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A development of a PCR-RFLP test system for the identification of mitochondrial lines of the *Pelophylax ridibundus* lake frog in Kazakhstan

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Abstract. *Background.* Molecular typing by PCR-RFLP method allows to identify the specific attribution of an organism that has a weak phenotypic difference. The main advantage of this research method is the capacity to analyze a large number of samples without applying the sequencing method. The purpose of the work is to develop a test-system for the identification the matrilines of marsh frogs of the *Pelophylax ridibundus* complex from Kazakhstan. *Materials and methods.* The analysis was based on the variability of the primary structure of the mitochondrial gene the second subunit of dehydrogenase (ND2) which is a species-specific marker. Subsequently a search for marker nucleotide substitutions specific for each lineage and recognition sites for the HaeIII and TasI restriction endonucleases was conducted. *Results.* As a result of the research, it was confirmed that on the territory of Kazakhstan inhabiting three main forms of lake frogs where two of them are native (Balkhash, Syrdarya), and invasive Anatolian form – *P.* cf. *bedriagae*. *Conclusions.* The authors' PCR-RLFP test technique is a straightforward and reliable tool for detecting mitochondrial lineages in the *Pelophylax ridibundus* complex marsh frogs and may be used successfully in mass screening investigations.

Keywords: restriction analysis, NADH dehydrogenase (ND2), mitochondrial DNA, *Pelophylax ridibundus* complex

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Разработка ПЦР-ПДРФ тест-системы для идентификации митохондриальных линий озерной лягушки комплекса *Pelophylax ridibundus* в Казахстане

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Аннотация. *Актуальность и цели.* Молекулярное типирование методом ПЦР-ПДРФ позволяет определить видовую принадлежность организмов, имеющих слабые фенотипические различия. Главное преимущество данного метода – это возможность анализировать значительные по объему выборки без применения методов секвенирования. Авторами была поставлена задача разработать тест-систему для идентификации озерных лягушек комплекса *Pelophylax ridibundus* в Казахстане. *Материалы и методы.* Анализ был основан на изменчивости первичной структуры митохондриального гена – второй субъединицы НАДН-дегидрогеназы, который является видоспецифичным маркером. В дальнейшем был проведен поиск маркерных нуклеотидных замен, специфичных для каждой линии, и сайтов узнавания для эндонуклеаз рестрикции HaeIII и TasI. *Результаты.* В результате проведенных исследований было подтверждено обитание трех форм озерной лягушки на территории Казахстана, две из которых – нативные (Балхаш, Сырдарья) и инвазивной анатолийской формы – *P.* cf. *bedriagae*. *Выводы.* Разработанная авторами методика ПЦР-ПДРФ анализа представляет собой простой и надежный инструмент для выявления митохондриальных линий у озерных лягушек комплекса *Pelophylax ridibundus* и может успешно использоваться в массовых скрининговых исследованиях.

Ключевые слова: рестрикционный анализ, НАДН-дегидрогеназа, митохондриальная ДНК, *Pelophylax ridibundus* комплекс

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Introduction

Pelophylax ridibundus (Pallas, 1771) is a species complex of marsh frogs, occupying territories from Europe and North Africa in the west to Central and Middle Asia in the east [1–3]. Nowadays, the taxonomy of many forms within the complex has not been sufficiently studied, in particular, the marsh frog from the Central Asian region needs to be clarified. Due to its high ecological plasticity, representatives of the *P. ridibundus* complex are adapted to extensive expansions of

new territories. To the territory of Kazakhstan, the marsh frog was unintentionally introduced at the end of the 20th century into the basins of the Irtysh and Ural rivers, as well as into fish farms, which was the beginning of resettlement in the adjacent territories [4–7]. Due to the difficulties in identifying marsh frogs of the *P. ridibundus* complex at the phenotypic level, over the past two decades, many approaches have been taken to study its genetic component.

Molecular genetic analysis of *P. ridibundus* forms was carried out on the basis of mitochondrial genes [8–9]. Several distribution points from the territory of Kazakhstan were also involved in these studies (Atyrau – western Kazakhstan, and Almaty – southeastern Kazakhstan). The results of the analysis showed the presence of two haplogroups on the territory of Kazakhstan – Central Asia 1 and Central Asia 2 with the nominal name *P. sp. novum* [10]. Haplogroup Central Asia 1 represents populations from the southern and eastern part of the Caspian Sea, Iran, Turkmenistan and Uzbekistan, while haplogroup Central Asia 2 included populations from Kyrgyzstan and the adjacent territory of Kazakhstan. Along with this, test systems were also developed for determining the cryptic forms of the marsh frog complex that involved various nuclear DNA (nuDNA) analyses, such as the PCR-RFLP-based method [11], the PCR method based on differences in length of serum albumin intron 1 (SAI-1) sequences [12], and several methods detected variation of microsatellite markers [13–16]. According to our recent studies [17], it was found that 3 forms of the marsh frog live in Kazakhstan, two of which are native: Balkhash form = (Central Asia 2), Syrdarya form – (Middle East) sensu C. Akin [9], and an invasive form *P.* cf. *bedriagae*. The distribution area of *P.* cf. *bedriagae* occurs in almost the entire territory of the country from west to east [9]. It has the ranges overlaps with Balkhash form on the east and southeast, and southeast, whereas Syrdarya form occupies the basin of the same name river in the south of Kazakhstan [17].

The purpose of this study is to develop a test system based on restriction analysis of the second subunit of the mitochondrial dehydrogenase gene ND2 for screening identification of Kazakhstani forms of the marsh frog.

Materials and methods

In total, 89 samples of the marsh frog of the *Pelophylax ridibundus* complex were analyzed from 21 localities in Kazakhstan, collected during the period of field expeditions 2009–2021. The toe clips fixed in 96 % ethanol were used as tissue samples. Extraction of genomic DNA was performed using the standard proteinase K lysing salt method [18]. The ND2 gene sequence was amplified with use of the universal primer ND2L1 5′-AAG CTT TTG GGC CCA TAC CCC-3′ [19] and a specific primer ND2H1 5′- GCA AGT CCT ACA GAA ACT GAA G-3′ [20]. Reaction mixture (25 μl) contained 50–100 ng of DNA of frogs, 0.5 μM of each primer, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 2.5 μl 10 × of PCR buffer (10 мМ Tris-HCl, pH 8.3, 50 мМ KCl), and 2 units of *Taq* polymerase (Thermo Scientific). PCR was performed at 95 °C for 30 sec, 60 °C (depending on the annealing temperature of primers) for 30 sec, and 72 °C for 90 sec (32 cycles).

Amplification products were exposed to restriction endonucleases BsuRI (HaeIII), and TasI (Tsp509I) (Fermentas). PCR fragments were digested according to the manufacturer's protocol by adding 2 units of enzyme activity directly to aliquots of amplification mixtures (4 μl). Obtained PCR products were

analyzed by electrophoresis in 6 % polyacrylamide gel (glass plate sizes 8 × 10 cm) with further dying by ethidium bromide for UV visualization. For molecular weight size markers, we used the DNA kit of pBR322 plasmid processed with restrictase HpaII (pBR/HpaII).

Results

The length of the amplified product of the mitochondrial ND2 gene was 1170 base pairs (bp), including primers. Analysis of the nucleotide sequence of the gene showed the presence of 6 restriction endonuclease HaeIII (GG$^{\vee}$CC) recognition sites, 2 of which were common for all three forms, and 4 sites for the Syrdarya and *P.* cf. *bedriagae* forms. In view of the insufficiently studied populations of marsh frogs from the territory of Kazakhstan, the "western" form, *P. ridibundus*, was used as a control group (Fig. 1, gel well 1). Upon treatment with HaeIII endonuclease in *P.* cf. *bedriagae*, 4 restriction sites were found, the fragments of which were digested at different lengths and segregating the form into the two matrilines, hereafter named as *P.* cf. *bedriagae* 1 and *P.* cf. *bedriagae* 2.*P.* cf. *bedriagae*1 is distinguished by the fragment with length of 515 bp, where as *P.* cf. *bedriagae* 2, characterized by additional fragments 148 and 367 bp (Fig. 1, gel wells 2–3, respectively). In the Syrdarya form, two slightly different matrilines were also noted, which, when treated with restriction enzyme, are split into 5 fragments of different lengths. Indicative length of digested fragment for line Syrdarya 1 was 432 bp, and for Syrdarya 2 were fragments of 375 + 367 and 65 bp respectively (Fig. 1, gel wells 4–5). The Balkhash form has three restriction sites, whereas feature fragment of which is cleaved at length of 511 bp (Fig. 1, gel well 6).

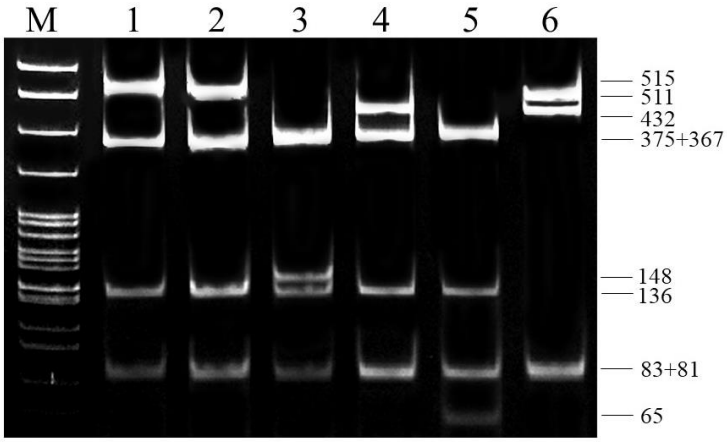

Fig. 1. Electropherogram of the ND2 gene restriction products by HaeIII endonuclease in the identification of three mitochondrial lineages of marsh frog *P. ridibundus complex* in Kazakhstan. Gel wells: 1 – *P. ridibundus*; 2–3 – *P.* cf. *bedriagae* 1, 2; 4–5 – Syrdarya 1, 2; 6 – Balkhash. On the right side – length of restriction fragment, bp; M – molecular lengths marker pBR/HpaII; Fragments less than 60 bp are not shown on the phoregram

The endonuclease HaeIII enable to identify the native forms but cannot distinguish *P. ridibundus*. Thus, the restriction analysis revealed a variation of the "eastern" form of *P.* cf. *bedriagae* 2 from Semey, indicated by the 2 additional marker fragments – 148 and 367 bp. The variation of the Syrdarya 2 linethatis characterized by the presence of fragments of 375 + 367 and 65 bp (Table 1).

Table 1

Lengths of the restriction fragments (bp) detected by HaeIII and TasI endonucleases of Kazakhstani marsh frogs

HaeIII endonuclease					
P. ridibundus	*P.* cf. *bedriagae* 1	*P.* cf. *bedriagae* 2	Syrdarya 1	Syrdarya 2	Balkhash
515	515	–	–	–	511
–	–	–	432	–	432
375	375	375+367	375	375+367	
–	–	148	–	–	–
136	136	136	136	136	
81	81	81	83+81	83+81	83+81
–	–	–	–	65	–
54	54	54	54	52	54
TasI endonuclease					
441	–	–	–	–	–
–	360	360	360	360	360
–	204	204	–	–	204
194	–	–	–	–	–
–	153	153	154	154	153
–	–	–	140	140	140
101+103	–	–	–	–	–
85	85	85	–	–	–
69	–	–	69+73	–	69

Endonuclease TasI ($^{\vee}$AATT) allows to identify the presence of the control "western form" of *P. ridibundus* among the studied forms. The number of recognition sites for this restriction enzyme in the ND2 gene sequences among the studied lines was 8, 2 of which were common to the three studied lines, the remaining 2–4 in different combinations formed fragments characterizing only of one or another line. A large number of cleavage sites made it possible to obtain specific restriction patterns for each genetic line, which are well visualized on electropherograms in the length zone from 69 to 360 bp (Fig. 2).

The common cleavage fragments for all three lines were 360, 154 + 153 bp. *P.* cf. *bedriagae* matrilines are not distinguishable by the TasI restriction enzyme, therefore fragment lengths were same and had no characteristic features(Fig. 2, gel wells 2–3), however, the Syrdarya 1 line had additional favorable fragment with length 69 + 73 bp. The absence of the 69 and 204 bp fragments for the line of Syrdarya 2 was specific factor (Fig. 2, gel wells 4–5). Balkhash line identified by the fragment of 204 bp in length (Fig. 2, gel well 6).

According to the results of TasI endonuclease analysis, among the studied samples of the Kazakh populations of the marsh frog, the "western" form of *P. ridibundus* was not found. An information about restriction fragments is given in the Table 1.

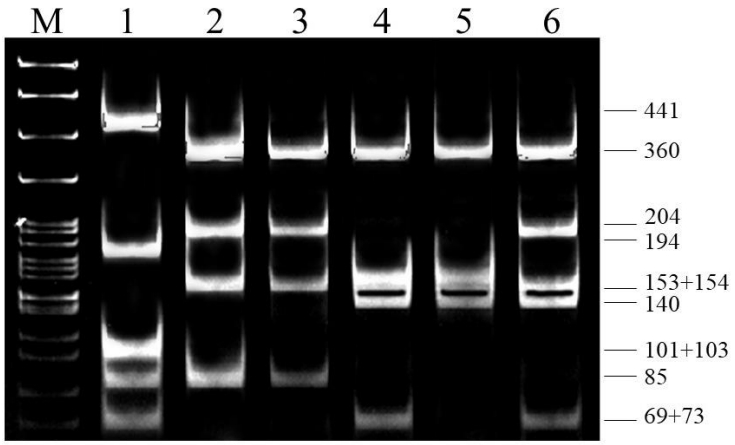

Fig. 2. Electropherogram of the ND2 gene restriction products by TasI endonuclease in the identification three mitochondrial lineages of the marsh frog *P. ridibundus complex* in Kazakhstan. Gel wells: 1 – *P. ridibundus*; 2–3 – *P.* cf. *bedriagae* 1, 2; 4–5 – Syrdarya 1, 2; 6 – Balkhash. On the right side – length of restriction fragment, bp; M – marker of molecular lengths pBR/HpaII. Fragments less than 60 bp are not shown on the phoregram

Conclusions

One of the main advantages of this research method is its low cost, which allows to avoid sequencing stage in some cases. Evaluation of the applicability of the PCR-RFLP method for the identification of mitochondrial lines of the marsh frog of *P. ridibundus* complex showed that nucleotide sequence of the ND2 gene has sufficient variability to allow selection of restriction endonucleases to identify three known lines. Moreover,the developed test-system serves as a universal method for determining the cryptic forms particularly with the overlapped habitats, and can also be used for mass analyses for scientific and monitoring purposes.

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