shabanov D. 2022. First finding of triploid hybrid
484   frogs *Pelophylax esculentus* (Anura: Ranidae) in Mozh river basin (Kharkiv region, Ukraine).
485   *Biodiversity, ecology and experimental biology,* 23:2 DOI: 10.34142/2708-5848.2021.23.2.04
486   Dubey S, Maddalena T, Bonny L, Jeffries DL, Dufresnes C. 2019. Population genomics of an
487   exceptional hybridogenetic system of *Pelophylax* water frogs. *BMC Evolutionaty Biology*, 19:164
488   DOI: 10.1186/s12862-019-1482-4
489   Dufresnes C, Mazepa G. 2020. Hybridogenesis in water frogs. *eLS*, 1:718-726 DOI:
490   10.1002/9780470015902.a0029090
491   Graf JD, Müller WP. 1979. Experimental gynogenesis provides evidence of hybridogenetic
492   reproduction in the *Rana esculenta* complex. *Experientia*, 35:1574–1576 DOI:
493   10.1007/BF01953200
494   Graf JD, Polls-Pelaz M. 1989. Evolutionary genetics of the *Rana esculenta* Complex. In: Dawley
495   RM, Bogart JP, eds. *Evolution and ecology of unisexual vertebrates.* Albany, New York: New
496   York State Museum Publications, 289–302.
497   Günther R, Uzzell T, Berger L. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia,
498   Salientia). *Mitteilungen des Zoologischen Museums Berlin*, 55:35–57.
499   Günther R. 1983. Zur populationsgenetik der mitteleuropäischen wasserfrösche des *Rana*
500   *esculenta*—synkleptons (Anura, Ranidae). *Zoologischer Anzeiger*, 211(1/2):43–54
501   Heppich S, Tunner HG, Greilhuber J. 1982. Premeiotic chromosome doubling after genome
502   elemination during spermatogenesis of the species hybrid *Rana esculenta. Theoretical and Applied*
503   *Genetics*, 61:101–104 DOI: 10.1007/BF00273874
504   Hoffman A, Plötner J, Pruvost NBM, Christiansen DG, Röthlisberger S, Choleva L, Mikulíček P,
505   Cogălniceanu D, Sas-Kovács I, Shabanov D, Morozov-Leonov S, Reyer HU. 2015. Genetic
506   diversity and distribution patterns of diploid and polyploid hybrid water frog populations
507   (*Pelophylax esculentus* complex) across Europe. *Molecular Ecology*, 24:4371–4391 DOI:
508   10.1111/mec.13325

509 Hotz H, Semlitsch RD, Gutmann E, Guex GD, Beerli P. 1999. Spontaneous heterosis in larval life-
510 history traits of hemiclonal frog hybrids. *Proceedings of the National Academy of Sciences,*
511 96(5):2171-6 DOI: 10.1073/pnas.96.5.2171

512 Ishishita S, Tsuboi K, Ohishi N, Tsuchiya K, Matsuda Y. 2015. Abnormal pairing of X and Y sex
513 chromosomes during meiosis I in interspecific hybrids of *Phodopus campbelli* and *P. sungorus.*
514 *Scientific reports*, 5(1):1-9 DOI: 10.1038/srep09435

515 Lenormand T, Engelstadter J, Johnston SE, Wijnker E, Haag CR. 2016. Evolutionary mysteries in
516 meiosis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 19;371(1706).
517 DOI: 10.1098/rstb.2016.0001

518 Mallet J. 2007. Hybrid speciation. *Nature*, 446:279–283 DOI: 10.1038/nature05706

519 Mazepa G, Doležálková M, Choleva L, Plötner J, Biriuk O, Drohvalenko M, Korshunov O,
520 Shabanov D, Wolf J, Perrin N. 2018. Distinct fate of the asexual genomes in two convergently
521 evolved *Pelophylax* hybridogenetic systems. *Sex uncovered: the evolutionary biology of*
522 *reproductive systems.* Roscoff (Brittany), France, 57.

523 McKee BD. 2004. Homologous pairing and chromosome dynamics in meiosis and mitosis.
524 *Biochimica et Biophysica Acta*, 1677:165–180 DOI: 10.1016/j.bbaexp.2003.11.017

525 Mikulíček P, Kotlík P. 2001. Two water frog populations from western Slovakia consisting of
526 diploid females and diploid and triploid males of the hybridogenetic hybrid *Rana esculenta*
527 (Anura, Ranidae). *Mitteilungen aus dem Museum fuer Naturkunde in Berlin Zoologische Reihe*,
528 77:59-64 DOI: 10.1002/mmnz.20010770110

529 Neaves WB, Baumann P. 2011. Unisexual reproduction among vertebrates. *Trends in Genetics*,
530 27(3):81-88 DOI: 10.1016/j.tig.2010.12.002

531 Ogielska M. 1994. Nucleus-like bodies in gonial cells of *Rana esculenta* [Amphibia, Anura]
532 tadpoles-a putative way of chromosome elimination. *Zoologica Poloniae*, 39:3-4.

533 Ogielska M, Bartmańska J. 1999. Development of testes and differentiation of germ cells in water
534 frogs of the *Rana esculenta*-complex (Amphibia, Anura). *Amphibia-Reptilia*, 20:251–263 DOI:
535 10.1163/156853899X00286

536 Plötner J. 2005. *Die westpaläarktischen Wasserfrösche: von Märtyrern der Wissenschaft zur*
537 *biologischen Sensation.* Bielefeld: Laurenti

538 Pruvost NBM, Hoffmann A, Reyer HU. 2013. Gamete production patterns, ploidy, and population
539 genetics reveal evolutionary significant units in hybrid water frogs (*Pelophylax esculentus*).
540 *Ecology and Evolution*, 3(9):2933–2946 DOI: 10.1002/ece3.687

541 Pruvost NBM, Mikulíček P, Choleva L, Reyer HU. 2015. Contrasting reproductive strategies of
542 triploid hybrid males in vertebrate mating systems. *Journal of Evolutionary Biology*, 28(1):189–
543 204 DOI: 10.1111/jeb.12556

544 Ragghianti M, Bucci S, Marracci S, Casola C, Mancino G, Hotz H, Guex GD, Plötner J, Uzzell T.
545 2007. Gametogenesis of intergroup hybrids of hemiclonal frogs. *Genetics Research*, 89:39–45
546 DOI: 10.1017/S0016672307008610

547 Ragghianti M, Guerrini F, Bucci S, Mancino G, Hotz H, Uzzell T, Guex GD. 1995. Molecular
548 characterization of a centromeric satellite DNA in the hemiclonal hybrid frog *Rana esculenta* and
549 parental species. *Chromosome Research*, 3(8):497–506 DOI: 10.1007/BF00713965

550 Renner O. 1916. Zur Terminologie des pflanzlichen Generationswechsels. *Biologisches*
551 *Zentralblatt*, 36:337–374

552 Reyer HU, Arioli-Jakob C. Arioli M. 2015. Post-zygotic selection against parental genotypes
553 during larval development maintains all-hybrid populations of the frog *Pelophylax esculentus.*
554 *BMC Evolutionary Biology*, 15(1):1-16 DOI 10.1186/s12862-015-0404-3

555 Rieger R, Michaelis A, Green MM. 1991. *Glossary of genetics classical and molecular, 5th edn.*
556 Springer, Berlin Heidelberg New York.

557 Rieseberg LH. Chromosomal rearrangements and speciation. *Trends in ecology & evolution*,
558 16(7):351-358 DOI: 10.1016/S0169-5347(01)02187-5

559 Rybacki M, Berger L. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland.
560 *Mitteilungen aus dem Museum für Naturkunde in Berlin. Zoologische Reihe*, 77:51-57 DOI:
561 10.1002/mmnz.20010770109

562 Salas N, Valetti J, Grenat P, Otero M, Martino A. 2014. Meiotic behavior of two polyploid species
563 of genus *Pleurodema* (Anura: Leiuperidae) from central Argentina. *Acta Herpetologica*, 9(1):109-
564 113 DOI: 10.1002/mmnz.20010770109

565 Schön I, Martens K, van Dijk P. 2009. *Lost sex. The evolutionary biology of parthenogenesis.*
566 Heidelberg, Germany: Springer.

567 Shabanov D, Vladymyrova M, Leonov A, Biriuk O, Kravchenko M, Mair Q, Meleshko O,
568 Newman J, Usova O, Zholtkevych G. 2020. Simulation as a Method for Asymptotic System
569 Behavior Identification (e.g. Water Frog Hemiclonal Population Systems). In: Ermolayev V,
570 Mallet F, Yakovyna V, Mayr HC, Spivakovsky A, eds. *Information and Communication*
571 *Technologies in Education, Research, and Industrial Applications. ICTERI 2019.*
572 *Communications in Computer and Information Science, vol 1175.* Springer, Cham. DOI:
573 10.1007/978-3-030-39459-2_18

574 Stöck M, Dedukh D, Reifová R, Lamatsch DK, Starostová Z, Janko, K. 2021. Sex chromosomes
575 in meiotic, hemiclonal, clonal and polyploid hybrid vertebrates: along the 'extended speciation
576 continuum'. *Philosophical Transactions of the Royal Society B*, 376(1833):20200103 DOI:
577 10.1098/rstb.2020.0103

578 Svinin AO, Litvinchuck SN, Borkin LJ, Rosanov JM. 2013. Distribution and population system
579 types of green frogs (*Pelophylax* Fitzinger, 1843) in Mari El Republic. *Current Study of*
580 *Herpetology*, 13(3/4):137-147.

581 Svinin AO, Dedukh DV, Borkin LJ, Ermakov OA, Ivanov AY, Litvinchuk JS, Zamaletdinov RI,
582 Mikhaylova RI, Trubyanov AB, Skorinov DV, Rosanov YM, Litvinchuk SN. 2021. Genetic
583 structure, morphological variation, and gametogenic peculiarities in water frogs (*Pelophylax*) from
584 northeastern European Russia. *Journal of Zoological Systematics and Evolutionary Research*,
585 59(3):646-662 DOI: doi.org/10.1111/jzs.12447

586 Torgasheva AA, Borodin PM. 2016. Cytological basis of sterility in male and female hybrids
587 between sibling species of grey voles *Microtus arvalis* and *M. levis*. *Scientific Reports,* 6:36564
588 DOI: 10.1038/srep36564

589 Tunner HG. 1973. Demonstration of the hybrid origin of the common green frog *Rana esculenta*.
590 *Naturwissenschafte*, 60:481–482 DOI: 10.1007/BF00592872

591 Tunner H. 1974. Die klonale Struktur einer Wasserfröschpopulation. *Journal of Zoological*
592 *Systematics and Evolutionary Research*, 12:309–14.

593 Tunner HG & Heppich S. 1981. Premeiotic genome exclusion during oogenesis in the common
594 edible frog, *Rana esculenta*. *Die Naturwissenschaften*, 68(4):207-208.

595 Tunner H, Heppich-Tunner S. 1991. Genome exclusion and two strategies of chromosome
596 duplication in oogenesis of a hybrid frog. *Naturwissenschaften*, 78:32–34 DOI:
597 10.1007/BF01134041

598 Tunner H, Heppich-Tunner S. 1992. A new population system of water frogs discovered in
599 Hungary. *Proceedings of the Sixth Ordinary General Meeting of the Societas Europaea*
600 *Herpetologica*, 19-23:453-460.

601 Uzzell T, Günther R, Berger L. 1977. *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic
602 system (Amphibia, Salientia). *Proceedings of the Academy of Natural Sciences of Philadelphia*,
603 128:147–171
604 Vinogradov AE, Borkin LJ, Günther R, Rosanov JM. 1991. Two germ cell lineages with genomes
605 of different species in one and the same animal. *Hereditas*, 114(3):245–251 DOI: 10.1111/j.1601-
606 5223.1991.tb00331.x
607 Vorburger C. 2001. Non-hybrid offspring from matings between hemiclonal hybrid waterfrogs
608 suggest occasional recombination between clonal genomes. *Ecology Letters*, 4:628-636 DOI:
609 10.1046/j.1461-0248.2001.00272.x
610 Zong E, Fan G. 1989. The variety of sterility and gradual progression to fertility in hybrids of the
611 horse and donkey. *Heredity*, 62(3):393–406 DOI: 10.1038/hdy.1989.54

Figure 1

Identification of ploidy level and genome composition of gonocytes, spermatocytes, and spermatids from *P. esculentus* males collected from the Mozh river basin

FISH with RrS1 probe helps distinguish pericentromeric regions only of *P. ridibundus* chromosomes (indicated by thin arrows). (A-C) Somatic cells (C), spermatids (B, C), and spermatocytes in meiosis I (A) and II (B) had only *P. ridibundus* chromosomes suggesting the presence of premeiotic genome elimination of *P. lessonae* genome and endoreplication of *P. ridibundus* genome. (D-J) Germ line cells (gonocytes, spermatocytes, and spermatids) with different ploidies suggesting the presence of premeiotic elimination and endoreplication of different genomes in various cell lines. Interphase cells (indicated by thick arrows) with a haploid set of *P. ridibundus* chromosomes (D, E, G, H, J) and with *P. lessonae* chromosomes (I). Mitotic metaphase cell with 13 *P. ridibundus* chromosomes and 13 *P. lessonae* chromosomes (E). Meiotic metaphase I with 13 bivalents of *P. ridibundus* (D, F, J) and 13 bivalents of *P. lessonae* (G). Meiotic metaphase II with 13 univalents of *P. ridibundus* (H) and 13 univalents of *P. lessonae* (I). Spermatids (indicated by arrowheads) with haploid set of *P. ridibundus* chromosomes (D, J) and *P. lessonae* (D, J). Scale bar = 10μm.
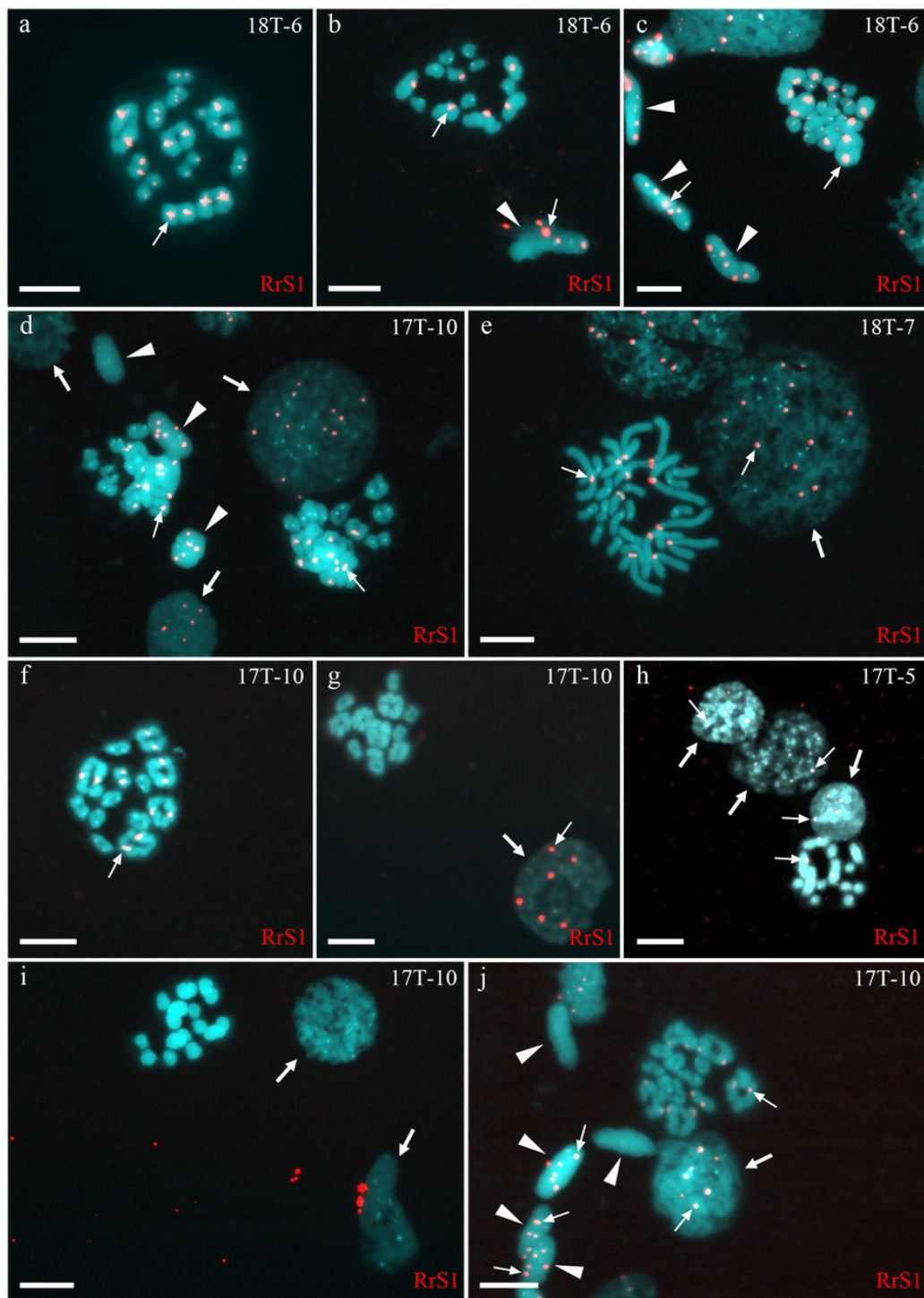
Figure 2

Identification of ploidy level and genome composition of gonocytes, spermatocytes and spermatids from particular *P. esculentus* male producing diploid spermatids collected from the Mozh river basin

Interphase cell nuclei (indicated by thick arrows) with diploid *P. ridibundus* chromosomal set (A, D). Mitotic metaphases with 26 *P. ridibundus* chromosomes (D), approximately 47 *P. ridibundus* chromosomes (B), approximately 40 *P. lessonae* chromosomes (C) and 13 *P. ridibundus* and 13 *P. lessonae* chromosomes. Meiotic metaphase I with 13 *P. ridibundus* bivalents (A, H), approximately 12 tetravalents (or mixture of bivalents and tetravalents) with chromosomes exclusive to *P. ridibundus* (E), and with approximately 11 tetravalents with chromosomes exclusive to *P. lessonae* (G). Meiotic metaphase I with a mixture of approximately 9 *P. lessonae* tetravalents and 4 *P. lessonae* bivalents as well as 4 *P. ridibundus* tetravalents and 4 *P. ridibundus* bivalents. Spermatids (shown by arrowheads) with at least 5 P. ridibundus chromosomes (designated as haploid *P. ridibundus* genome) (B, H), with only *P. lessonae* chromosomes (designated as haploid or diploid *P. lessonae* genome) and at least 14 *P. ridibundus* chromosomes and at least 17 *P. ridibundus* chromosomes (designated as diploid *P. ridibundus* genome) (F, H). *P. ridibundus* chromosomes identified using FISH-based detection of pericentromeric RrS1 repeats (indicated by thin arrows). Scale bar = 10μm
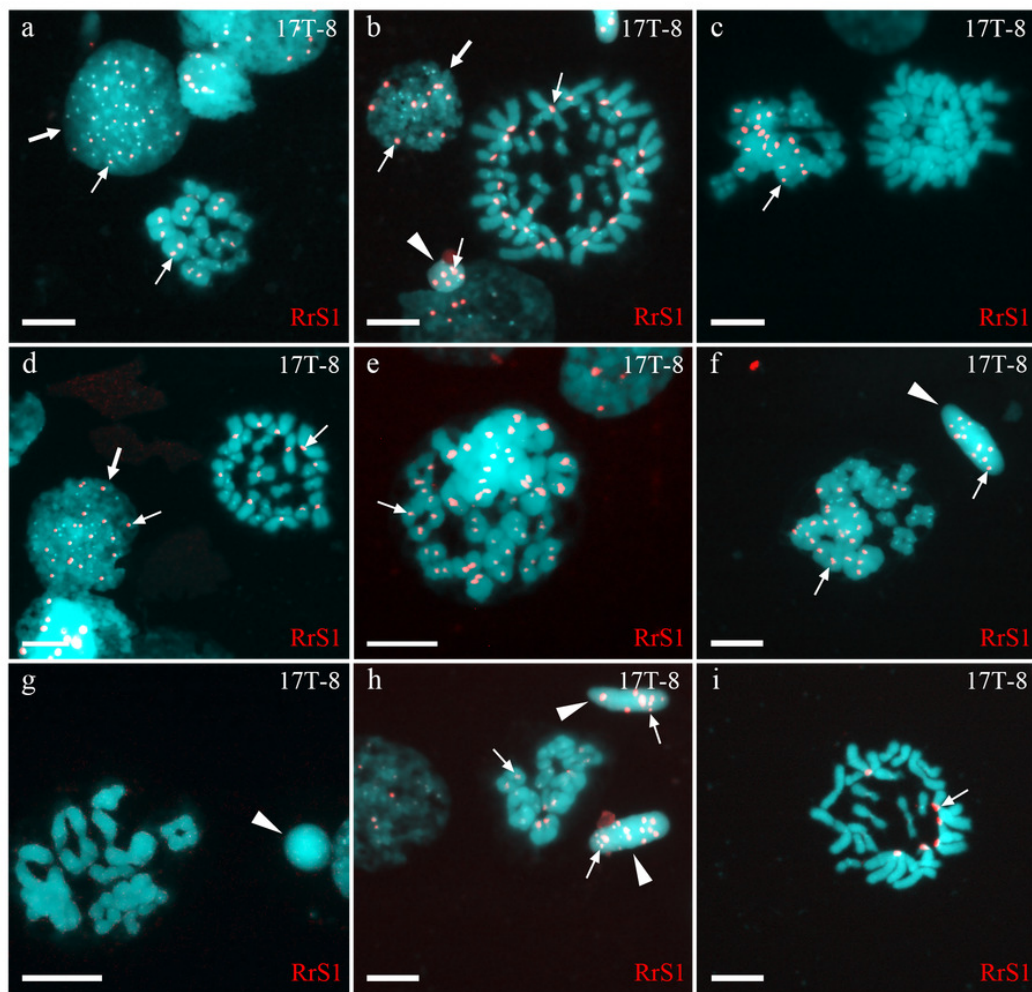
Figure 3

Relative number of normal and aneuploid chromosomal plates during mitosis (A, C) and meiosis (B, D) from hybrid frogs collected from the R-E system of the Mozh river (A, B) and Iskiv pond (C, D)

R – genome of *P. ridibundus*, L – genome of *P. lessonae*; aneuploidy – number of chromosomes more or less 13 bivalents or univalents.
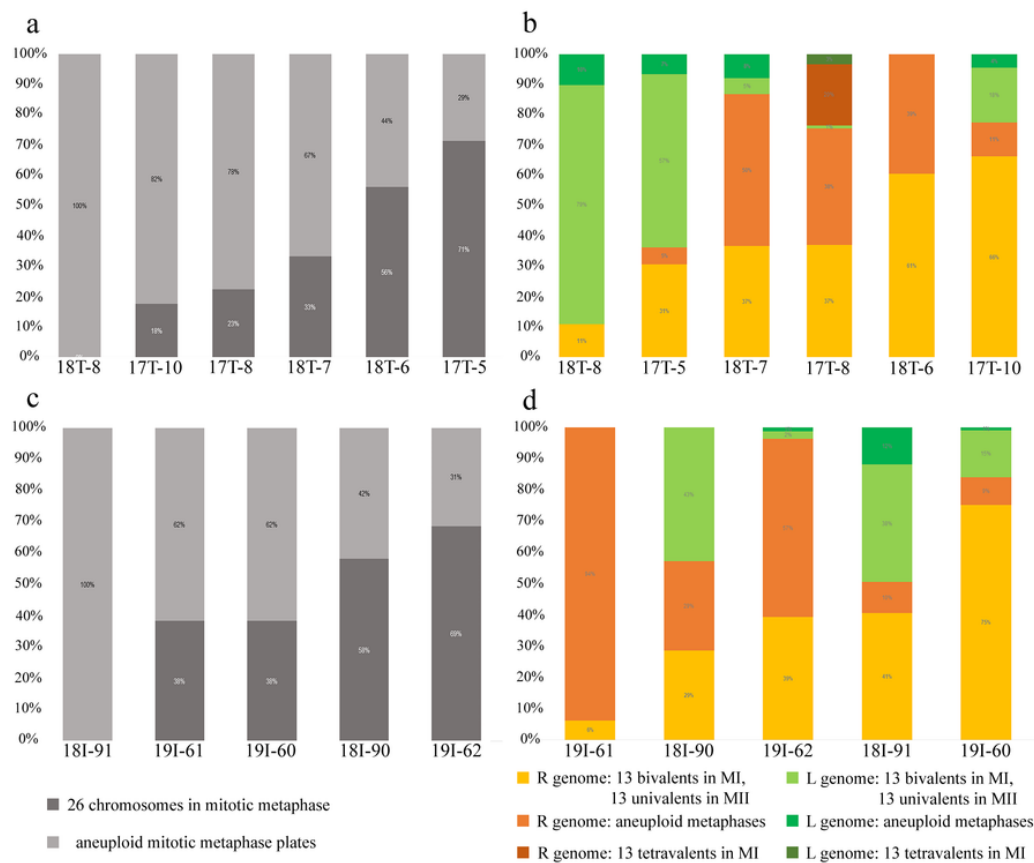
Figure 4

Suggested gametogenic pathways in sexual species and hybrid males from studied R-E-HPSs

Pathway I: Genome elimination and endoreplication ('classical' hybridogenesis). During classical genome elimination, one of the parental genomes is eliminated before meiosis, whereas the other is endoreplicated, allowing the restoration of the diploid chromosome set. These cells undergo meiotic division with 13 bivalents during meiosis I and 13 bivalents during meiosis II. Subsequent spermatids bear the genomes of only one parental species (*P. ridibundus* or *P. lessonae*). Pathway II: Genome elimination of one of the parental species (*P. ridibundus* or *P. lessonae*) during meiosis. This type of gamete formation also involves the elimination of only one parental genome. However, it occurs directly during meiosis. After meiotic divisions I (13 bivalent stages) and II (13 univalent stages), spermatids bear the endoreplicated genome. Pathway III: The genomes of different parental species were eliminated from different germline populations. Therefore, some gonocytes bear only *P. ridibundus* chromosomes, whereas some cells have *P. lessonae* chromosomes only. Germ cells with both parental genomes duplicated and formed two types of parental species bivalents (2n = 26). After meiosis II, the spermatids were from both parental species (*P. ridibundus* and *P. lessonae*). Pathway IV: Diploid sperm formation. Two rounds of endoreduplication of one parental species genome resulted in the formation of tetravalents, bearing four sets of *P. ridibundus* or *P. lessonae* genomes in meiosis I. Such cells, which have undergone meiosis II, bear a double chromosome set (RR, LL, or even RL). Pathway V: Abnormal meiosis. Due to disruptions during the elimination of *P. ridibundus* or *P. lessonae* genome, there are no vital spermatids, so the individual is sterile.

Mitosis   Meiosis   Spermatids

Way I:
no genome
elimination

Way II:
non-clonal genome
elimination

endoreduplication

Way III:
different genomes
elimination in
different cell lines

endoreduplication

endoreduplication

endoreduplication

Way IV:
different genomes
elimination in
different cell lines
+
endoreduplication

endoreduplication

endoreduplication

Way V:
abnormal
elimination

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