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METHODS AND RESOURCES

Multiplex PCR assay to distinguish among three widespread brown frog species

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Abstract
Here we present a multiplex PCR assay to distinguish three widespread brown frog species, *Rana temporaria*, *R. arvalis* and *R. amurensis*, based on amplified fragment length. The system consists of four primers to the mitochondrial cytochrome c oxidase subunit I gene, with one universal reverse primer and three species-specific forward primers. Therefore, the PCR gives a fragment of a known specific length, which can be identified by electrophoresis in standard agarose gel. The efficiency of the developed assay was confirmed using both pre-identified and unidentified samples.

Keywords Eurasia · Ranidae · *Rana* · Siberia · Species identification

The Siberian wood frog (*Rana amurensis* Boulenger, 1886), the moor frog (*R. arvalis* Nilsson, 1842), and the common frog (*R. temporaria* L., 1758) are three widespread Eurasian frog species that occur in sympatry in some areas, with two or all three species being present in the same habitats. *R. arvalis* has a wide distribution in countries of Northern, Eastern, and South-Eastern Europe, with a spread through Siberia (Stugren 1966; Loman 1978; Kuzmin 2012). *R. temporaria* is found in Northern, Central, and Western Europe, and parts of Southern Europe and the European part of Russia (up to Urals and West Siberia) (Loman 1978; Kuzmin et al. 2008; Kuzmin 2012). *R. amurensis* inhabits eastern Asia including northeastern China regions, Korea, and Mongolia, and Russia (West and East Siberia, Far East) (Kuzmin 2012; Kuzmin et al. 2017). Thus, *R. arvalis* and *R. temporaria* have a vast territory of coexistence in Europe, while *R. arvalis* and *R. amurensis* range overlap covers West Siberia, western parts of East Siberia and Transbaikalia. All three species have an area of sympatry in West Siberia. Their eggs, tadpoles, and juvenile individuals are difficult to distinguish visually. Although the species are widespread in general, they are Red Listed in some countries. *R. temporaria* has vulnerable status in Romania, Italy, Slovenia, Greece, and is "strictly protected" in Serbia (Heatwole and Wilkinson 2015). *R. arvalis* is endangered in Romania and vulnerable in Slovenia (Heatwole and Wilkinson 2015). *R. amurensis* is protected in Mongolia (Terbish, 2006). *Rana arvalis* is Red Listed in four regions of Russia, *R. temporaria* in 12 regions, and *R. amurensis* in 10 regions (Kuzmin 2012). In many of these areas the species occur in sympatry in different combinations. Thus, a fast and cheap method for their identification in the areas of coexistence, applicable to eggs, larvae and juveniles would be valuable for ecological research and species surveillance.

Primer design was performed based on the alignment of the cytochrome c oxidase subunit I sequences of the studied species. In total, 117 sequences were obtained from GenBank (NCBI) and Barcode of Life Data System (BOLD) (Supplementary).

The regions with species-specific substitutions were found in the alignment and used for the design of species-specific forward primers. For the universal reverse primer, a conservative region was used to make the common start

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Table 1 Primers developed for the multiplex PCR assay to distinguish between *Rana amurensis*, *R. arvalis* and *R. temporaria*

Species	Primer name	Primer sequence (5'-3')	PCR product size [a]
Universal primer	HRun	TCTACTGATGGRCCTGCGT	
R. arvalis	LRar	TAATTTTCTTCATAGTCATGCCT	237
R. amurensis	LRam	TTGGAGCCCCTGATATAGCT	121
R. temporaria	LRte	CCCTAAGCCTACTYATTCGG	336

[a] in a combination with the universal primer

point for each PCR with the whole set of primers (Supplementary). Its position was chosen to yield PCR products with sufficient relative length differences to be separated and easily distinguishable by agarose gel electrophoresis. The developed primers are given in Table 1.

First, the assay was validated on the several samples of a known species identity. Samples were collected by ventral skin swabs using sterile collection swabs. Specimens of the *R. temporaria* were caught in Mari El Republic (56.79 N, 47.782 E and 56.80 N, 47.90 E). *R. amurensis* individuals were caught in the Omsk region (55.23 N, 73.01 E) and Khanty–Mansi Autonomous Okrug (61.16 N, 72.85 E). Samples of *R. arvalis* came from the Tyumen region (57.26 N, 65.18 E). All frogs were released at the place of capture immediately after sampling. To test the developed protocol on frog eggs, a single egg per clutch was collected from the clutches of unknown species across different sites in the Tyumen region. Total DNA isolated by QIAamp DNA Mini Kit (QIAGEN) according to manufacturers' instructions.

Initial optimization of PCR conditions was performed in 25 µl volume reactions containing 12.5 µl of BioMaster HS-Taq PCR-Color (2×) reaction mix (Biolabmix, Russia), 5 pmol of each of four primers, 5 ng DNA. Amplification was performed in a Mastercycler nexus gradient (Eppendorf) thermocycler with an initial denaturation step for 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, gradient of annealing temperatures from 50.4 to 63.6 °C for 20 s, 72 °C for 45 s, and 72 °C for 5 min for a final elongation. Visualization of PCR products was done via electrophoresis in 1.5% agarose gel with addition of ethidium bromide. Gels were run for 30 min at 100 V in 1x TAE buffer. For estimation of the length, 100 + bp DNA ladder (Evrogen, Russia) was used. For identification of egg samples, PCR was performed

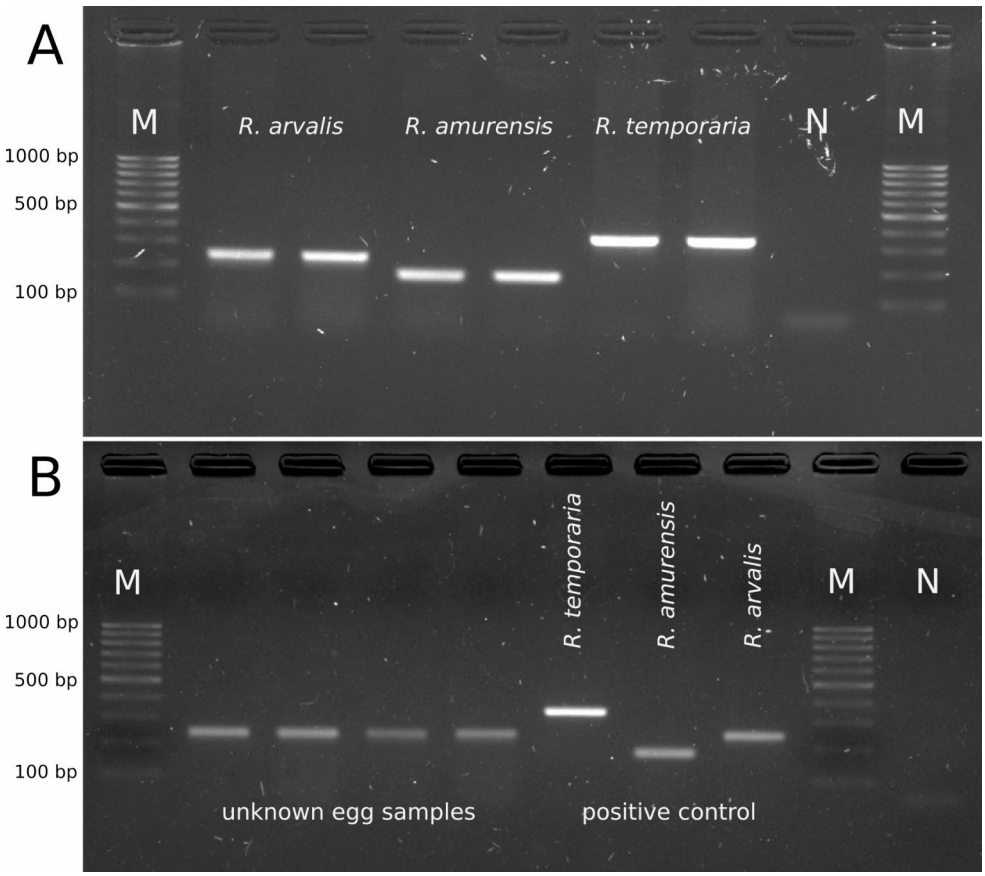

Fig. 1 **A.** Agarose gel showing PCR multiplex amplification for three species of brown frogs; **B.** Agarose gel electrophoresis for unidentified samples of frog eggs. M - DNA size ladder, N - no DNA template control

with the same reaction mixture and conditions, except using the selected for the assay annealing temperature.

The PCR products obtained with the control samples were of expected length (Fig. 1A). However, at the lower temperatures, several non-specific products that could complicate the interpretation of the results appeared with *R. amurensis* samples. Thus, an annealing temperature of 62 °C was selected for the further use. Length of the PCR products for all tested unidentified samples was the same and based on the length all samples belonged to *R. arvalis* (Fig. 1B).

In conclusion, the developed multiplex PCR is a simple and fast diagnostic tool for identification of the three sympatric *Rana* species.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12686-023-01338-y.

Author contributions ES conceived and designed the study. OE designed the assay and primers. AS, AL and ES conducted the fieldwork. PC and AL conducted the experiments. PC and ES analyzed the data. PC wrote the initial draft of the manuscript. All authors discussed the results, contributed to the draft, read the final manuscript, and gave approval for publication.

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Data Availability NA.

Code Availability No custom codes were used.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Ethical approval from Biomedical Ethic Committee of Tyumen State University (record no. 4\15.12.2021).

Consent to participate/consent of publication NA (no human participants in the study).

Clinical trials registration NA.

Gels and blots/image manipulation No manipulations were done.

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