Research Article

A record of alien *Pelophylax* species and widespread mitochondrial DNA transfer in Kaliningradskaya Oblast’ (the Baltic coast, Russia)

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Abstract

Alien species can strongly impact local environments and compete against native species, which can lead to their extinction. Marsh frogs of the *Pelophylax ridibundus* complex are one of the most invasive amphibians in Northern Eurasia. It was previously thought that three water frog species of the genus *Pelophylax* (the marsh frog, *P. ridibundus*, the pool frog, *P. lessonae* and their hemiconal hybrid, the edible frog, *P. esculentus*) inhabited Kaliningradskaya Oblast’ along the Russian Baltic coast. However, based on our study of the intron-1 of the nuclear serum albumin gene, two other marsh frog species were detected (the Balkan marsh frog, *P. kurtmuelleri*, and the Anatolian marsh frog, *P. c.f. bedriagae*) as well as putative hybrids between *P. ridibundus* and *P. c.f. bedriagae*. The majority of individuals of *P. ridibundus* and hybrids between *P. ridibundus* and *P. c.f. bedriagae* had mitochondrial (mt) DNA of *P. lessonae*, while all others featured the *P. kurtmuelleri* mtDNA. The prevalence of *P. lessonae* mtDNA haplotypes in populations of *P. ridibundus* from the Baltic Coast of Russia suggests that local individuals of the latter species originated from crosses between *P. esculentus* individuals. Two hypotheses could explain the records of *P. kurtmuelleri* and *P. c.f. bedriagae* in the region. The establishment of local populations of the first species could have occurred via postglacial dispersal from the Balkan refugium. The origin of local *P. c.f. bedriagae* could be an occasional introduction of individuals from the Ponto-Caspian region. Since our study is preliminary (19 individuals), in the future it would be important to continue the study of water frogs in Kaliningradskaya Oblast’ and neighboring countries by applying multiple genetic markers. Additional genetic markers will enable researchers to study routes of dispersal and introductions of marsh frogs, to clarify peculiarities of their hybridization and distribution, and to evaluate the impact of *P. kurtmuelleri* and *P. c.f. bedriagae* on the reproduction success of hybridogenous populations and abundance of local amphibians.

Key words: *Pelophylax* c.f. bedriagae, *Pelophylax kurtmuelleri*, invasive species, introduction, hybridolysis, hybridization, postglacial dispersal

Introduction

Alien species, which were introduced by man outside their natural ranges, can strongly impact local environments and compete against native species, which can lead to their extinction (Kraus 2009, 2015; Bucciarelli et al. 2014). Several nonnative amphibian species are known in Europe, among
which the most well known are the African clawed frog, *Xenopus laevis* (Daudin, 1802), and the American bullfrog, *Lithobates catesbeianus* (Shaw, 1802) (Ficetola et al. 2007; Measey et al. 2012).

The genus *Pelophylax* Fitzinger, 1843 consists of about 22 water frog species distributed predominantly throughout the Palearctic (Frost 2020). The taxonomic status of some of them is under discussion (i.e., Lymberakis et al. 2007; Largen and Spawls 2010). The most complicated situation is with marsh frogs of the *P. ridibundus* complex, which includes numerous closely-related cryptic lineages (Plötnner and Ohst 2001; Akin et al. 2010; Plötnner et al. 2012). Several of these lineages (e.g., Syrdaryan, Anatolian, Euphrates, Cilician, Iranian) are yet to receive a formal taxonomic description (Mezhzherin and Peskov 1992; Plötnner and Ohst 2001; Pesarakloo et al. 2016). Marsh frogs are considered one of the most invasive amphibians of Northern Eurasia (Zeisset and Beebee 2003; Duysebaeva et al. 2005; Bashinskiy et al. 2018; Bellati et al. 2019). Several cryptic marsh frog species were introduced to European countries (Supplementary material Table S1).

For example, the Balkan marsh frog, *P. kurtmuelleri* (Gayda, 1940), was recorded in the Czech Republic, Switzerland, France, Italy, Ukraine, and some regions of Russia (Lanza 1962; Bellati et al. 2013; Laghi et al. 2013; Akin Peksen 2015; Dufresnes et al. 2017, 2018; Bellati et al. 2019; Bisconti et al. 2019; Ivanov 2019; Vershinin et al. 2019), despite the fact that the native range of the frog is restricted to the Balkan Peninsula (Figure 1). Some authors have indicated the presence of alleles and/or haplotypes of the species in the Baltic Region in Latvia, Lithuania and Poland (Plötnner et al. 2008; Hauswaldt et al. 2012; Kolenda et al. 2017).

Another species, the Anatolian marsh frog (*P. cf. bedriagae*), was introduced to Italy, Belgium, France, Switzerland, Germany, and some
regions of Russia (Holsbeek et al. 2008, 2009, 2010; Ohst 2008; Dubey et al. 2014; Dufresnes et al. 2018; Lyapkov et al. 2018; Bellati et al. 2019; Ivanov 2019; Vershinin et al. 2019), including the vicinities of St. Petersburg City in the Baltic region of Russia (Ohst 2008; Akin et al. 2010). The species naturally occurs in western Iran, Turkey, the Caucasus, Bulgaria, eastern Greece, western Kazakhstan, southern and eastern Ukraine, the Crimea, and the Volga River region of Russia (Figure 1).

Hybridization between various water frog species is quite common (Plötner et al. 2010). Some events can lead to mitochondrial (mt) DNA transfer into other species. Such transfer is mediated by fertile hybrids that transmit their maternal mtDNA to the paternal gene pool via backcrosses with males of the paternal parental species (Plötner et al. 2008). In western Poland, Spolsky and Uzzell (1984) were the first to reveal individuals of the marsh frog, *P. ridibundus* (Pallas, 1771), with mtDNA of the pool frog, *P. lessonae* (Camerano, 1882). Plötner et al. (2008) noted that 34% of individuals of *P. ridibundus* in Europe were characterized by the *P. lessonae* mtDNA. In Belgium, Ukraine and European Russia numerous populations of *P. ridibundus* possessed mtDNA of *P. cf. bedriagae* and vice versa, i.e. *P. cf. bedriagae* can have the mtDNA of *P. ridibundus* (Holsbeek et al. 2008, 2009; Ermakov et al. 2013, 2014; Ivanov et al. 2015; Svinin et al. 2015; Hoffmann et al. 2015; Zamaletdinov et al. 2015). The mtDNA of the Karpathos marsh frog, *P. cerigensis* (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994), was found in a population of *P. cf. bedriagae* from Kaş in southwestern Turkey (Ohst 2008; Akin et al. 2010; Plötner et al. 2012). Sánchez-Montes et al. (2016) reported that two populations of *P. ridibundus* in northwestern Spain (Prades and Oix) have the mtDNA of the Iberian frog, *P. perezi* (López-Seoane, 1885). In southern France (Lac de Condamine), Dufresnes et al. (2017) found an introduced population of *P. kurtmuelleri* with the mtDNA of *P. perezi*. Finally, in a population from European Russia, Ivanov et al. (2019) recently described a case of mtDNA transfer from *P. cf. bedriagae* into *P. lessonae*.

Three species of water frogs (*P. ridibundus*, *P. lessonae*, and their hemiagonal hybrid, the edible frog, *P. esculentus* (Linnaeus, 1758)) inhabit Kaliningradskaya Oblast’ of Russia (the northern part of the historical East Prussia) (Litvinchuk et al. 2015). The pool and edible frogs are widespread and form hybridogenous systems throughout Kaliningradskaya Oblast’. The marsh frog is rarer. Its populations in the region are located on the northern border of the species range. The species distribution is restricted to the westernmost part of the Oblast’ and consists of two isolated parts. The northern part extends along the shores of the Curonian Lagoon and Neman River, where the species forms mixed hybridogenous systems with *P. esculentus* and sometimes *P. lessonae* (Litvinchuk et al. 2015). The southern part is located near the Vistula Lagoon where local marsh frogs did not usually co-occur with *P. esculentus* and *P. lessonae* (Litvinchuk et al.
Table 1. List of individuals studied, numbers and names of localities, years when individuals were collected, numbers of specimens stored in herpetological collections of the Zoological Institute of Russian Academy of Sciences (ZISP), presence of other water frog species (esc is Pelophylax esculentus and les is P. lessonae) in the locality, geographic coordinates ("Lat" is latitude and "Long" is longitude), results of marsh frog species identification (rid is P. ridibundus, kurt is P. kurtmuelleri, bedr is P. cf. bedriagae, and rid/bedr are individuals which have alleles of both P. ridibundus and P. cf. bedriagae), and accession numbers of sequences. nDNA is nuclear and mtDNA is mitochondrial DNA markers. SAI-1 is the intron-1 of the nuclear serum albumin gene and ND2 is the subunit 2 of mitochondrial NADH dehydrogenase gene.

| N  | Locality   | Date   | ZISP number | Other species | Coordinates | Results | Accession numbers |
|----|------------|--------|-------------|---------------|-------------|---------|------------------|
|    |            |        |             |               | Lat, N      |         |                  |
|    |            |        |             |               | Long, E     |         |                  |
| 1  | Sovetsk    | 2014   | 14233       | esc+les       | 55.094      | 21.844  | –                 | –               |
| 2  | Morskoe    | 2018   | 14244       | –             | 55.228      | 20.920  | rid               | les             |
| 3  | Rybachiy   | 2014   | 14237       | esc           | 55.157      | 20.844  | rid               | kurt            |
| 4  | Rybachiy   | 2018   | 14241       | esc           | 55.157      | 20.844  | rid               | kurt            |
| 5  | Rybachiy   | 2018   | 14242       | esc           | 55.157      | 20.844  | rid               | les             |
| 6  | Zelenogradsk | 2018  | 14243       | esc           | 54.952      | 20.484  | rid               | les             |
| 7  | Kaliningrad | 2014  | 7025.702    | –             | 54.691      | 20.512  | rid/bedr          | les             |
| 8  | Kaliningrad | 2014  | 14236       | –             | 54.691      | 20.512  | bedr              | kurt            |
| 9  | Ushakovo    | 2014   | 14238       | –             | 54.612      | 20.243  | kurt              | kurt            |
| 10 | Baltiysk   | 2002   | 7025.538    | –             | 54.635      | 19.874  | rid               | kurt            |
| 11 | Baltiysk   | 2002   | 7025.539    | –             | 54.635      | 19.874  | kurt              | –               |
| 12 | Baltiysk   | 2014   | 7025.605    | –             | 54.635      | 19.874  | rid/bedr          | les             |
| 13 | Baltiysk   | 2018   | 14240       | –             | 54.635      | 19.874  | rid               | les             |
| 14 | Mamonovo   | 2014   | 14230       | –             | 54.449      | 19.952  | rid               | les             |
| 15 | Mamonovo   | 2014   | 14231       | –             | 54.449      | 19.952  | rid/bedr          | les             |
| 16 | Mamonovo   | 2014   | 14232       | –             | 54.449      | 19.952  | rid               | les             |
| 17 | Mamonovo   | 2014   | 14234       | –             | 54.449      | 19.952  | rid               | –               |
| 18 | Mamonovo   | 2014   | 14235       | –             | 54.449      | 19.952  | rid               | –               |

2015). In addition, a presumably introduced population of the marsh frog (syntopic with P. esculentus) is known from the southern part of the Oblast in fish ponds near the town of Pravdinsk (Borkin et al. 1986; Litvinchuk et al. 2015).

No molecular studies have been specifically conducted on marsh frogs from the Baltic Coast of Russia. Therefore, the aim of our paper was to study the genetic variation, using a multilocus approach, of local marsh frogs and describe records of alien marsh frog species and interspecies mtDNA transfer.

Materials and methods

Pieces of femur muscle from herpetological collections of the Zoological Institute of Russian Academy of Sciences (fixed by 96% ethanol and stored in 70% ethanol) were used as tissue samples. We studied 19 marsh frog specimens collected from 2002 to 2018 in eight localities from Kaliningradskaya Oblast’ (Table 1, Figure 2). The DNA was extracted by the standard salt-extraction method (Aljanabi and Martinez 1997).

The primary identification of alleles of the intron-1 of the nuclear serum albumin gene (SAI-1) of three marsh frog species (P. ridibundus, P. kurtmuelleri and P. cf. bedriagae) was performed using the methods described by Hauswaldt et al. (2012) and Ermakov et al. (2019). The method described by Ermakov et al. (2019) was used to identify the mtDNA (the COI gene fragment) of P. ridibundus and P. cf. bedriagae. To identify
haplotypes of *P. kurtmuelleri*, we used an endonuclease restriction analysis. The COI gene fragment (744 bp) was amplified using UTF 5′-TGT AAA ACG ACG GCC AGT TCT CAA CCA AYC AYA ARG AYA TYG G-3′ and UTR 5′-CAG GAA ACA GCT ATG ACT ARA CTT CTG GRT GKC CRA ARA AYC A-3′ (Lissovsky et al. 2010) primers at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s (30 cycles). The PCR reaction mixture (25 μL) contained 50–100 ng of DNA, 0.5 μM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 2.5 μL 10×PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), and 2 units of *Taq* polymerase (Thermo Scientific). The PCR fragments obtained were digested with the restriction endonuclease *Bme*1390I (site CCTGG at 5917 position in the *P. kurtmuelleri* mitochondrion (NC_026895); Hofman et al. 2016) for 2–4 h at 37 °C (2–4 enzyme units to 2–4 μl of amplification mixture). After restriction obtained fragments had different lengths: 388 bp for *P. kurtmuelleri* and 354 bp for *P. ridibundus* and *P. cf. bedriagae*.

Selective sequencing was used to verify the primary identification results. The nuclear SAI-1 gene fragment was sequenced in eight specimens and the subunit 2 of mitochondrial NADH dehydrogenase (*ND2*) gene in 10 specimens (Table 1). Sequencing of fragments was performed on an ABI
3500 automatic sequencer (Applied Biosystems) using the BigDye® Terminator 3.1 (Applied Biosystems) kit, and the same primers that were used for amplification. The ND2 gene sequence (1038 bp) was amplified with use of the universal primer ND2L1 5'-AAG CTT TTG GGC CCA TAC CCC-3' (Meyer 1993) and a developed specific primer ND2H1 5'-GCA AGT CCT ACA GAA ACT GAA G-3'. The following amplification methods were used: initial denaturation for 1 min at 95 °C, followed by 32 cycles of 94 °C for 30 s, 60 °C for the ND2 and 53 °C for the SAI-1 pair of primers for 30 s, 72 °C for 60 s, and final extension for 5 min at 72 °C. The PCR reaction mixture proportions were the same as for amplification of the COI gene fragment. The sequences obtained have been deposited in GenBank (ND2 gene no. MN271951–MN271956 and SAI-1 gene no. MN497957–MN497961).

The nucleotide sequences were aligned both with BioEdit (Hall 1999) software and manually. We used MEGA v. 7.0. software (Kumar et al. 2016) for data processing. For constructing the phylogenetic tree, the maximum likelihood (ML) method was used. The most appropriate DNA substitution model for the datasets was established using jModelTest 2.1.10 (Posada 2008). The ML trees were created with the Hasegawa-Kishino-Yano model for ND2 gene, gamma distributed (HKY+G) (–lnL = 3239.07, BIC = 8313.13) and Tamura-Nei model for SA gene (T92) (–lnL = 1300.32, BIC = 3611.79). Node support values in phylogenetic trees were estimated according to bootstrap support (500 replicates).

Maps of native ranges of *P. ridibundus*, *P. kurtmuelleri*, *P. cf. bedriagae*, and *P. esculentus* (Figure 1) are based on previously published nuclear (n) DNA data (Ohst 2008; Akin et al. 2010; Plötner et al. 2012; Ermakov et al. 2013, 2014, 2016a, b; Akin Peksen 2015; Ivanov et al. 2015, 2019; Svinin et al. 2015; Zamaletdinov et al. 2015; Fayzulin et al. 2017, 2018; Kolenda et al. 2017; Kukushkin et al. 2018; Ivanov 2019).

**Results**

Despite the fact that our data are preliminary (only 19 individuals were studied), based on the analysis of the nuclear SAI-1 fragment, we were able to detect alleles of the following three marsh frog species in Kaliningradskaya Oblast’ (Table 1): *P. ridibundus* (13 individuals; 68%; six localities), *P. kurtmuelleri* (n = 2; 11%; two localities) and *P. cf. bedriagae* (n = 1; 5%; three localities). The remaining three individuals (16%; three localities) contained alleles of both *P. ridibundus* and *P. cf. bedriagae*. Putative hybrids of *P. kurtmuelleri* with other marsh frog species were not found.

The following peculiarities in geographical distribution of the species were revealed. Populations distributed in the Curonian Lagoon and Neman River (localities 1–4 in Figure 2) only featured *P. ridibundus* nDNA alleles. Populations located around the Vistula Lagoon (localities 5–8 in Figure 2) were composed of individuals with nDNA alleles of all three marsh
frog species. Ponds in Yuzhnyi park in the center of Kaliningrad City (Figure 3A) were inhabited by *P. cf. bedriagae* and their hybrids with *P. ridibundus* (Figure 4). An individual of *P. kurtmuelleri* was collected in a drainage channel in Ushakovo settlement (Figure 3B). The system of shallow quarry ponds in the vicinities of Mamonovo Town (Figure 3C) were populated by *P. ridibundus* and their putative hybrids with *P. cf. bedriagae*. Individuals of *P. kurtmuelleri*, *P. ridibundus*, and putative hybrids *P. ridibundus* and *P. cf. bedriagae* inhabited a brakish fort moat in the Vistula Spit in Baltiysk Town (Figure 3D).

According to our data, marsh frogs in Kaliningradskaya Oblast’ possessed the mtDNA of the following two species only: *P. kurtmuelleri* (n = 8; 42%; 5 localities) and *P. lessonae* (n = 11; 58%; 6 localities). Only two individuals identified by the nuclear SAI-1 fragment as *P. kurtmuelleri* had the conspecific mtDNA. The other 17 individuals were characterised by non-conspecific mitochondrial genomes. The majority of individuals of *P. ridibundus* (n = 8; 62%; five localities) had the mtDNA of *P. lessonae*. Five individuals of *P. ridibundus* (38%; three localities) and an individual of *P. cf. bedriagae* carried the *P. kurtmuelleri* mtDNA. All three putative
hybrids (*P. ridibundus* × *P. cf. bedriagae*) had the mtDNA of *P. lessonae*. Marsh frogs with mtDNA haplotypes of *P. lessonae* only were detected in three localities (Figure 2b: 2, 4 and 8), *P. kurtmuelleri* in two localities (1 and 6), and both species in three localities (3, 5 and 7). The *P. lessonae* mtDNA was more frequent in populations located around the Vistula Lagoon (67%) than near the Curonian Lagoon and in Neman River (43%).

The phylogenetic analysis based on the nuclear *SAI-1* gene fragment (Figure 5) showed that local *P. kurtmuelleri* alleles were most similar to the diversity found in Poland. The individual of *P. cf. bedriagae* from Kaliningradskaya Oblast’ was similar to Anatolian and West-Kazakhstan individuals of the species. The genetic differences between *P. ridibundus* and *P. kurtmuelleri* (*p*-distance 0.8 ± 0.3%) were less than between *P. cf. bedriagae* with *P. ridibundus* and *P. kurtmuelleri* (4.3 ± 0.7% and 4.8 ± 0.8%, respectively).

The phylogenetic analysis of the mitochondrial *ND2* gene fragment (Figure 6) showed that the *P. kurtmuelleri* mtDNA from Kaliningradskaya Oblast’ was closely related to haplotypes sequenced in conspecific individuals from Macedonia, as well as in *P. ridibundus* from Latvia, Ukraine and Romania. The *P. lessonae* mtDNA found in marsh frogs from the Baltic Coast of Russia was quite similar to the mtDNA in European populations of the pool frog. The genetic differences between the mtDNA clades of *P. ridibundus* and *P. kurtmuelleri* were 1.2 ± 0.3%. *P. lessonae* differed from *P. ridibundus* and *P. kurtmuelleri* on 14.1 ± 0.1% и 14.3 ± 0.1%, respectively.
Figure 5. Dendrogram of phylogenetic relationships within *P. kurtmuelleri*, *P. ridibundus* and *P. cf. bedriagae* inferred from sequence analysis of the nuclear DNA S4I-1 gene by the maximum likelihood (ML) method. Bootstrap support values higher than 80% are shown. Full circles represent our data and empty circles were data obtained from GenBank.

Discussion

The history of marsh frog populations in Kaliningradskaya Oblast’ is unknown. These populations might have existed in the region for a long time. The first reliable record could be attributed to Muhling (1898), who found “*Rana esculenta* var. *ridibunda*” in Baltiysk (“Pillau”) Town. Le Roi (1903) suggested that water frogs from the Rybachiy (“Rossitten”) settlement might belong to “*Rana esculenta* var. *ridibunda*”. Pagast (1941) mentioned records of marsh frogs (“*Rana ridibunda*”) in the vicinities of Kaliningrad.
Figure 6. Dendrogram of phylogenetic relationships among haplotypes within *P. kurtmuelleri*, *P. ridibundus*, and *P. lessonae* inferred from sequence analysis of the mitochondrial ND2 gene by the maximum likelihood (ML) method. Bootstrap support values higher than 80% are shown. Full circles represent our data and empty circles were data obtained from GenBank. Triangles indicate specimens of *P. lessonae*, while circles indicate specimens of the *P. ridibundus* complex.
Alien *Pelophylax* species on the Baltic Coast

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Königsberg”) City, the Vistula (“Frischen”) and Curonian (“Kurischen”) lagoons. Thus, we can assume that, at a minimum, *P. ridibundus* has inhabited the region for more than a century. However, it is obvious that the age of the Baltic Coast populations of *P. ridibundus* should be much older because the species inhabits some islands in the Baltic Sea, which have been isolated from the mainland for more than 9,000 years (Ojaveer 2017).

Two hypotheses could explain the origin of *P. kurtmuelleri* and *P. cf. bedriagae* in the region. They could be recently introduced or a relic of previous distributions of species in northern Europe. Some indirect evidence supports the latter proposal. Records of two isolated caudate amphibian species with a more southern distribution exist in the region (Litvinchuk 1996; Jakóbik et al. 2019). These are the Alpine newt, *Ichthyosaura alpestris* (Laurenti, 1768), and the fire salamander, *Salamandra salamandra* (Linnaeus, 1758). However, these records are exclusively associated to relic beach forest massifs. Additionally, the Baltic populations of the green toad, *Bufo* *viridis* (Laurenti, 1768), bear mtDNA of a southern species, the Anatolian *B. sitibundus* (Pallas, 1771). Perhaps, expanding populations of *B. viridis* captured the *B. sitibundus* mtDNA in Balkan refugium before its postglacial dispersal throughout the Baltic Region (Dufresnes et al. 2019). The same capture of genes of *P. kurtmuelleri* and *P. cf. bedriagae* appears possible for *P. ridibundus* which have a glacial refugium in the Balkans.

This hypothesis of a genetic exchange between species before a postglacial dispersal seems most plausible for *P. kurtmuelleri*, whose distributional range in the Balkans overlaps with *P. ridibundus* (Figure 1). Recent records of alleles and/or haplotypes of *P. kurtmuelleri* in populations of *P. ridibundus* through European Russia, Ukraine, Belarus’, Lithuania, Latvia, and Poland (Plötner et al. 2008; Hauswaldt et al. 2012; Kolenda et al. 2017; Lukonina et al. 2019; Vershinin et al. 2019) could support this proposal. However, the relic hypothesis seems unlikely for *P. cf. bedriagae* because no records of alleles and haplotypes of the species were found in Poland or the Baltic Republics located around Kaliningradskaya Oblast’ (Plötner et al. 2008; Hauswaldt et al. 2012; Kolenda et al. 2017). Therefore, an occasional introduction seems to be more credible for *P. cf. bedriagae*.

Marsh frogs are often introduced as a food source (i.e., consumption of frogs legs), to stock garden ponds, a result of dispersal through newly created waterways, laboratory animals for teaching and study at universities, and occasionally with fish fry (Duysterbaeva et al. 2005; Kuzmin 2013; Bisconti et al. 2019). The first three pathways of introductions are unrealistic for the species studied here because Kaliningradskaya Oblast’ has not recently created any large water channels and local people do not use marsh frogs as a food source or to stock garden ponds. However, the last two pathways could be introduction vectors for these species in this region. The Baltiyskiy Federal University (Kaliningrad) is the only educational...
institution which uses local and regionally collected water frogs for teaching purposes, and frogs could be released into natural water bodies in Kaliningrad. Similar marsh frog introductions were documented in 1961 in Ust’-Kamennogorsk (Duysebaeva et al. 2005), in 1970 in Gorno-Altaisk (Yakovlev and Malkov 1985; Yakovlev 1987), and in 1972 in Yakutsk (Belimov and Sedalischev 1980). Usually, marsh frogs are collected from the Volga River delta, which has the highest abundances in Russia, for teaching in universities located in the North-Western Region of European Russia. According to Ivanov (2019), based on the analysis of the nuclear SAI-1 fragment, marsh frogs from the Volga River delta region (Republic of Kalmykia and Astrakhanskaya Oblast’, Russia) are represented by both *P. ridibundus* and *P. cf. bedriagae* (and their hybrids). Therefore, the Volga River delta region could be a source for the introduction of *P. cf. bedriagae* to Kaliningradskaya Oblast’.

The second realistic pathway for the introduction of *P. cf. bedriagae* to the Baltic Region of Russia is an occasional release of tadpoles with juvenile fish. Since the 1940s the European carp (*Cyprinus c. carpio*) has been intensively aquacultured throughout Russia. For example, thousands of fish were released between 1953 and 1955 into the Curonian Lagoon in Kaliningradskaya Oblast’ (Kudersky 2001; Khainovsky and Ulianov 2015). The native range of the European carp is the Ponto-Caspian region (Tsepkin 2003), where the majority of fish fry rearing ponds are located; fry produced in the Ponto-Caspian region are then transported throughout Russia to stock fish farms. Marsh frog introductions related to fish reservoir stocking have been previously observed from the Altayskiy Kray and Alakol and Issyk-Kul lakes in the 1960s (Yakovlev and Malkov 1985; Duysebaeva et al. 2005; Kuzmin 2013) and the Krasnoyarskiy Kray and Republic of Khakassia in Siberia in the 1970s and 1980s (Chuprov 2013). Marsh frogs from the Reftinskoe Reservoir in the Ural Mountains (Russia) were occasionally introduced with fish fry in the 1970s from Krasnodarskiy Kray in the Western Caucasus (Ivanova and Berzin 2019). Both *P. ridibundus* and *P. cf. bedriagae* (and their hybrids) inhabit the Ponto-Caspian Region of Russia (Ermakov et al. 2014; Ivanov et al. 2015; Ermakov et al. 2016a, b; Ivanov 2019). Therefore, the region could be a source for introduction of *P. cf. bedriagae* to Kaliningradskaya Oblast’.

Marsh frogs prefer open landscapes. Since the start of the Holocene, the eastern part of the Baltic Region has been covered by closed forest massifs (Smirnova and Turubanova 2004), which are usually populated by *P. lessonae* and *P. esculentus*. The prevalence of the *P. lessonae* mtDNA in marsh frogs from the Baltic Coast of Russia may suggest that the territory was originally inhabited by mixed populations of *P. lessonae* and *P. esculentus*, in which the latter species usually produced gametes of *P. ridibundus* (reviewed by Plötner 2005). Sporadically, viable individuals of *P. ridibundus* may be produced in such populations as a result of crosses between *P. esculentus*.
individuals (hybridolysis; see details in Plötner 2005 and Dubey et al. 2019). Usually, *P. ridibundus*, which overwinters in water, do not survive in waterbodies populated by *P. lessonae* and *P. esculentus* (both of which, as a rule, overwinter on land) due to freezing of the water and low concentrations of dissolved oxygen (Berger 1984; our data). However, *P. ridibundus* tolerates brackish water and can survive in deep lagoons of the Baltic Sea (Litvinchuk et al. 2015). Such individuals resulting from hybridolysis should carry the mtDNA of *P. lessonae* because the parental *P. esculentus* have perpetuated through hybridogenesis with *P. lessonae* only. Similar populations of *P. ridibundus* carrying the *P. lessonae* mtDNA were found in the Czech Republic, Slovakia, Switzerland, Germany, Poland, and the Danish Island of Bornholm in the Baltic Sea (Plötner et al. 2008; Hofman et al. 2012; Mikulíček et al. 2014; Dubey et al. 2014; Hoffmann et al. 2015; Hawlitschek et al. 2016; Dufresnes et al. 2018). Populations of Kaliningradskaya Oblast’ are now the northeasternmost records of this phenomenon.

The presumed hybridization of *P. kurtmuelleri* and *P. ridibundus* (which carried the *P. lessonae* mtDNA) in the Baltic Region of Russia leads to coexistence of mtDNA genomes of both *P. kurtmuelleri* and *P. lessonae* in local populations of *P. ridibundus*. The absence of the *P. cf. bedriagae* mtDNA in local marsh frog populations could indicate selection due to local environmental conditions and/or drift. Individuals of *P. cf. bedriagae* (and their hybrids) with the mtDNA of *P. kurtmuelleri* have been previously detected in introduced marsh frog populations in Belgium (Holsbeek et al. 2008, 2009).

The release of alien water frogs in Kaliningradskaya Oblast’ can have several negative consequences, but the threat of genetic introgression is the greatest among them. Hybridization between the alien species (*P. cf. bedriagae*) and native species (*P. ridibundus* and presumably *P. kurtmuelleri*) can be common here. The result of this is replacement or local extinction of native species by introgressive hybridization (see Blackburn et al. 2014). Moreover, such hybridization can impact the persistence of local water frog hybridogenetic systems (Holsbeek et al. 2010; Dufresnes et al. 2017; Fayzulin et al. 2018), since *P. esculentus* strictly reproduces successfully with only “true” *P. ridibundus* and *P. lessonae*. As shown from laboratory crosses, a low frequency of nuclear alleles of *P. cf. bedriagae* among parent *P. ridibundus* from Mariy El Republic (Russia) disturbed germ cell development in hybridogenous *P. esculentus* (Dedukh et al. 2019). Additional evidence of the negative impact of *P. cf. bedriagae* and *P. kurtmuelleri* on hemiclonal reproduction of *P. esculentus* can provide data about their distribution. *P. esculentus* is absent in the Balkan mountainous regions (Figure 1), which is the only region where *P. kurtmuelleri* is found, and in the eastern part of European Russia, where *P. ridibundus* bears some portion of *P. cf. bedriagae* alleles (Fayzulin et al. 2018). In Kaliningradskaya Oblast’, the presence of *P. cf. bedriagae* and *P. kurtmuelleri* in water bodies surrounding the Vistula
Lagoon could led to the disappearance of local hybridogenic population systems (unlike the Curonian Lagoon, where alleles of both these species are absent).

In addition, reproductive barriers between marsh frog species could be altered under the new conditions. The viability of interspecific crossings between various water frog species have been widely tested by several authors (see Plötner et al. 2010), and *P. ridibundus*, *P. kurtmuelleri* and *P. cf. bedriagae* can successfully hybrize in the Eastern Balkans (Hotz et al. 2013). We assume that releases of alien species can favor production of new hybrid genotypes, whose invasive potential can be higher than those of parental species. If they do not suffer from intrinsic incompatibilities, hybrids might acquire an increased potential for local adaptations and resistance to diseases, higher survival, growth and development rates (Grant and Grant 1992; Frankham et al. 2002; Seehausen 2004).

The establishment and spread of *P. kurtmuelleri* and *P. cf. bedriagae* populations in the Baltic Coast of Russia could also threaten the persistence of native amphibians via competitive interactions in terms of food resources and breeding sites; they can also prey on larvae, juveniles and adults. For example, the introduction of marsh frogs in north-eastern Kazakhstan and Western Siberia (Russia) reduced the abundance of two native anurans: tetraploid green toad, *Bufotes pewzowi* (Bedriaga, 1898), and the moor frog, *Rana arvalis* Nilsson, 1842 (Berezovikov 2008; our data). However, it should be noted that in Kaliningradskaya Oblast’ *P. kurtmuelleri* and *P. cf. bedriagae* inhabit human-made waterbodies which are unsuitable for life and reproduction of most local amphibians.

Since our study is preliminary (19 individuals), in the future it would be very important to continue the study of water frogs in the Baltic Region of Russia and neighboring countries by applying multiple genetic markers. This would enable researchers to study routes of dispersal and introductions of marsh frog species, to clarify peculiarities of their hybridization and patterns of distribution, and to evaluate the impact of *P. kurtmuelleri* and *P. cf. bedriagae* on reproduction success of hybridogenous populations and abundance of local amphibians.

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**Supplementary material**

The following supplementary material is available for this article:

**Table S1.** Frequencies of occurrence of alleles and haplotypes (in %) of various water frog species in European presumably invasive populations.

This material is available as part of online article from:

http://www.reabic.net/journals/bir/2020/Supplements/BIR_2020_Litvinchuk_etal_SupplementaryMaterial.xlsx