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SHORT COMMUNICATIONS

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NEW MULTIPLEX PCR METHOD FOR IDENTIFICATION OF EAST EUROPEAN GREEN FROG SPECIES AND THEIR HYBRIDS

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A molecular multiplex PCR method for identification of East European green frog species (*Pelophylax ridibundus*, *P.* cf. *bedriagae* and *P. lessonae*) and their hybrids was developed. This simple and rapid method can be used for identification of species-specific mitochondrial and nuclear DNA. The method is based on species-specific differences in primary structure of the subunit 1 of the mitochondrial cytochrome C oxidase gene (*CO*I) and the intron-1 of the nuclear serum albumin gene (*SA*I-1). Based on the method, we analyzed numerous individuals of these species and their hybrids from East European Plain, the Crimea, the Caucasus, the Ural, as well as introduced populations from Western Siberia and the Kamchatka. In all cases, identification of species performed by use of the multiplex PCR method coincided with results of study of primary nucleotide sequences.

Keywords: *Pelophylax ridibundus*; *Pelophylax* cf. *bedriagae*; *Pelophylax lessonae*; *Pelophylax esculentus*; rapid species identification; water frogs; cytochrome C oxidase; serum albumin.

An identification of European green (or water) frog species is usually related to difficulties due to their morphological similarity. However, their precise identification is necessary for study of local amphibian diversity, species invasions, and, finally, development of regional conservation strategies. Three green frog species are known from Eastern Europe: the marsh frog, *P. ridibundus*, the pool frog, *P. lessonae*, and their hemiclonal hybrid the edible frog, *P. esculentus*. Additionally, the Anatolian marsh frog (*P.* cf. *bedriagae*) was recently revealed in some regions of Eastern Europe (Ermakov et al., 2014; Zamaletdinov et al., 2015).

The special problem is numerous invasions of green frog species to various parts of Eurasia (Holsbeek et al., 2008, 2009; Holsbeek and Jooris, 2010; Leuenberger et al., 2014; Dufresnes et al., 2016, 2017; Lyapkov et al., 2018). For example, the Anatolian (*P.* cf. *bedriagae*) and the Balkan (*P. kurtmuelleri*) marsh frogs were recently found in Switzerland and Belgium (Holsbeek et al., 2008, 2009; Dubey et al., 2014). In Switzerland, an invasive the Italian pool frog (*P. bergeri*) replaced a native pool frog, *P. lessonae* (Leuenberger et al., 2014; Dufresnes et al., 2016). New molecular data, apart of indication of exogenous green frog species, allowed also to reveal potential existing of new hybridogenetic complexes (Dufresnes et al., 2017) or even traces of extinct species in Europe (Dubey and Dufresnes, 2017).

For reliable determination of water frog species (*P. ridibundus*, *P. lessonae*, and *P. esculentus*), various molecular-genetic methods were previously proposed. As a rule, they are involved various nuclear DNA (nuDNA) analyses, such as the PCR-RFLP-based method (Patrelle et al., 2011), the PCR method based on differences in length of serum albumin intron-1 (*SA*I-1) sequences (Hauswaldt et al., 2012), and several methods

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detected variation of microsatellite markers (e.g., Arioli et al., 2010; Leuenberger et al., 2014; Herczeg et al., 2017; Stakh et al., 2018). However, these methods have some limitations. For example, they are usually not able to determine *P.* cf. *bedriagae*, which is widespread in Eastern Europe (Ermakov et al., 2014; Zamaletdinov et al., 2015). Additionally, it was previously proposed the multiplex PCR method based on differences in primary sequences of the subunit 1 of NADH dehydrogenase (*ND*1) gene (Holsbeek et al., 2010) which allowed determining mitochondrial DNA (mtDNA) of three green frog species (*P. ridibundus*, *P. lessonae*, and *P. esculentus*). Unfortunately, an error in published sequences of species-specific primers makes impossible to use this technique.

Here, we described new multiplex PCR method for determination of all East European green frog species. It is based on differences between these species in DNA sequences of the subunit 1 of mitochondrial cytochrome C oxidase gene (*CO*I, 657 bp) and the intron-1 of nuclear serum albumin gene (*SA*I-1, 838 bp). Data about nucleotide sequences of these both fragments of genes were previously published (Plötner et al., 2009, 2012; Hofman et al., 2012, 2016) and their sequences were placed in such databases as GenBank NCBI (www.ncbi.nlm.nih.gov) and BOLD (www.boldsystems.org). This is allowed us to analyze numerous individuals originated from various parts of geographical ranges of these species.

Differences in sequences of the *CO*I gene fragment between *P. ridibundus* ($n = 39$) and *P.* cf. *bedriagae* ($n = 31$) were 5.3 ± 0.9% of nucleotide substitutions and between *P. lessonae* ($n = 16$) on the one hand and these two species on the other hand were 14.3%. Differences in primary structure of the *SA*I-1 gene fragment between *P. ridibundus* ($n = 22$) and *P.* cf. *bedriagae* ($n = 23$) were 3.5 ± 0.9% of nucleotide substitutions. Lengths of the *SA*I-1 fragment of the pool frog on the one hand and two species of the marsh frog on the other hand were different. The length of the fragment was approximately on 550 bp shorter in *P. lessonae* than in both *P. ridibundus* and *P.* cf. *bedriagae* (Plötner et al., 2009; Hauswaldt et al., 2012).

To test our multiplex PCR method, we used toe clips as tissue samples, which were fixed by 96% ethanol in a field. DNA was extracted by standard salt-extraction method (Aljanabi and Martinez, 1997) combined with lysis by proteinase K. PCR mixtures for mtDNA and nuDNA were separately made. In each mtDNA and nuDNA PCR mixtures, primer concentrations were equal. PCR was performed at 94°C for 30 sec, 60 and 62°C (for *SA*I-1 and *CO*I, respectively) for 30 sec, and 72°C for 30 sec (30 cycles). Each PCR reaction mixture (12 μL) contained 30 – 50 ng of DNA of frogs, 0.25 μM of each primer, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1.2 μL 10× of PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), and 1 unit of *Taq* polymerase (Thermo Scientific). Obtained products of PCR were analyzed by electrophoresis in 6% polyacrylamide gel (glass plate dimensions 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 *Hpa*II (pBR/*Hpa*II).

For identification of each East European green frog species, we designed a kit with one common and three species-specific primers for both mtDNA and nuDNA markers (Table 1), which should meet following conditions: (1) one of substitution replacements on 3′-end of species-specific primer and (2) nearly similar annealing temperatures should be for all four primers. For the study of mtDNA, the common reverse primer COIR-Pu was used. For detection of *P. lessonae*, the forward primer COIF-Pl was applied, which allowed to obtain PCR products with length of 294 bp. Primers COIF-Pr and COIR-Pb were used for identification of *P. ridibundus* (214 bp) and *P.* cf. *bedriagae* (440 bp) mtDNA (Table 1 and Fig. 1: lanes 1 – 3). For the study of nuDNA, the common forward primer SA1F-Pu was applied. This is the modified primer Pal-SA-F1 which was previously proposed by Hauswald et al. (2012). Species-specific reverse primers SA1R-Pl (109 bp), SA1R-Pr (210 bp) and

TABLE 1. Primers of Multiplex PCR Test Systems for Identification of Green Frog Species

Primer	Position	Sequence (5′ – 3′)	Annealing temperature, °C	PCR product length, bp	Specificity
COIR-Pu	624-601	CCTGCRGGATCAAAAAATGTTGT	63.6	—	All three species
COIF-Pl	329-349	GAACTGTGTACCCCCCACTAG	63.7	294	*P. lessonae*
COIF-Pr	409-429	GCTGGGGTTTCATCAATTCTG	61.8	214	*P. ridibundus*
COIF-Pb	183-204	CTTTGGAAATTGACTCGTGCCA	63.8	440	*P.* cf. *bedriagae*
SA1F-Pu	25-59	CCATACAAATGTGCTAAGTAGGTT	61.3	—	All three species
SA1R-Pl	140-119	TACCGTACCGATATTTGTATGC	60.2	109	*P. lessonae*
SA1R-Pr	245-221	GATACAAATGATACATTCCCACCT	61.0	210	*P. ridibundus*
SA1R-Pb	450-429	TTGTTCCCTATACTAAGGTCAC	59.3	415	*P.* cf. *bedriagae*

SA1R-Pb (415 bp) were used for detection of *P. lessonae*, *P. ridibundus*, and *P.* cf. *bedriagae* nuDNA, respectively (Table 1 and Fig. 1: lanes 4 – 6). Hybrid individuals were represented on gels by two bands (heterozygotes), which were specific for each parental species (Table 1 and Fig. 1: lanes 7 – 9). Differences between amplified species-specific fragments were high (about 100 bp), which allowed us simple visual identification of species and their hybrids after electrophoresis of PCR products in polyacrylamide gel (Fig. 1).

Our new multiplex PCR method was verified by selective sequencing of the *SA*I-1 gene fragment of nuDNA and the subunit 2 of NADH dehydrogenase (*ND*2) gene fragment of mtDNA in 64 and 108 specimens, respectively (Lyapkov et al., 2018; our unpublished data). In all cases, identification of species performed using our multiplex PCR method coincided with results of study of primary nucleotide sequences.

Based on our multiplex PCR method, we successfully identified 1029 individuals of *P. ridibundus* and *P.* cf. *bedriagae*, as well as 146 individuals of *P. esculentus* from East European Plain, the Crimea, the Caucasus, the Ural, as well as introduced populations from Western Siberia and the Kamchatka. We observed all six possible mtDNA/nuDNA marker combinations for two species of marsh frogs (*P. ridibundus* and *P.* cf. *bedriagae*). The R/RR combination was found in 23% of cases, B/BB in 21%, R/RB in 8%, B/RB in 20%, R/BB in 3%, and B/RR in 25%. Thus, individuals with all species-specific markers were less than one half (44%). All other combinations were individuals with introgression of alien mtDNA or hybrids (28% for both cases).

Three combinations of markers were revealed in hybridogenetic *P. esculentus*: L/RL (65%), R/RL (23%), and B/RL (6%). Other hybrids between marsh and pool frogs had two rare combinations: R/BL (3%) and B/BL (2%). Thus, our data let us to suggest the hypothesis of "de novo" hybrid production in East European Plain. *Pelophylax esculentus*, which live together with marsh frogs carrying out *P.* cf. *bedriagae* alleles, can occasionally get them (Svinin et al., 2015).

Thus, our originally designed test system for molecular identification of East European green frog species is simple, quick and reliable method which we can recommend using to analyze large samples.

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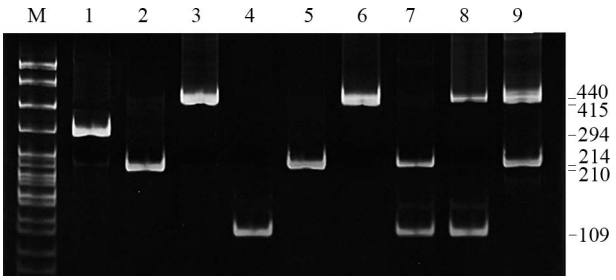

Fig. 1. Electrophoregram of products of multiplex PCR with species-specific primers for *CO*I mtDNA (lanes 1 – 3) and *SA*I-1 nuDNA (lanes 4 – 9). Lanes in polyacrylamide gel: 1, 4, *Pelophylax lessonae*; 2, 5, *P. ridibundus*; 3, 6, *P.* cf. *bedriagae*; 7, *P. esculentus*; 8, hybrid between *P. lessonae* and *P.* cf. *bedriagae*; 9, hybrid between *P. ridibundus* and *P.* cf. *bedriagae*. M is a marker of molecular length. Lengths of PCR products (in bp) are shown on the right side of electrophoregram.

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