

SHORT  
COMMUNICATIONS

## Mitochondrial Heteroplasmy in Marsh Frog (*Pelophylax ridibundus* Pallas, 1771)

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**Abstract**—The population features of nuclear and mitochondrial genomes of *P. ridibundus* Pallas, 1771 and related species of green frogs from Nizhny Novgorod and Sverdlovsk oblasts were investigated for the first time. The existence of the R–E–L population system on the territory of Nizhny Novgorod oblast was confirmed. The presence of heteroplasmy was found in all examined samples of marsh frogs, as well as in hybrid, edible, frogs. These findings indicate the presence of hybridization and introgressions that took place in the history of the studied forms of each of the species. Considerable heterogeneity of animals from the territory of Sverdlovsk oblast in the nuclear and to a higher degree in the mitochondrial genome was demonstrated, which with high probability indicates that the populations existing in this territory originated from multiple introductions. The obtained data characterize the state of population systems, as well as the history of the formation of the modern phylogeographic pattern of green frogs on the studied territories.

**Keywords:** heteroplasmy, marsh frog, hybridization, mitotypes, nuclear genome

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Marsh frog is one of the widespread species of the European green (water) frog complex. The species inhabits a wide area, including North Africa, the Near East, Central Asia, the Caucasus, and Europe. As is known, in Europe, *Pelophylax ridibundus* (Pallas, 1771) is a part of a hybridogenetic complex, the *P. esculentus* complex, which is characterized by hybridization, hemiclinal (or meroclinal) inheritance, polyploidy, and variable composition of population systems [1]. On the eastern slope of the Urals, there is only one species of the green frog complex, *P. ridibundus*, which on the basis of molecular genetic data, is now considered to be a complex of cryptic species [2].

In the last 40 years, in Asian Russia, the expansion of marsh frog outside its natural range to the east has been observed. The sources of random introduction are fish farms and medical and biological institutions that use this species in their experiments. The spread of marsh frog to the northeast has become possible owing to human industrial activity and the ubiquitous presence of thermal anomalies of anthropogenic and natural origin (Middle Urals, Yakutsk, Altai, Siberia, Kamchatka). At present, *P. ridibundus* is a typical syn-

anthropic amphibian species in the Urals. Of both Europe and the eastern part of the marsh frog range in Russia, it is characteristic that the eastern form of marsh frog (*P. cf. bedriagae*) more often becomes the invader [3–5]. There is a hypothesis that individuals with the mitochondrial genome of the eastern type (*P. cf. bedriagae*) are tolerant to anthropogenic transformation of the environment, and for this reason, these forms move to the east and north [6].

S.N. Litvinchuk and J. Plötner pointed out (personal communication) that *P. ridibundus* from the eastern slope of the Middle Urals with respect to the mitochondrial *ND3* gene coincided with animals from Kharkov, Kiev, Zhytomyr, Kherson, Odessa, and Voronezh oblasts. At the same time, J. Plötner notes that the relationships within the *ridibundus* group cannot be fully clarified on the basis of the analysis of the mitochondrial *ND2* and *ND3* genes [7]. To obtain a complete pattern of phylogenetic relationships (the systematic status of the form and its relationships with the others) of the green frog complex of the Palearctic, along with the analysis of mitochondrial DNA, nuclear markers should be taken into consideration.

During 2017–2018 in Nizhny Novgorod and Sverdlovsk oblasts, green frogs were captured to determine the *P. ridibundus* forms inhabiting these territories. Among 89 individuals studied, 25 marsh frogs, 58 pool frogs, and 6 edible frogs were identified. Using molecular genetic methods, nine adult *P. ridibundus* individuals from the Ural region (4 specimens, Yekaterinburg, 2017; 5 specimens, Sukhoi Log, 2018) and 16 *P. ridibundus* individuals from Nizhny Novgorod oblast (15 specimens, Nizhny Novgorod, 2018, and 1 specimen, Dzerzhinsk, 2017) were examined. In addition, the nuclear and mitochondrial genomes of *P. lessonae* (21 specimens) and of all hybrid *P. cl. esculentus* individuals (6 specimens) were examined.

DNA was extracted from fresh tissue samples taken from the first two phalanges of the fingers using the PureLink® Genomic DNA Kit (Invitrogen). Molecular genetic analysis was performed in the laboratory of the Amphibian Research Center, Hiroshima University (Japan). A series of PCR with a standard set of reagents was performed to select and optimize primers. A fragment (981 bp) of the mitochondrial DNA cytochrome b gene (*Cyt b*) and a fragment (206 bp) of intron 1 of the nuclear genome serum albumin gene (*SAI* gene) were amplified and their sequences were determined. PCR was carried out using KAPATaq EXtra HotStart ReadyMix with dye (Genetics, Japan) as follows: 1 µL of DNA was amplified in 25 µL of the reaction mixture containing 12.5 µL of 2× buffer and 0.5 µL of each primers (primer concentration 12.5 µM) at 94°C for 40 s, 55°C for 20 s and 72°C 1 min for 35 cycles using LifeEco (Bioer technology).

Amplification of the cytochrome b gene was carried out using forward 5'-CTC CTG GGA GTC TGC CTA ATC-3' (L *Cytb* (F) [8]) and reverse 5'-GTC TTT GTA GGA GAA GTA TGG-3' primers [8]. Intron 1 of the serum albumin gene was amplified with forward 5'-TCC ATA CAT GTG CTA AGT AGG TT-3' and reverse 5'-GAC GGT AAG GGG ACA TAA TTCA-3' primers [9]. DNA was sequenced using an ABI PRISM 3130 XL genetic analyzer (Applied Biosystem, USA).

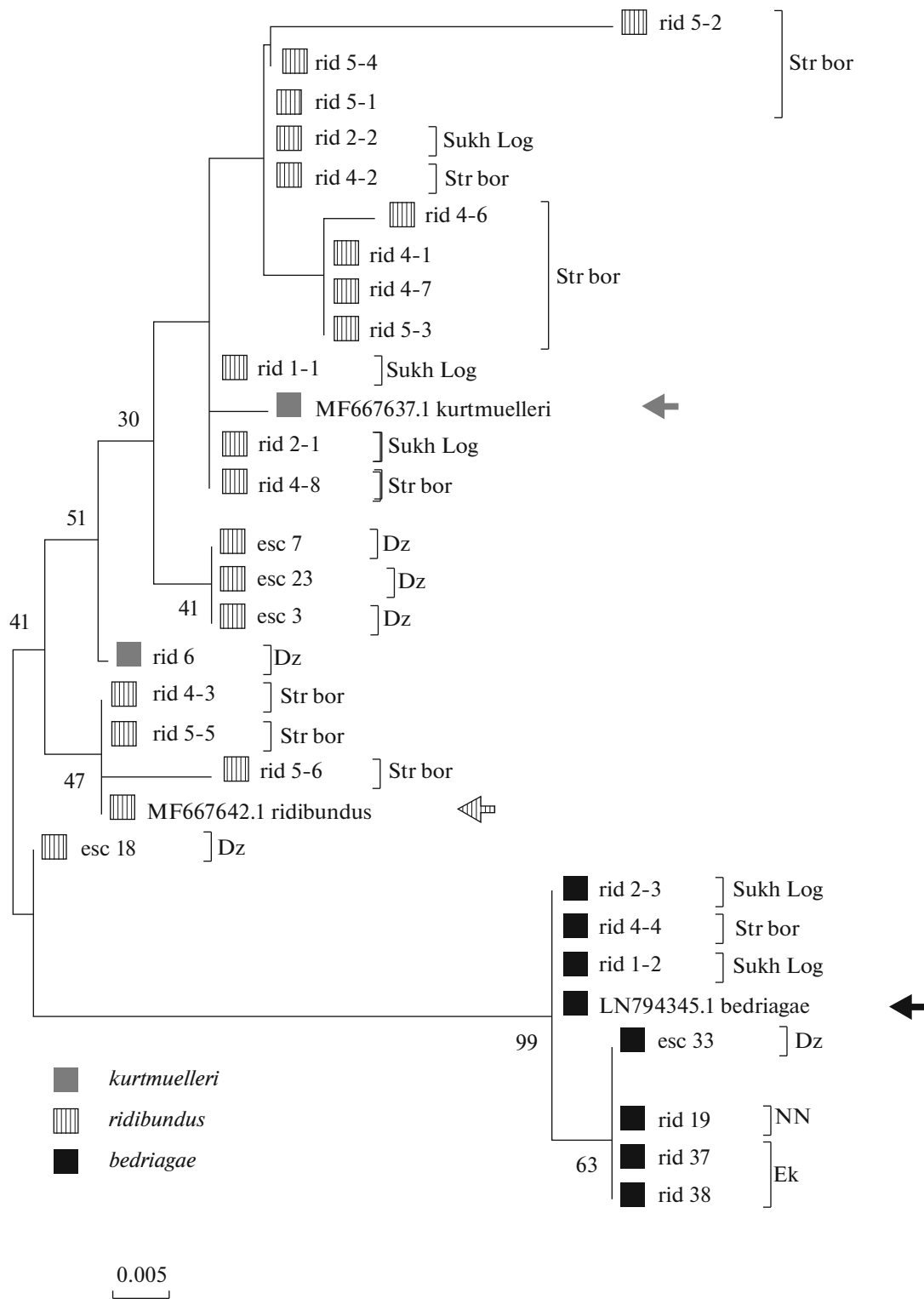
The species ratio in the Nizhny Novgorod samples was 74% pool frogs; 18% marsh frogs, and 8% edible frogs. These findings support the existence in Nizhny Novgorod oblast (in the city of Nizhny Novgorod and the city of Dzerzhinsk) of the population system belonging to the R–E–L (*ridibundus*–*esculentus*–*lessonae*) type, where the proportion of *P. esculentus* is less than 10% [10].

The territory of Sverdlovsk oblast is inhabited exclusively by marsh frogs. It was demonstrated that, with respect to nuclear DNA marker sequences, animals from the Yekaterinburg population corresponded to the eastern groups of *P. cf. bedriagae*. Only one individual (F19) fell into the clade of Nizhny Novgorod samples (Striginsky Bor). The second sample of marsh frogs from Sverdlovsk oblast (city of Sukhoi Log) was

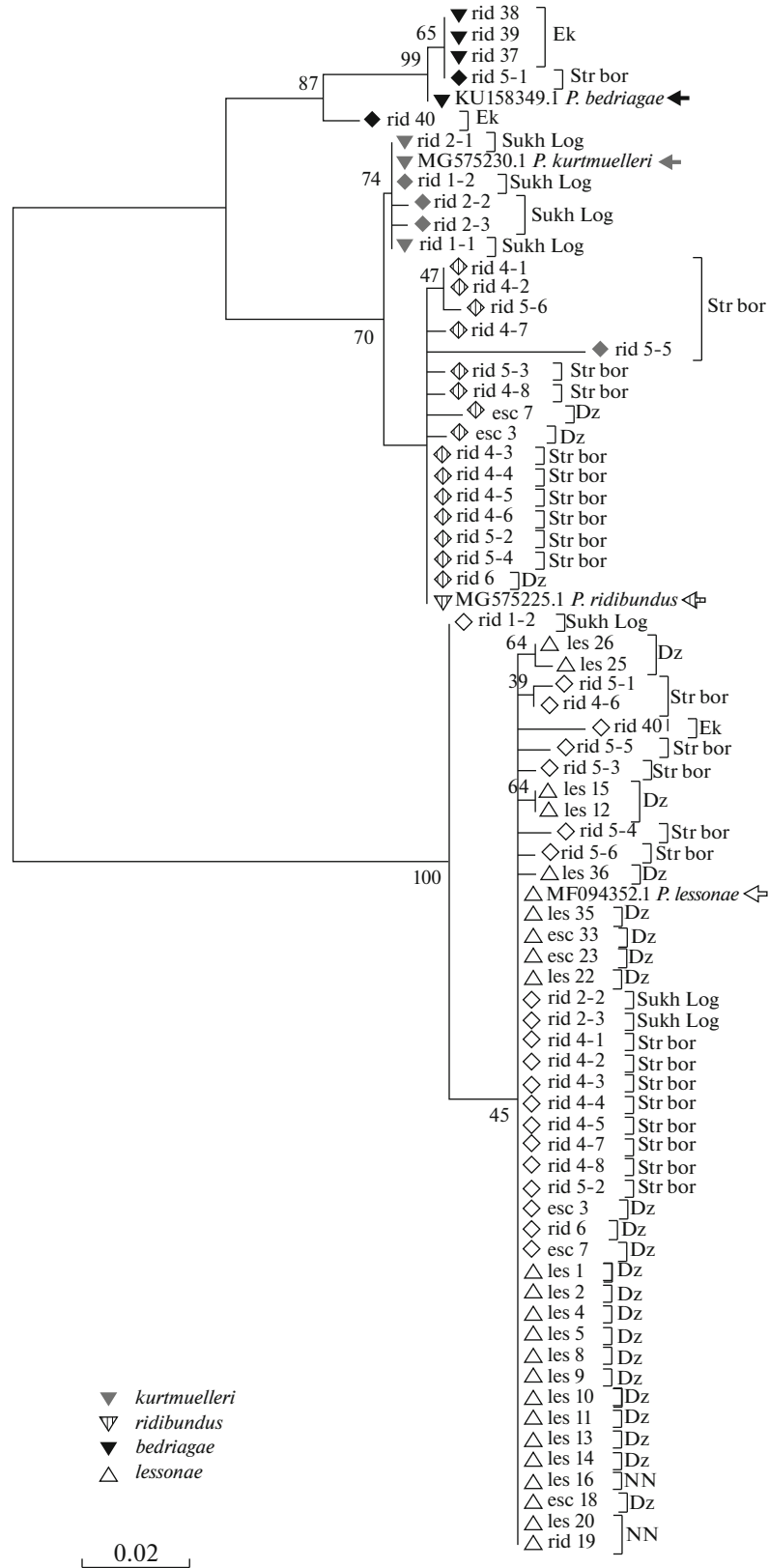
heterogeneous with respect to the nuclear genome and consisted of two individuals (rid 1-2 and rid 2-3) belonging to the “eastern” form of *P. ridibundus* (*P. cf. bedriagae*), while three individuals (rid 1-1, rid 2-1, and rid 2-2) belonged to the “western” form (*P. ridibundus* sensu lato or *P. kurtmuelleri*). According to the mitochondrial DNA marker sequences (*Cyt b*), three out of four animals (rid 37, rid 38, and rid 39) from the Yekaterinburg sample corresponded to the “Anatolian” *P. bedriagae* inhabiting the eastern part of the range. In one individual (rid 40), heteroplasmy was detected, which suggested crossing with pool frog and introgression, which could have occurred in the history of the studied forms. However, this individual also showed slightly lower homology with the sequence of *P. bedriagae*, which enables one to suggest its different origin from different lines. The mitochondrial genomes of all individuals from the Sukhoi Log sampling (rid 1-1–rid 2-3) have mtDNA of the “Balkan” form of *P. kurtmuelleri*, while in three animals (rid 1-2, rid 2-2, and rid 2-3) heteroplasmy is detected (the presence of the marsh frog haplotype). Heteroplasmy, i.e., the presence of more than one type of mitochondrial genome in the organism, is often found in both animals and plants [11], as well as in hybridogenetic complex of *Pelophylax* spp. [12].

With respect to the nuclear genome, marsh frogs from the suburb of Nizhny Novgorod (Striginsky Bor), with the exception of one individual (rid 19, Fig. 1) belonging to “eastern” *P. bedriagae*, carry the markers of the “western” form (*P. ridibundus*). The mitochondrial genomes of this sample (rid 4-1–rid 6), with the exception of one individual (rid 19) having a combination of the *P. bedriagae* and *P. lessonae* haplotypes, carry “western” mtDNA in combination with the *P. lessonae* haplotype (Fig. 2). One marsh frog (rid 19, Fig. 2), caught there in 2017, with the “western” nuclear genome, turned out to be a carrier of mtDNA with the haplotype of pool frog. This finding is the evidence of the introgression of genetic material (mtDNA) from pool frog, which is often detected in the center of the range [13]. A single *P. ridibundus* individual (rid 6) from the outskirts of Dzerzhinsk was the carrier of heterogeneous nuclear genome of the Balkan form and *P. kurtmuelleri* with mtDNA heteroplasmy (*P. lessonae* and *P. ridibundus*).

Five hybrid *P. esculentus* individuals were identified on the basis of serum albumin gene intron 1 and two other genes, *SOX3* and *Rhodopsin*. In two animals (esc 3 and esc 7), mtDNA heteroplasmy (*P. ridibundus* and *P. lessonae*) was detected, while the other three frogs (esc 23, esc 33, esc 18; Fig. 2) had only the *P. lessonae* haplotype. The nuclear genomes of the hybrids (esc 3, esc 7, esc 18, and esc 23) in five cases contained DNA markers of the “western” form of marsh frog, *P. ridibundus* or *P. kurtmuelleri*, and in one individual (esc 33), haplotype of *P. bedriagae* was identified (Fig. 2).



**Fig. 1.** Standard ML dendrogram of the distances of marsh frogs and hybrids (*P. esculentus*) inferred from the sequence data on first intron of the serum albumin gene (*SAI1* gene) and constructed using the maximum likelihood algorithm. The arrows indicate standard gene sequences for the corresponding forms taken from GenBank. Species: rid, lake frog; esc, edible frog; les, pool frog; numbers indicate the individual number. Habitats: NN, Nizhny Novgorod; Dz, Dzerzhinsk; Ek, Yekaterinburg; Str bor, Striginsky Bor; Sukh Log, Sukhoi Log.



**Fig. 2.** The distances of the studied *Pelophylax* individuals obtained by the method of maximum likelihood on the basis of the cytochrome *b* gene (*Cyt b*). The arrows indicate standard sequences of the gene for the corresponding forms taken from GenBank. The diamond indicates heteroplasmy. For designations, see the caption to Fig. 1.

Hence, green frog samples from Nizhny Novgorod oblast are characterized by a great variety of combinations of nuclear genomes (RR, BB, KK, RL, BL, LL: R, *ridibundus*; B, *bedriagae*; K, *kurtmuelleri*; L, *lessonae*) and fewer variants of mitochondrial heteroplasmy (RL, BL). On the other hand, specimens from Sverdlovsk oblast (represented by a smaller number of animals) demonstrated high heterogeneity of the mitochondrial genome (BB, RL, KK, KL) under a lower variety of nuclear genome variants (BB, RR). High mitotype diversity of Middle Ural populations of marsh frogs indicates the repeated introductions of animals from different sources, as we suggested in our earlier study [14].

It is known that stealing of alien mitochondria of one of the parental species during hybridogenesis and subsequent introgression can increase the adaptive potential in the forms accepting these mitochondria [11, 15].

Thus, the obtained data characterize the state of population systems and also the history of the formation of the modern phylogeography of green frogs from the studied territories.

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#### COMPLIANCE WITH ETHICAL STANDARDS

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

#### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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