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Molecular Differentiation of Three Loach Species (Pisces, Cobitidae) Based on the Nuclear 5S rDNA Marker*

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The diversity of the 5S rDNA fragment among three loach species: *Cobitis taenia*, *C. elongatoides* and *Sabanejewia aurata* was investigated using universal PCR primers for this gene. Three amplification products were obtained: 220 bp length for *C. taenia* and *C. elongatoides*, and 330 bp for *S. aurata*. Two amplicons with the same length (in *Cobitis*) were digested with *TaqI* restriction endonuclease. This enzyme found one restriction site T/CGA in the *C. elongatoides* fragment, while in the case of *C. taenia* no cleavage effect was observed. On this basis we constructed an easy and cheap method for loach species discrimination. It seems adequate for effective support of conservation initiatives for endangered loaches.

Key words: *Cobitis*, PCR-RFLP, *Sabanejewia,* species identification, 5S rDNA.

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The spined loach *Cobitis taenia*, the Danubian loach *Cobitis elongatoides* and the golden loach *Sabanejewia aurata* are considered as protected species and in many cases are distributed in the same water bodies (BĂNĂRESCU 1992). Although loaches from the genus *Cobitis* occur in many European waters, the geographical and taxonomic status of *S. aurata* is unclear. KOTTELAT (1997) classifies all subspecies of the genus *Sabanejewia* described in Europe as the monotypic species *S. balcanica* and suggests that true *S. aurata* are distributed only in northern Iran (Caspian Sea basin).

Some loaches, particularly from the genus *Cobitis*, exist in diploid-polyploid complexes and often exhibit very similar sets of morphological features (VASIL'EV *et al*. 1989; BOROŃ & DANILKIEWICZ 1998; BOHLEN & RÁB 2001). Moreover, these loaches show a relatively high level of flexibility in adaptation to local environmental conditions. At the same time, phenotypic species-specific characters may be partly modified toward unification and bring some problems in proper identification of fish individuals (BOROŃ 2003). Thus, identification of *C. taenia* based on only morphological features may erroneously classify different loach species, especially *C. elongatoides* (JANKO *et al*. 2007; JELEŃ *et al*. 2008; KOTUSZ 2008).

A main limitation in active protection of a species is incomplete knowledge about its geographical distribution combined with uncertain taxonomic status. Genetic methods used for *Cobitis* species identification are mainly based on chromosome examination (BOROŃ 2003; BOROŃ *et al*. 2006) or molecular analysis of mitochondrial DNA sequences (DOADRIO & PERDICES 2001; CULLING *et al*. 2006), but rarely involve nuclear DNA (JANKO *et al*. 2007). Nuclear DNA markers, underestimated and sometimes ignored, should be developed for loach identification. There is a growing need in fish research, especially in protected species, to extend the identification tool set and establish new, stable and reliable methods.

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Ribosomal genes are organized in the genome of vertebrates in two families of 45S rDNA (transcriptional unit encoding 28S, 5,8S and 18S rRNA) and 5S rDNA. They both have a tandemly repeated structure. The minor gene class consists of a conserved 120 bp coding region and a variable NTS unit, which usually show species-specific characters (LITTLE & BRAATEN 1989). We searched for differences between *C. taenia*, *C. elongatoides* and *S. aurata* within this nuclear DNA fragment.

The results of the present study offer a relatively simple and fast method for distinguishing the three loach species based on the 5S ribosomal DNA unit and PCR-RFLP technique. We hope our results will be helpful in many aspects of biological research conducted on these interesting and rather mysterious fish species.

Material and Methods

Sampling

Fin clips of five specimens from each investigated species were collected (*C. taenia* from lake Klawój, Poland, *C. elongatoides* from the Psovka River, Czech Republic and *S. aurata* from the Bug River, Poland), preserved in 96% ethanol and stored at -20°C. All individuals were first analyzed morphologically and cytogenetically in order to determine their species status.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from the fin clips of all specimens according to the method described by WALSH *et al*. (1991) with some modifications. Briefly, small pieces of tissue were placed in an eppendorf tube with 500 μl of 10% Chelex 100 solution (Biorad, USA) and Proteinase K (MP Biomedicals, USA). Incubation was conducted in 55°C for 3h with periodical shaking. After centrifugation at 2000 RPM for 10 minutes, 200 μl of the supernatant was transferred into a new tube and placed in -20°C. The content of DNA was checked on a 1.5% agarose electrophoresis gel (Prona, Spain) containing 1 μg ml $^{-1}$ of ethidium bromide (MP Biomedicals, USA).

For amplification of the 5S rRNA gene fragment we used the primer pair: 5S-1 (5'-TAC GCC CGA TCT CGT CCG ATC-3') and 5S-2 (5'-CAG GCT GGT ATG GCC GTA AGC-3') (KOMIYA & TAKEMURA 1979) and a GeneAmp PCR System 2700 (Applied Biosystems, USA) thermal cycler with the following reaction conditions: 5 minutes 94°C, 30 cycles of 1 minute 94°C, 30 s 55°C and 1 minute 72°C, and a final extension at 72°C for 5 minutes. The PCR reaction was carried out in a 50 μl reaction volume containing 10 μl of 5X Flexi Reaction Buffer (Promega, USA), 100 μM of each dNTP (Promega, USA), 3 mM of $MgCl_2$ (Promega, USA), 1.25 U GoTaq Flexi DNA Polymerase (Promega, USA), 20 pM of each primer, 5 μl of DNA template, and dH_2O. The sizes of PCR products were determined on a 1.5% agarose electrophoresis gel (Prona, Spain) containing 1 μg ml $^{-1}$ of ethidium bromide (MP Biomedicals, USA). A molecular weight marker O'Gene Ruler 50 bp (Fermentas, Canada) was used for size determination.

PCR products were purified using the Clean-Up System (A&A Biotechnology, Poland) according to the manufacturer's manual, and then sent to the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland for direct sequencing by an ABI3730 Genetic Analyzer (Applied Biosystems, USA). All sequences were analyzed using Mega software v.4.0 (TAMURA *et al*. 2007), and have been deposited in the GenBank database under the following accession numbers: HQ456227 (*C. taenia*), HQ456228 (*C. elongatoides*) and HQ456229 (*S. aurata*).

RFLP analysis

The obtained nucleotide sequences were used for preparation of restriction site maps using NEBcutter software v. 2.0 (VINCZE *et al*. 2003). The proper restriction endonuclease for RFLP analysis was selected on this basis. DNA digestion was carried out in a 31 µl mixture containing 1 μl PCR product, 2 μl of 10X buffer TaqI, 10 U *TaqI* restriction enzyme (Fermentas, Canada), 27 μl dH_2O at 65°C for 3h. A total of 8 μl of the reactant mixture was run on a 1.5% agarose gel (Prona, Spain) and visualizated under UV illumination in UVIsave Gel Documentation System (UVItec, UK).

Results and Discussion

The applied DNA preparation method produced an optimal quantity of double-stranded template for amplification in each specimen. The PCR reaction resulted in the three following products: 220 bp in *C. taenia* and *C. elongatoides*, and 330 bp in *S. aurata* (Fig. 1). Sequencing yielded data on the exact nucleotide sequence and length of each 5S rDNA fragment (Fig. 2). Based on the restriction maps, we found that the enzyme *TaqI* was able to differentiate sequences in *C. taenia* and *C. elongatoides* by cutting the second one between 140 and 141 nucleotides within a T/CGA fragment (Fig. 2). After enzymatic digestion we observed no cleavage effect in *C. taenia* and three fragments in *C. elongatoides* (Fig. 1). We found a characteristic RFLP pattern in *C. elongatoides* (two large bands

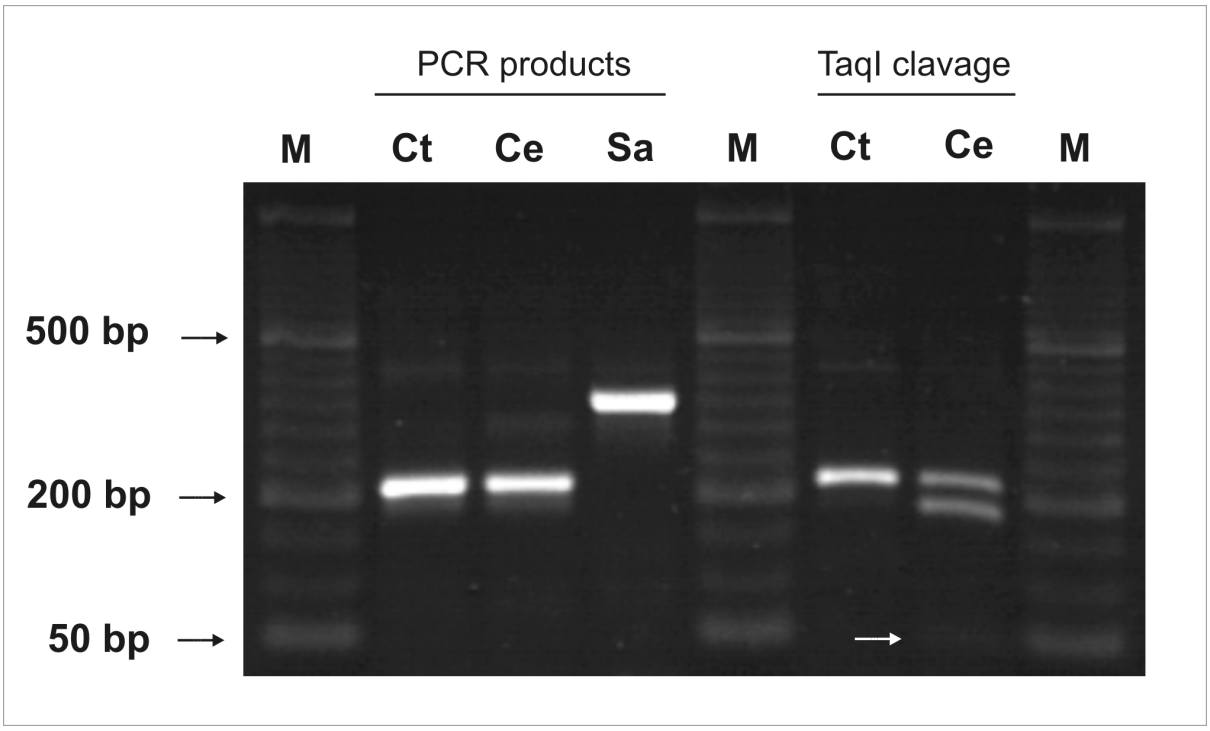

Fig. 1. Electrophoresis of the 5S rDNA fragment in a 1.5 % agarose gel after PCR amplification and *TaqI* digestion (M – weight DNA marker, Ct – *C. taenia*, Ce – *C. elongatoides*, Sa – *S. aurata*); white arrow indicates a 61 bp fragment in the RFLP pattern of *C. elongatoides*.

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              *        20         *        40         *        60         *        80
Ct : GGACATGGTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCATTTTGAATGGGCCAAAA :  83
Ce : GGCAGCAGTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCATTTTGAATGGGCAAAAA :  83
Sa : GGCACAGTCGGGCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTTTTTGGGTTTCCTTCCTTA :  83

            *       100         *       120         *       140         *       160
Ct : AGCCCCTATAAGCTTTTGCTGAGGTTTAACCGATTCCACTGGTGTGTAAGTGCCTCTCGGACGGCGGCCGCTTTCGCTTACGG : 166
Ce : AGCCCCTATAAGCTTTTGCTGATGTTCAGGCATTGCCATTGGTCTTGAAGTGCCTTTCGAACGGCGGCCGCTTTCGCTTACGG : 166
Sa : ATTGTCGGATGCCTGCGCTTTGCCCAGGCTCACTTTTCACCCTTCTACGTTCCTCTTCTTTGCGATGACTGGTGCATTTGTGG : 166

          *       180         *       200         *       220         *       240
Ct : CCATACCAGCCTGA------------------------------------------------------------------- : 180
Ce : CCATACCAGCCTAA------------------------------------------------------------------- : 180
Sa : ATGTGTTTTTTACAAGGCAAGGGGTCCGTTGGGGTTGCAGTCGCAGAGCAAAACGCTCGTCTGTGCATGTATATAGGAAAAAC : 249

        *       260         *       280         *       300         *       320         *
Ct : ------------------------------------------------------------------------------- :   -
Ce : ------------------------------------------------------------------------------- :   -
Sa : CCGGGAAGGCTATGCTCAAAGGGCCCGGCAAAAAAGCCCCTTTCACTTACCGCCATATCCAGCCTGACCATACCAGCCTGA : 330
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Fig. 2. 5S rDNA sequences of *C. taenia* (Ct), *C. elongatoides* (Ce) and *S. aurata* (Sa); T/CGA motif recognized by the *TaqI* restriction enzyme is marked by a black frame.

and one small band with low intensity, indicated by the white arrow in Fig. 1).

The structure of 5S rDNA makes this fragment very useful for species identification, including fishes (PENDAS *et al*. 1995; CÉSPADES *et al*. 1999; CARRERA *et al*. 2000; ARANISHI & OKIMOTO 2004). The simplest way to identify a species using 5S rDNA is to detect length differences among species (CARRERA *et al*. 2000). Between closely related species, we cannot often expect differences in the size of this DNA fragment, but usually we find some nucleotide changes along the sequence (PENDAS *et al*. 1995).

In our study, gel electrophoresis was inadequate for simple differentiation of all 5S rDNAs, because the PCR products from *C. taenia* and *C. elongatoides* had a similar size (Fig. 1). Thus, we have used enzymatic digestion with *TaqI* for both undifferentiated fragments. In the course of restriction enzyme analysis we observed three DNA bands of 180, 161 and 61 bp in *C. elongatoides*, whereas the *TaqI* enzyme did not find any restriction site in the *C. taenia* sequence (Figs 1 & 2). Within the RFLP pattern observed in all Danubian loach specimens, the biggest band equals the entire amplified product of the 5S rRNA gene. This indicates that only a part of the DNA was digested by the *TaqI* enzyme and the rest was not recognized by this restrictase. A small amount of DNA could explain the visual effect of low intensity in the band of 61 bp length. The observed RFLP pattern of two different products of 5S rRNA in the Danubian loach suggests the effect of heterozygosity in the studied locus. The same RFLP pattern was also found in specimens from another population of the Danubian loach (KIRTIKLIS, unpublished data). We do not know the real origin of this mutation in the examined species, but the occurrence of different variants of 5S rDNA is common in fishes (for review see MESSIAS *et al*. 2003) and some changes of these ribosomal fragments have originated early in the evolution of the vertebrate genome (KOMIYA *et al*. 1986; LITTLE & BRAATEN 1989; LEAH *et al*. 1990). Perhaps we are observing a rather similar model of diversification in the case of the Danubian loach? We cannot confirm or exclude this presumption today, because it is very unlikely to determine the sequence of 5S rDNA from pre-glacial parental lineages of *C. elongatoides*. However, we suggest a wider screening of this genome fragment for a comparison among specimens from different populations.

In conclusion, the result of PCR-RFLP technique using 5S rDNA fragments in loaches is discernible and does not procure any ambiguities during its application. Thus, the method described herein proved to be useful in the determination of the mentioned species, it seems easy to use and generates low costs. Proper fish identification can help to protect these endangered species, while the results of this study extend the collection of available species identification techniques.

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