

Distribution and Origin of Two Forms of the Marsh Frog *Pelophylax ridibundus* Complex (Anura, Ranidae) from Kamchatka Based on Mitochondrial and Nuclear DNA Data

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Abstract—The formation of the first populations of the marsh frog (the *Pelophylax ridibundus* complex) near Petropavlovsk-Kamchatsky and in the Paratunka River valley must have been the result of human introduction in the late 1980s. At present, more than 20 localities of this species are recorded in Kamchatka. For a more precise definition of the taxonomic status of *P. ridibundus* sensu lato, samples from five populations (altogether, 30 individuals) from southeastern and central Kamchatka are analyzed using molecular methods. In all frogs, a mitochondrial DNA type specific for the “eastern” form (=the Anatolian *P. cf. bedriagae*), but not for the “western” form (=the Central European *P. ridibundus*), is revealed. However, the results of nuclear DNA analysis of marsh frogs from Kamchatka reveal alleles specific for both of the forms, “eastern” and “western,” with a frequency ratio of about 2 : 1. The results of sequencing the mitochondrial *ND2* gene and nuclear *SAT-1* gene suggest that the “ancestor” individuals might have been introduced into Kamchatka from the Volga–Don interfluvium or Ciscaucasia. The absence of both haplotype and nucleotide diversity in the samples studied suggests a single successful introduction that involved a low number of frogs stemming from a single locality.

Keywords: marsh frog, *Pelophylax ridibundus*, *Pelophylax cf. bedriagae*, NADP-dehydrogenase, serum albumin, Kamchatka Peninsula

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INTRODUCTION

The marsh frog, *Pelophylax ridibundus* (Pallas 1771), was first found on Kamchatka Peninsula in the neighborhood of Petropavlovsk-Kamchatsky at the end of the 1980s. It is believed that several attempts at its introduction took place. Eventually, the successful colonization occurred in the basin-cooler of thermal power plant no. 2 (TPP-2) and the canal that flows out from it and serves for hot water removal, and the shallow parts of Khalaktyrskii Lake (Sheyko and Nikanorov, 2000). Presently, more than 20 places of inhabitation of this species are known on the Kamchatka Peninsula (for more information see Lyapkov, 2016). Local residents reported to one of the authors that the sound of frogs was also recognized in the region of hot water evacuation in the Paratunka River Valley at the end of the 1980s. The first long-term observations after the annual activity of frogs, reproduction, and development of eggs and tadpoles were first carried out in the neighborhood of the villages of Paratunka and Termalny in 2006–2011 (Bukhalova and Veligura, 2007; Shnurkova and Pisareva, 2007). However, there

are no data from which region and of how wide an area of natural inhabitation these specimens were introduced. It is not clear as well whether they invaded the pools near the TPP-2 and the Paratunka River Valley independently or one after the other. Moreover, there are fragmentary data on the formation of populations of this species in other regions of Kamchatka, including those located far from the TPP-2 and the Paratunka Valley after the end of the 1980s, because of their dispersion by people (see the review by Lyapkov, 2014a, 2016). The situation is even more complicated by the fact that presently the marsh frog is considered as a complex of several species on the basis of molecular genetic studies (Borkin et al., 2004; Plötner, 2005; Plötner et al., 2008, 2012). Within its wide inhabitation areal in Russia, the marsh frog is represented by two morphologically similar, but genetically different forms: the “western,” i.e., the Central European *P. ridibundus* and the “eastern,” i.e., the Anatolian *P. cf. bedriagae* (Akin et al., 2010; Ermakov et al., 2013, 2014, 2016, 2016a; Zaks et al., 2013; Zamaletdinov et al., 2015; Ivanov et al., 2015; Svinin et al., 2015).

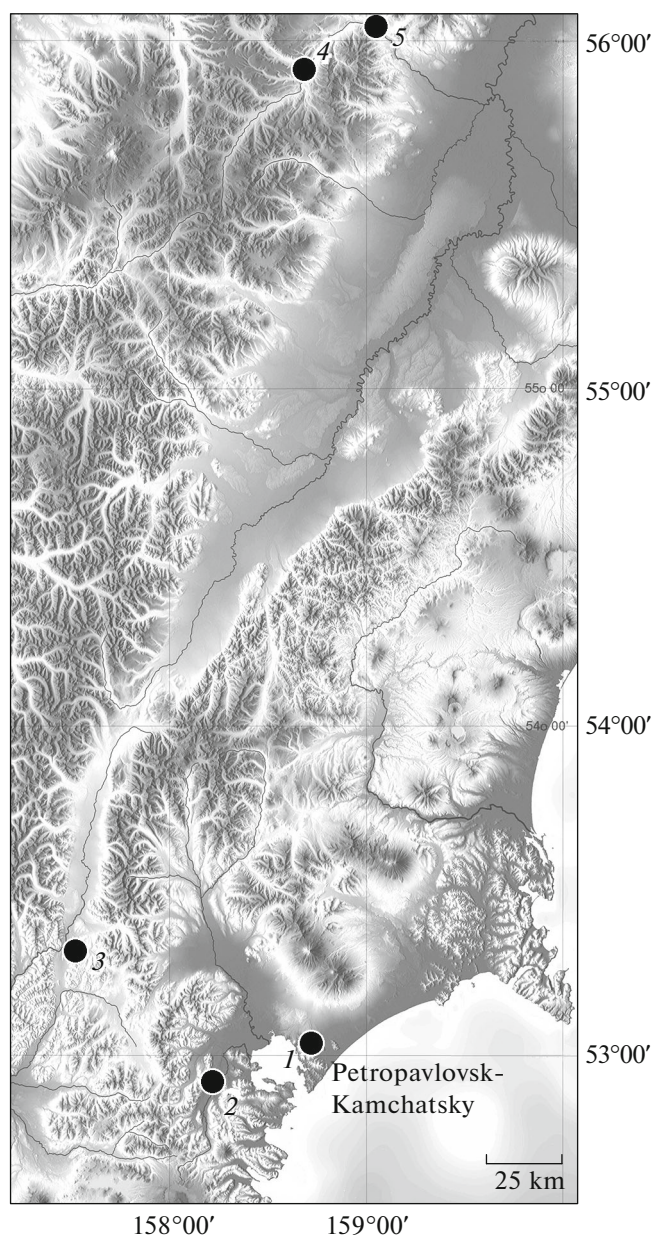


Fig. 1. Fragment of the map of Kamchatka on which the points of material collection are shown: 1, “TPP-2” and “Khatarylskoe”; 2, “Helios”; 3, “Malka”; 4, “Esso”; 5, “Anavgai.”

Therefore, the goals of this work were the following: (1) collection and molecular-genetic genotyping of specimens collected in some places in Kamchatka, where marsh frogs are often found in order to find out, which form, “western” or “eastern” they belong to; (2) to reveal the putative region within the natural inhabitation areal of this species, from which the specimens of the first populations in Kamchatka near TPP-2 and the Paratunka Valley were introduced.

MATERIALS AND METHODS

The experimental material was collected in June and the beginning of July 2014 in the southeastern and central parts of Kamchatka (Fig. 1). In total, 30 specimens collected in five areas were analyzed by methods of molecular genetics. Brief descriptions of the ecotopes, as well as their coordinates and sample sizes (n), are given further.

(1) TPP-2 and Khalaktyrskoe Lake are artificial basins of TPP-2 of Petropavlovsk-Kamchatsky, and Khalaktyrskoe Lake is the basin-cooler of TPP-2. It is a small artificial pool (150 × 70 m; 3 m deep) into which hot water is evacuated year-round. The water from this basin flows out through a narrow (about 5 m) and shallow (not more than 1 m) canal about 1.5 km long (53.015162° N, 158.743691° E, $n = 3$). Frogs were also collected in shallow creeks of the lake located nearby and restricted from hot water inflow (53.012167° N, 158.749247° E, $n = 5$).

(2) “Helios” is a small pond into which hot water is evacuated year-round from a recreation center located in the Paratunka Valley (53.002983° N, 158.269953° E, $n = 5$).

(3) “Malka” is a complex of small warm puddles, which are not frozen in winter and are formed in places where warm water is evacuated in the recreation area. They are located on both banks of the Kluchevka River, 5 km from the village of Malka (53.324009° N, 157.538454° E, $n = 7$).

(4) “Esso” is a complex of small puddles located in the area of hot water evacuation in the village of Esso (55.927075° N, 158.679454° E, $n = 5$).

(5) “Anavgai” is a small cold spring in the village of Anavgai, which contains weakly warmed regions in places where natural hot springs and natural warm water from heating systems of houses and greenhouses are evacuated (56.052489° N, 158.986569° E, $n = 5$).

The molecular genetic analysis of mitochondrial and nuclear DNA markers (mtDNA and nDNA) was performed in two stages. At the first stage, screening diagnostics of the whole sample ($n = 30$), which allowed us to estimate the form, “eastern” or “western,” which the haplotypes of mtDNA and nDNA of marsh frogs belong to, was carried out as described in (Ermakov et al., 2013; Zaks et al., 2013; Hauswaldt et al., 2012), omitting the sequencing. At the second stage, the mtDNA, which encodes the second subunit of NADH-dehydrogenase (*ND2*, 1038 bp), and nDNA encoding the intron 1 fragment of serum albumin (*SAL-1*, 638 bp) were sequences in all specimens ($n = 30$) and in homozygous specimens ($n = 20$) respectively in order to confirm the data of screening obtained with the primers used in (Plötner et al., 2008, 2009, 2012; Akin et al., 2010). The sequences obtained have been deposited in GenBank (no. KX503311–KX503322). The phylogenetic analysis was carried out with the MEGA 6.06 program (Tamura et al., 2013), using the original sequencing data and the sequences deposited

Table 1. Data of the screening typing aimed at analysis of the distribution of mt- and nDNA types in marsh frogs from Kamchatka

Population (the number of the point on the map in Fig. 1 is shown in brackets)	<i>n</i>	<i>COI</i> mtDNA		
		B		
		<i>SAI-1</i> nDNA		
		BB	BR	RR
TPP-2 (1)	3	1	2	–
Khatarlykskoe (1)	5	2	1	2
Helios (2)	5	4	1	–
Malka (3)	7	–	5	2
Esso (4)	5	2	1	2
Anavgai (5)	5	5	–	–
Total	30 (100%)	14 (47%)	10 (33%)	6 (20%)

R is the nDNA alleles of the “western” form of lake frog; B is nDNA alleles and mtDNA haplotypes of the “eastern” form; *n* is the sample size.

Table 2. Ratio of the “western” (R) and “eastern” (B) mtDNA haplotypes and alleles of nDNA in marsh frogs collected in the regions studied

Region	<i>n</i>	mtDNA		<i>n</i>	nDNA		Source
		R, %	B, %		R, %	B, %	
Kamchatka	30	–	100	60	37	63	This work
Caucasus	93	–	100	186	22	78	Ermakov et al., 2016, 2016a
Middle Volga region	171	32	68	342	86	14	Ermakov et al., 2014; Zaks et al., 2013
Upper Volga region	90	83	17	180	94	6	Svinin et al., 2015; Zamaletdinov et al., 2015
Upper Oka region	34	100	–	68	74	26	Ivanov et al., 2015

in the GenBank NCBI (www.ncbi.nlm.nih.gov), and our own unpublished data. Screening of the primary sequences most similar to those of marsh frogs from Kamchatka (less than 1% nucleotide substitutions) was performed with the BLAST algorithm (www.blast.ncbi.nlm.nih.gov). The methods of neighbor-joining (NJ) and maximal likelihood (ML) were applied in order to reconstruct the phylogenetic relationships between the identified haplotypes.

To calculate the haplotype frequencies, it was taken into account that the mtDNA is haploid and may formally be considered as one allele. Therefore, the numbers of specimens and alleles were equal. Nuclear DNA is, on the contrary, diploid and contains two alleles of each gene. Therefore, the portions of the alleles and samples of one form or another were different and depended on the ratio of homo- and heterozygous specimens (Table 2). The differences in allele frequencies were assessed by the χ^2 -test for the 2×2 tables using the STATISTICA v. 10 program (StatSoft).

RESULTS

It was shown that all marsh frogs from Kamchatka contained the “eastern” form of specific mtDNA markers (Table 1). However, the analysis of nuclear DNA showed that the marsh frogs from Kamchatka contained not only the “eastern” form of specific alleles of the *SAI* gene, but also the “western” form of specific alleles, the ratio of which was close to 2 : 1. Almost half of the specimens studied (47%) were shown to be homozygous and were identified as the “eastern” form. The other 33% and 20% referred to hetero- (BR) and homozygotes (RR) of the “western” form (Table 1). The observed ratio of homo- and heterozygous specimens does not contradict the data predicted by the Hardy–Weinberg principle ($\chi^2 = 2.38$, $df = 1$, $p = 0.991$). The absence of the “western” type mtDNA in the general sample suggested that the specimens, which carry both nuclear alleles specific for the “western” form, occur as a result of cleavage after crossing of heterozygotes. This suggestion is consistent

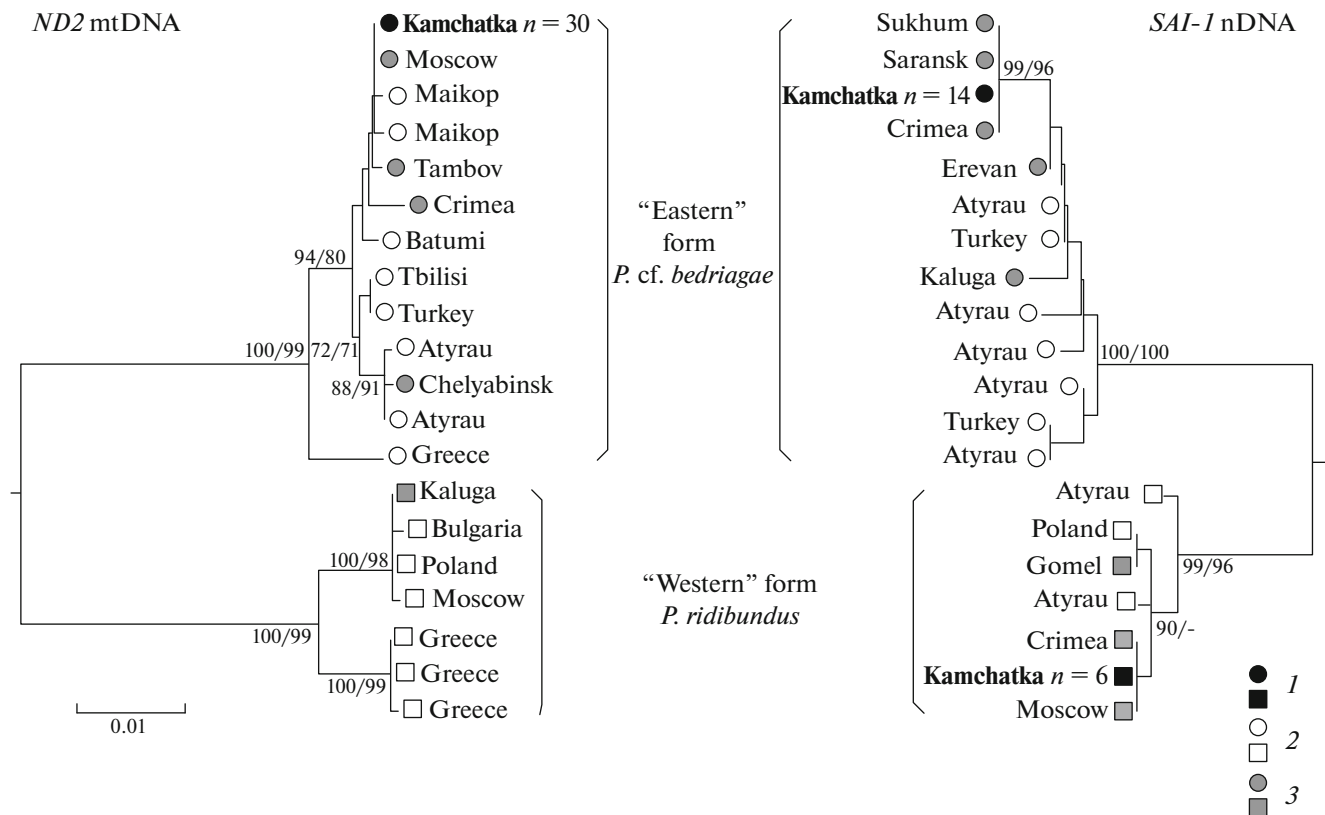


Fig. 2. Dendrograms demonstrating the connection between the mt- and nDNA haplotypes of marsh frogs from Kamchatka (1) and frogs from other regions (2, the GenBank database, 3, our own unpublished data). Numbers at the bottom of the clusters show the stability of the branch knots (%) from 1000 bootstrap replications (the NJ and ML methods). The scale represents the genetic distances (p -distance) between haplotypes.

with the fact that all six homozygous specimens of the RR-type were found in populations that contained heterozygous specimens.

The results obtained revealed the mismatch in the frequencies of mt- and nDNA marker distribution in populations of marsh frogs from Kamchatka, namely, the simultaneous absence of “western” mtDNA haplotypes and the presence of these haplotypes in nuclear genomes. Although the analysis of mtDNA revealed the presence of the *P. cf. bedriagae* haplotypes only in the general sample, the analysis of nuclear DNA markers showed the presence of 37% alleles of *P. ridibundus*. Differences in the frequencies of mt- and nDNA alleles were shown to be significant ($\chi^2 = 12.64$, $p = 0.0004$).

The data of sequencing of the mt- and nDNA markers fully corresponded to the data of screening analysis, on the basis of which the forms of marsh frogs were identified. All 30 sequences of the mitochondrial *ND2* gene referred to the “eastern” form; 14 out of 20 sequences of the nuclear *SAI-1* gene fragment also corresponded to the *P. cf. bedriagae*. The remaining six sequences corresponded to the “western” form

(Fig. 2). Both mitochondrial and nuclear genes were represented by one haplotype for each form studied, and both markers analyzed showed no genetic variability.

The phylogenetic reconstructions showed in Fig. 2 allowed us to reveal the mt- and nDNA haplotypes closest to frogs from Kamchatka.

A haplotype identical by the mtDNA marker was found in the Moscow suburbs. Sequences that differed by 1–3 nucleotide substitutions were found in Tambov region, the Black Sea coast of the Caucasus (Maikop, Batumi), and Crimea. The marsh frog haplotypes, which were found eastwards from the Volga River (Atyrau, Chelyabinsk), Central Transcaucasia (Tbilisi), and Turkey, were combined in an individual cluster and demonstrated less similarity with the sequences from Kamchatka.

To the north and east of Oka-Don Lowlands (Moscow, Saransk), on the Black Sea Coast (Sukhum) and in Crimea, the haplotypes equal to those from Kamchatka by the nDNA marker, which is less variable in comparison with the mtDNA marker, were found for each of the two forms of marsh frogs.

DISCUSSION

The marsh frog is an ecologically plastic polymorphous species, which occupies a huge areal, which also increases because of multiple introductions (Kuzmin, 2012, pp. 223–228). Use of molecular genetic analysis allowed us to verify the species of frogs introduced in Kamchatka. The species was formerly identified by G.A. Lada on the basis of morphological analysis of only a small number of specimens from the population of TPP-2 (Sheiko and Nikanorov, 2000). Study of marsh frogs from Kamchatka revealed their specific traits and allowed us to approach the task of the origination of these most eastern populations and outline the territory within the native areal from which the introduction took place.

First, all specimens studied were shown to contain the mtDNA markers typical for the *P. cf. bedriagae*. Haplotypes of the “eastern” form of the natural range of the “*ridibundus*-complex” were typical for Eastern Turkey, the Caucasus, and Crimea (Akin et al., 2010; Akin Peksen et al., 2014; Ermakov et al., 2016, 2016a). They also dominate in the Middle and Upper Volga Region (Ermakov et al., 2013, 2014; Zaks et al., 2013; Zamaletdinov et al., 2015; Svinin et al., 2015). The mtDNA, which is specific for *P. ridibundus* only, was found in marsh frogs from Central and Eastern Europe (Plötner et al., 2008), and in the Upper Oka region (Kaluga region, Table 2) (Ivanov et al., 2015). This leads to the conclusion that the Don River is the apparent western border of the “eastern” type of mitochondrial haplotype distribution on the East European Lowland. The sole exception is the one *P. cf. bedriagae* haplotype, which was found in southeastern Ukraine near Kharkov (Hoffmann et al., 2015).

Second, the “eastern” form of specific nDNA markers prevails over the “western” one. It was shown (Table 2) that most of the *P. cf. bedriagae* alleles were found in the marsh frog populations of the Caucasus and neighboring regions (Abkhazia, Adygea, Armenia, Georgia, Dagestan, and Krasnodar region) (Ermakov et al., 2016, 2016a). The portion of the “eastern” allelic forms decreases northwards, whereas the portion of the “western” form, conversely, increases. According to the nDNA analysis, “pure” populations of any of the forms of frogs are apparently absent on the whole territory studied from the Caucasus to the Upper Volga region. The alleles of both forms were found in all places of sample collection, in which the yield was more than five specimens. Homo- and heterozygous specimens were also found, and their portion varied from 9 to 29%. Therefore, a population from any region studied may be considered as the donor on account that a small number of founder-specimens were introduced and the bottleneck effect took place.

Third, both haplotypic and nucleotide diversity were absent in the sample studied. Zero values of both parameters of the genetic marker variability in the

marsh frogs from Kamchatka evidence the small number of specimens introduced in Kamchatka, i.e., the founder effect, and may be considered as proof that the specimens for introduction originated from the same place and, apparently, only one introduction was successful.

Fourth, the successful introduction of marsh frogs was to a certain degree supported by predominance of the “eastern” form of specific molecular markers. It is known that the specimens that carry the *P. cf. bedriagae* markers in their genomes participate in active invasion of green frogs into new regions that may lead to the supplanting of autochthonous species (Holsbeek et al., 2008; Dubey et al., 2014; Plötner et al., 2015). Moreover, we showed that the frequency of the “eastern” form of alleles is increased in the anthropogenic water ponds in the Middle Volga region (Penza, Samara, and Saratov regions, and the Republic of Tatarstan) as compared with the same parameter in natural water ponds. In the northern Volga region (Mari-El Republic), all specimens found that carried the mt- or nDNA of the “eastern” form referred to the anthropogenic landscapes (Ermakov et al., 2013, 2014; Zamaletdinov et al., 2015; Svinin et al., 2015).

Therefore, the data available on the principles of geographical distribution of the mtDNA haplotypes of the two forms of marsh frogs and the revealed similarity of primary nucleotide sequences of the mt- and nDNA markers of marsh frogs from Kamchatka with homologous sequences of the specimens originating from other regions allow us to outline the territory from which the specimens were introduced into Kamchatka. This territory included the Volga–Don interfluvium, the Black Sea coast of the Caucasus, and Crimea. Moreover, the presence of identical gene sequences in the marsh frogs from Kamchatka and the Moscow suburbs in all three variations possible (the *ND2* gene is specific for the “eastern” form and the *SA1* gene is specific for each of the two forms, Fig. 2) does not exclude the possibility that the donor-population might have been located in Moscow region.

According to the aforesaid, the exact time of population of the warmed water basins of TPP-2 and the Paratunka Valley remains unclear. It is also unclear whether the water basins near the TPP-2 and Paratunka Valley were populated independently or if one of these areas was populated first and the other was invaded later on by the transfer of frogs from the first area. The data on the haplotype composition of these two forms do not clarify these questions. Reports about the first findings of marsh frogs in small hot basins in the recreation zone near the village of Malka have appeared quite recently. The first report was published on the Internet in May 2012, and it is most likely that frogs were transferred from one of the more southern populations (Fig. 1). However, the high similarity of populations from TPP-2 and the Paratunka Valley does not allow us to find out exactly which pop-

ulation the invasion came from. Scientists from Bystrinskii Natural Park (the Sychevs, personal communication) reported that dozens of adult specimens were collected from the population of TPP-2 and released into the warm flows of a private recreation center, which is located 40 km from Esso, in the mid 2000s. After successful introduction, in 2008, only about ten specimens were transferred from this population to Esso, where a new population also formed. Moreover, in Esso marsh frogs populated the hot puddles and main hot flows already in 2010.

Anavgai was populated last, and, judging by their genetic homogeneity (i.e., the presence of the “eastern” form markers, Table 1) the number of founder-specimens was rather small. It is also noteworthy that well-known cases of the formation of new spatially isolated populations as a result of delivery of a small number of specimens by people indirectly confirm the possibility that the first population in Kamchatka was formed by a limited introduction. The data of genotyping also do not contradict another possible scenario of the occurrence of the first population of this species in Kamchatka. It is known that there is an area in Khalaktyrskii Lake that never becomes frozen because of the evacuation of cold water into it from TPP-2 and because of which the lake has successfully been introduced with European carp, the populations of which reach high values here. The first marsh frog tadpoles could have been delivered together with young fishes. If this is the case, the success of frog introduction is due to the high number of tadpoles delivered. A similar introduction of species was observed in the case of seeding of several warmed water basins of Sverdlovsk region (Fominykh and Lyapkov, 2011). The next stage of the species distribution in Kamchatka could have been an intentional introduction into some hot water basin in the Paratunka Valley by transferring adult frogs to it. Since the hot water basins of the Paratunka Valley and the places of warm water delivery are located close to one another (minimal distance less than 1 km), the migrating frogs could further populate all ecotopes known to date.

The formation of the marsh frog populations in Kamchatka, which are characterized by high variability of their life cycle observed even within the limits of one population in water basins located close to one another (first of all, the number of the year-around reproduction cycles that depend on the volume of hot water delivered and the duration of the activity season; for more information, see Lyapkov, 2014, 2014a), is a unique case of successful introduction and wide distribution of a species on the basis of their high plasticity. We also observed a high level of variability in the size and rate of metamorphous growth of frogs, which could have belonged to either different or the same populations (Lyapkov, 2016a). It may be suggested that the presence of nuclear haplotypes of both forms of marsh frog (except Anavgai), as well as the diversity

of haplotypes in each population studied, also sustains this high plasticity.

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