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The first evidence of triploidy among *Pelophylax esculentus* (Linnaeus, 1758) (Anura: Ranidae) in the Chornobyl Exclusion Zone

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Abstract. *Pelophylax esculentus* complex hemiclonal systems are a unique evolutionary object due to intricate mechanisms of their sustainability at genomic, gamete, and developmental levels. Chornobyl Exclusion Zone (Ukraine) presents a no less unique object of nature evolution under the unprecedented radiation impact and human pressure decrease. By measuring erythrocyte lengths, we report the first findings of triploid hybrid frogs *P. esculentus* from two localities in Chornobyl Exclusion Zone, where diploid hybrids only were reported before. The presence of triploids implies high complexity of the local water frog population systems, enabling new research opportunities.

Key words. Water frog, hybrid, population system, erythrocyte.

Hybridogenetic species complexes are great models for studying the evolution of reproduction due to hybrids' capacity to reproduce themselves. The *Pelophylax esculentus* complex is one of the most studied (Dedukh & Krasikova 2021). It consists of pool frog *Pelophylax lessonae* (Camerano, 1882), marsh frog *Pelophylax ridibundus* (Pallas, 1771), and their hemiclonal hybrid, edible frog *Pelophylax esculentus* (Linnaeus, 1758), whose range approximately coincides with the range of pool frogs (Hoffmann et al. 2015). Hybrids that transmit parental genomes clonally are presented as both sexes and two ploidies (Tunner 1974; Berger 1977; Plötner 2005; Jakob 2007). Hybrids typically coexist with parental and other hybrid forms in mixed population systems, leading to formation of a new hybrid generation. Each such system has evolved the mechanisms to maintain its composition, like different ontogenetic strategies and selective survival (e.g., Berger 1973; Hoffmann et al. 2015; Shabanov et al. 2015). It is caused by the variety of ways different forms contribute to reproduction, mainly the types of gametes: not only haploid L and R (from hybrids and parental species), but also occasionally diploid LL, RR, and LR gametes from hybrids (Dedukh et al. 2013; Pruvost et al. 2015). The most complicated mechanisms are in the systems with triploids since they include the highest number of forms (parental species, 2n and 3n hybrids) balancing with each other (Pruvost 2013; Mikulíček et al. 2015). In Ukraine, such complex systems were known to be widespread in the eastern part only, in Siverskyi Donets river basin (Borkin et al. 2004; Shabanov et al. 2020).

Chornobyl Exclusion Zone per se possesses many opportunities in herpetology, at least due to long-term decreased anthropogenic pressure. Its radioactive contamination is already known for affecting amphibians (Gashchak et al. 2009), though some species remain thriving under its impact (Burraco et al. 2021a; Burraco 2021b). Radiation also affects amphibian populations. For instance, the changes in local evolution were described for *Hyla orientalis* Bedriaga, 1890 in the Zone, though without obvious harmful consequences (Car et al. 2022). However, the impact of some registered effects is not so clear. Water frogs of the *P. esculentus* complex from the area of the Chornobyl fallout impact in Belarus (Briansk region) were shown to have reduced genome size (Vinogradov & Chubinishvili 1999). Despite the found influence on their genomes (Rozanov et al. 1990; Vinogradov et al. 1990), all the previously examined water frogs from the Zone were diploids. Therefore, it was assumed that only diploid population systems exist here (S. Litvinchuk, St. Petersburg, pers. comm.). Herein, we report the evidence on the previously unrecorded presence of triploid *P. esculentus* in the Chornobyl Exclusion Zone.

The amphibian survey in the Chornobyl Exclusion Zone was carried out on 9–12 of August 2021 in collaboration with and legal permission from Chornobyl Radiation and Ecological Biosphere Reserve (http://zapovidnyk.org.ua) and aimed to explore the *Pelophylax* populations within the Zone. The region of the study included: the Prypiat River (in Chornobyl; 51.272483, 30.244840) and its floodplain (51.341432, 30.199178), the Uzh River (51.273852, 29.741894) and

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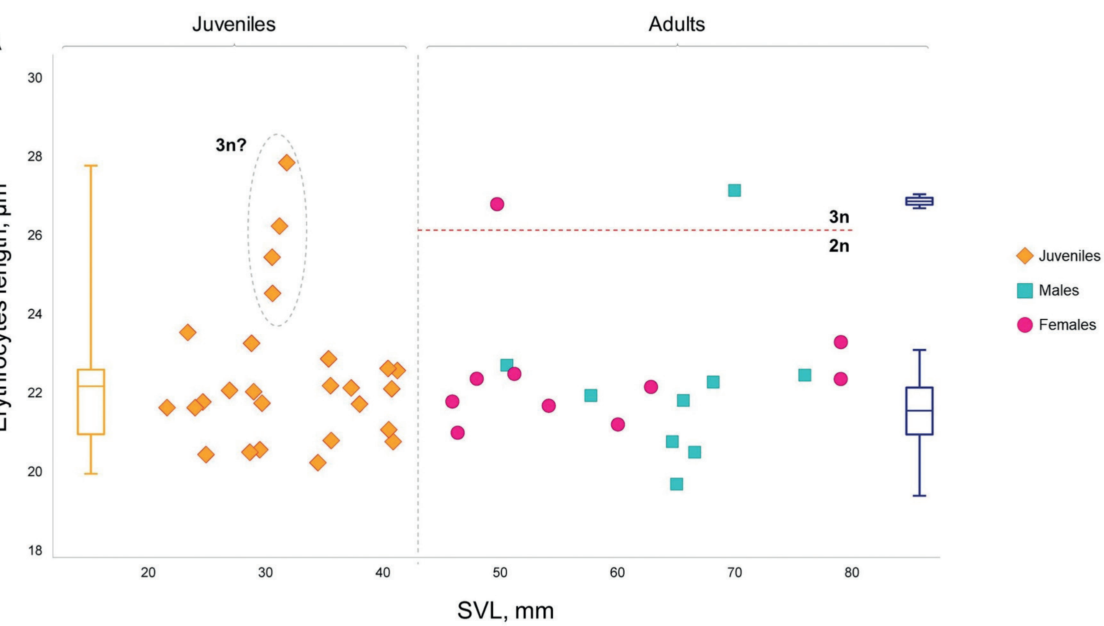
its floodplain (51.256711, 30.222522), isolated old melioration channels (51.245596, 30.169778) and those in Ilia River valley (51.278037, 29.808185), and the bypass channel of Chornobyl nuclear plant's cooling pond (51.396829, 30.141884). The frogs were caught by hands, dip-net, and using flashlights at night. Species and sex were identified morphologically. The presence of vocal sacs and nuptial pads indicated males, while species were identified by body coloration, metatarsal tubercle shape, and hindlimbs proportions. Juveniles had weakly expressed species- and sex-specific features so they were described as a homogenous group. Snout-vent length (SVL) was measured via scale photographing and later measured on photographs. The blood samples were taken from each frog by cutting the fingertips; wounds were treated with the antibiotic Vetbicilin-3 (Basalt, Ukraine). We prepared the air-dried blood smears and photographed and measured ~50 erythrocytes for each frog using a Leica DFC3000 G camera with Leica LASX Software. Triploid water frogs have about 1.5-fold more DNA in nuclei than diploids, so the ploidy of *P. esculentus* frogs can be estimated by mean erythrocytes length (Ogielska et al. 2004; Bondareva et al. 2012). It is also known that the exact cut-off value of erythrocyte length for triploids should be separately estimated for a particular population system since it varies among them (though often about 26 μm) (Drohvalenko et al. 2019). Techniques used in the capture and sampling sought to minimize animal suffering according to Directive 2010/63/EU (protection of animals used for scientific purposes). All the frogs were released after the sampling.

The total catch was 52 frogs: 1 tadpole (late development stage), 31 juveniles, and 19 adults (10 females, 9 males). Adult hybrids were found in Prypiat and Uzh floodplains, in Ilia River, old melioration channels, and bypass channel of cooling pond.

The distribution of collected frogs by SVL and erythrocytes lengths are shown in Fig.1. Adults are visually divided into two groups without any transient state. The vast majority (with a mean of 21.54 μm, 95% CI [21.06, 22.02]) belong to diploids. Two spike values exceeding the considered cut-off value putatively mean the triploidy

A



B

	Mean (95% CI)	Median (IQR)	Min	Max
Juveniles	**22.16** (21.39, 22.93)	**21.81** (20.94, 22.58)	19.94	27.76
Putatively **2n** juveniles	**21.48** (21.03, 21.93)	**21.50** (20.59, 21.93)	19.94	23.33
Putatively **3n** juveniles	**25.87** (23.57, 28.17)	**25.70** (25.06, 26.52)	24.35	27.75
Adults 2n	**21.54** (21.06, 22.02)	**21.69** (20.93, 22.16)	19.38	23.08
Adults 3n	**26.86** (24.6, 29.12)	**26.86** (26.77, 26.94)	26.68	27.03

Fig. 1. A. The distribution of frogs from the Zone by their mean erythrocyte lengths vs body length (SVL). Red dash line marks putative cut-off value for adult triploids; dash circle marks juveniles of doubtful ploidy. Boxplots denote means (middle line), IQRs (boxes) and extreme values (whiskers). **B**. The sample parameters for different groups of studied frogs.

of these individuals (male from bypass channel and female from Prypiat floodplain, with 27.03 and 26.67 µm, respectively). Although the gap between small-cell and large-cell individuals is obvious, more individuals should be examined to specify the erythrocyte length ranges for diploids and triploids, as well as their possible overlapping.

The distribution of erythrocyte sizes for juveniles looks more intricate. As there is no gap but rather a continuous set of cell lengths from small to large, we cannot confidently define the cut-off value for juveniles, so we treated them on the graph as a single group (mean 22.16 µm, 95% CI [21.39, 22.93]). It is known for certain systems (Drohvalenko et al. 2021) that cell length distribution could vary much between adults and juveniles, widely overlapping in the last. Worth noting that the juveniles with the largest cells (dash circle Fig. 1; mean 25.87 µm, 95% CI [23.57, 28.17]) originated from the bypass channel, where the triploid male was assumingly found. We thus can carefully suggest that these juveniles are a new generation of triploids. At the same site, there were also caught three individuals with tails remnants (still in the course of metamorphosis), which SVL weren't measured – they, however, had the smallest erythrocytes: 19.79, 19.71, and 18.80 µm. No significant differences were found between different ages, neither among diploids (Student's t-test p=0.354) nor triploids (Mann-Whitney's p = 0.533).

Due to the late summer survey, the catch of frogs appeared relatively small and insufficient to claim neither the exclusive presence of triploids in two localities only nor the exact composition of the studied systems. Nevertheless, every presence of hybrids *P. esculentus* probably means the presence of the parental species, which they depend on – and triploids presence implies the presence of diploid hybrids, producing 2n-gametes (Biriuk et al. 2016). In certain locations, the presence of some forms could be suggested by habitat preferences (like *P. lessonae* in swampy channels; [Pysanets, 2014]) or behavioral features (like *P. ridibundus* possibly migrating to bypass channel from Prypiat at breeding; [Wells, 2007]). The local absence of any form could indeed be just an artifact.

The features of triploid *P. esculentus* from the Chornobyl Exclusion Zone are planned to be examined using precise methods. Their very presence here would mean the existence of more complicated hemiclonal population systems there than was supposed before. At least the L-E-R system with triploids (in the bypass channel) is already more complex than the most complex system studied in Ukraine (Meleshko et al. 2014). They could become new models of interspecies evolution due to a combination of their inherent hemiclonality phenomenon and external radiation influence. The absence of previous data on triploids in the Zone could possibly be explained by samples too small to record non-numerous triploids. Or, we can hypothesize the recent emergence of triploids due to local population systems evolution – in that case, the role of radiation in this phenomenon is yet to be studied.

Our next step is to focus on obtained material deeply. As the collection of new bigger samples has become quite complicated due to ongoing war and following hazardous aftermath, the application of advanced techniques available for ploidy and genomes identification (karyology, microsatellite analysis, FISH) is scheduled.

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