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Characterization of Three Species from the Subfamily Leuciscinae (Pisces, Cyprinidae) Using the Nuclear ITS-1 rDNA Spacer*

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Fish species from the subfamily Leuciscinae are an important part of the European ichthyofauna. The abundance of this fish group has decreased in some natural populations because of human impact and partly by interspecific hybridization. The objective of the present study was to use the ITS-1 rDNA spacer for identification of the European chub, the common dace and the ide. The examination was conducted using the PCR-RFLP technique. PCR products of closely-related species were discriminated using *HinfI* and *SmaI* restriction endonucleases. Characteristic RFLP patterns observed in this study offer a simple method for distinguishing the species, thus providing an additional method of identification useful in fish management, biodiversity conservation and aquaculture.

Key words: ITS-1, *Leuciscus*, PCR-RFLP, rDNA, species identification, *Squalius*.

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According to the present freshwater fishes literature, the European chub *Squalius cephalus* (former name: *Leuciscus cephalus*), the common dace *Leuciscus leuciscus* and the ide *Leuciscus idus* are widely distributed throughout Europe (BANARESCU 1992). Formerly, these three species were considered as closely related fishes within the genus *Leuciscus*, living in the same water bodies. However, some morphological, meristic, karyological and molecular features have contested the classification of the described species within the genus (BOGUTSKAYA & ZUPANCIC 1999; DURAND *et al*. 2002; BOROŃ *et al*. 2009). As a result of the above mentioned disagreement, two equivalent genus names (*Leuciscus* and *Squalius*) exist in the literature for the European chub. Proper identification of these three species, based mainly on morphological characters, is not always easy, especially in the case of young individuals. In addition, these fishes can produce natural interspecific and intergeneric hybrids, occurring in the same waters as the parental species (WHEELER 1978; WITKOWSKI & BŁACHUTA 1989). Thus, it is very important to apply simple genetic markers for the discrimination of these species and as support for distinguishing them from their hybrids. These fishes are considered a natural and essential component of many freshwater ecosystems, and they also have some practical meaning. The common dace is used as a bioindicator in degraded rivers in which the composition of the ichthyofauna has changed due to human impact (WOJDA *et al*. 1993). The ide is one of the species bred in aquaculture with other cyprinids used for commercial purposes (WITKOWSKI & BŁACHUTA 1989). Furthermore, the European chub and the ide are important for anglers. The foregoing reasons point at the need of having some simple and low-cost identification method for these three fish species. Proper species identification is key to biodiversity conservation in natural freshwater ecosystems and aquaculture management.

The present paper focuses on the use of the PCR-RFLP technique for distinguishing the ide, the common dace and the European chub in order to develop a new DNA marker especially useful

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for identification of morphologically undifferentiated specimens at the early stages of development. Such identification is a very important step in fish management. In this study we use the internal transcribed spacer 1 (ITS-1) sequence for characterization of these three fish species.

In eukaryotes, including fish species, there are two classes of ribosomal genes, described as major- and minor ribosomal genes. These sequences are tandemly repeated as multiple copies of transcription units (for review see: VERA *et al*. 2003; PISANO & GHIGLIOTTI 2009). The ITS-1 spacer, as well as ITS-2, is a part of the major ribosomal DNA gene unit which encodes 18S, 5,8S and 28S rRNA. In general, the sequences of both internal transcribed spacers are more variable than ribosomal coding gene sequences because they accumulate numerous substitutions after species divergence (HILLIS & DIXON 1991). The ITS-1 spacer separates the 18S subunit from the 5,8S subunit (Fig. 1) and its sequence often shows quite high nucleotide variation among closely related species (NWAKANMA *et al*. 2003). The ITS-2 spacer, placed between subunits 5,8S and 28S, is less polymorphic than the ITS-1 fragment and is often used for molecular analysis among higher eukaryote taxa. In consequence, the ITS-1 region is widely use for phylogenetic analysis (COLLEMAN & VACQUIER 2002; WYATT *et al*. 2006) and also for the identification of closely related species (FERNANDEZ *et al*. 2001; HIDEYUKI *et al*. 2004).

Moreover, the combination of the ITS-1 spacer sequence and PCR-RFLP analysis is used as a simple and efficient marker for species identification, including fishes (CHOW *et al*. 1993).

Material and Methods

All experiments were approved by the Local Ethics Committee in Olsztyn (No. 65/2007 from 24 July 2007).

Sampling

Fin clips from 29 specimens of *S. cephalus,* 21 specimens of *L. idus* and 22 specimens of *L. leuciscus* from the Odra and the Wisła Rivers (Poland) were used as material for investigation. Tissue samples were preserved in a 96% ethanol solution and stored at -20ºC.

Each individual was previously examined and assigned to a morphological species.

DNA extraction

Total DNA was extracted from all collected fin clips using the DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA content was checked on a 1.5% agarose electrophoresis gel (Prona, Spain) containing 1 μg ml^{-1} of ethidium bromide (MP Biomedicals, USA). Obtained DNA was stored at -20ºC.

PCR amplification and sequencing

The primer set (ITS1F: 5'- AGG TTT CCG TAG GTG AAC CT - 3' and ITS1R: 5'- AGT GTC GAT GAT CAA TGT GTC CTG – 3') described by WYATT *et al*. (2006) was used for ITS-1 amplification. PCR was conducted in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, USA) with the following conditions: 1 minute 94°C, 35 cycles of 1 minute 94°C, 1 minute 55°C and 1 minute 72°C, and the final extension at 72°C for 7 minutes. The PCR was carried out in a 50 μl reaction volume containing 25 μl Master Mix (Promega, USA), 2 μl of DNA template, 20 pmol of each primer and dH_2O. PCR product size was determined on a 1.5 % agarose gel containing 1 μg ml^{-1} of ethidium bromide (MP Biomedicals, USA), using O' Gene Ruler 50 bp molecular weight marker (Fermentas, Lithuania).

PCR products were purified using the GeneElute PCR Clean-Up Kit (Sigma, USA) according to the manufacturer's instructions, and then sent to the Institute of Biochemistry and Biophysics, Polish

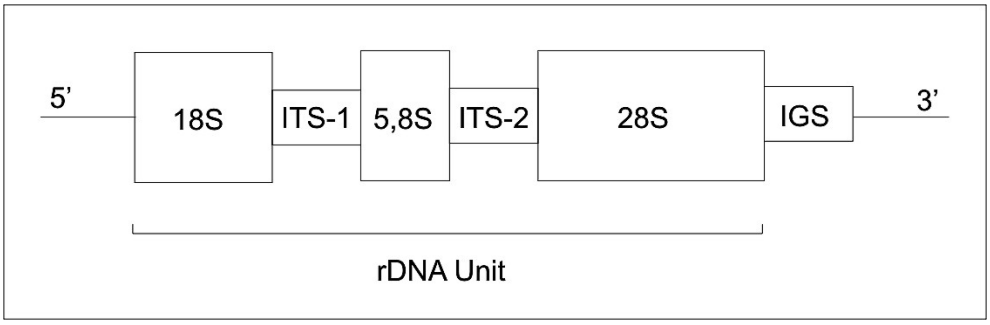

Fig. 1. General organization of the ribosomal DNA unit. The internal transcribed spacers are described as ITS-1 and ITS-2, the ribosomal genes are indicated as 18S, 5,8S, 28S and the intergenic spacer is marked IGS.

Academy of Sciences in Warsaw, Poland for direct sequencing by an ABI3730 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were analyzed using MEGA software v. 4.0 (TAMURA *et al.* 2007), and then they were confirmed by the Basic Local Alignment Search Tool (BLAST) (ZHANG *et al.* 2000). The sequences were deposited in the GenBank database under following numbers: JQ352764, JQ352765 (*S. cephalus*), JQ352766, JQ352767 (*L. idus*), JQ352768, JQ352769 (*L. leuciscus*).

RFLP

Two-step RFLP analysis, based on *HinfI* and *SmaI* endonucleases, was carried out according to the manufacturer's protocol (Fermentas, Lithuania). The first restriction digestion was performed on the amplicons of the European chub, the ide and the common dace in a 31 μl mixture containing 10 μl of PCR product, 2 μl of 10X buffer HinfI, 10 U *HinfI* restriction enzyme, 17 μl dH_2O at 37°C for 3h. An 8 μl restriction mixture was run on a 1.5 % agarose gel and visualized under UV light in the UVIsave Gel Documentation System (UVItec, UK). The second step of RFLP analysis was carried out on the PCR products of the common dace and the ide using the *SmaI* restriction enzyme under the same reaction conditions.

Results

The DNA extraction technique allowed obtaining an optimal quantity of double-stranded template for amplification in each fish specimen. PCR of the ITS-1 region produced three fragments: 465 bp in *S. cephalus*, 480 bp in *L. leuciscus* and 460 bp in *L. idus* (Fig. 2). After the first digestion using the *HinfI* endonuclease, three restriction patterns were observed: 70/395 bp for *S. cephalus*, 70/160/230 bp for *L. idus* and 70/165/240 bp for *L. leuciscus* (Fig. 3). In the second step of RFLP analysis, one CCC↓GGG restriction site recognized by *SmaI* was found in the ITS-1 spacer of *L. idus* (restriction pattern: 215/245 bp). We did not observe any digestion effect in the ITS-1 fragment of *L. leuciscus* (Fig. 4).

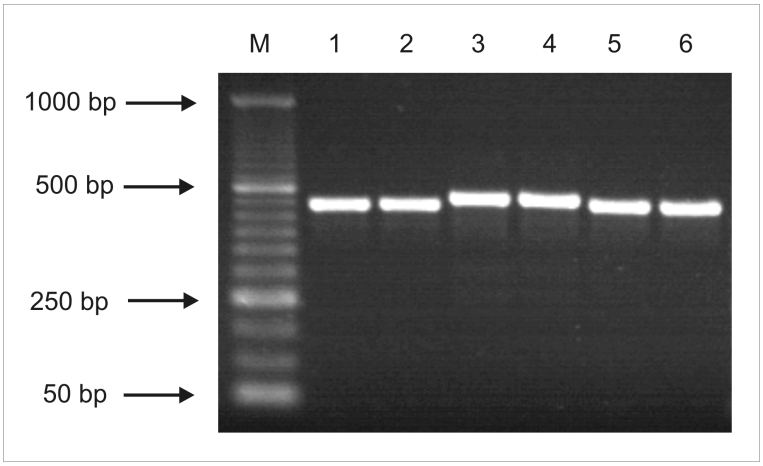

Fig. 2. Electrophoresis of the ITS-1 PCR products: 465 bp in the European chub (1-2), 480 bp in the common dace (3-4) and 460 bp in the ide (5-6); M – molecular weight marker.

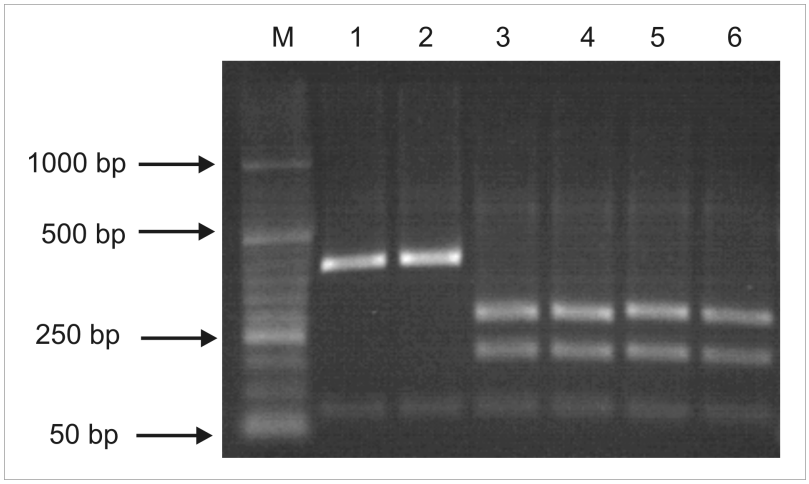

Fig. 3. *HinfI* restriction profile of the ITS-1 PCR products: 70/395 bp in the European chub (1-2), 70/160/230 bp in the ide (3-4) and 70/165/245 bp in the common dace (5-6); M – molecular weight marker.

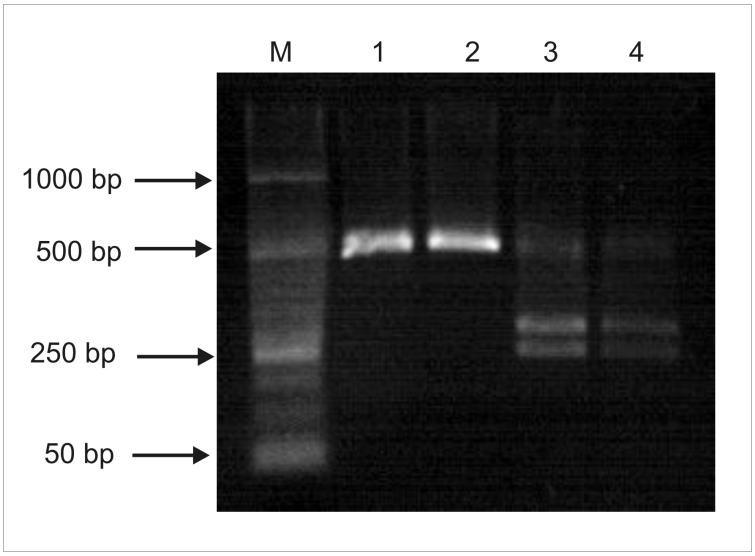

Fig. 4. *SmaI* restriction profile of the ITS-1 PCR products: 480 bp in the common dace (1-2) and 215/245 bp in the ide (3-4); M – molecular weight marker.

Discussion

The results of this study show that the amplified PCR product of the ITS-1 spacer has a different size in each studied species. We did not expect any differences in the ITS-1 size of the ide, the common dace and the European chub, because there are few data in the literature about any length variation of this region among closely-related fish species. In our opinion, the observed interspecific size differences of the analyzed rDNA region are not sufficient for routine identification of these three fish species. In addition, standard resolution of the agarose gel electrophoresis, routinely used in many laboratories, is often too low to separate two DNA fragments with similar length. In many cases, this limitation causes problems with reading and interpreting results. However, we have found some differences regarding the nucleotide sequence of the studied DNA fragment. This polymorphism allowed discrimination of each examined DNA fragment using RFLP analysis. The use of restriction endonucleases in order to obtain differences in DNA fragments of similar length is common and often reported in the literature. Nucleotide variation within the ITS-1 region among closely-related fish species was reported by REED *et al*. (2000) in some coregonids from North America. In our study, the application of the *HinfI* endonuclease for RFLP analysis of this rDNA spacer resulted in a different genotype for each species (Fig. 3). However, restriction patterns for the ide and the common dace were very similar, but a second step in the RFLP analysis based on the *SmaI* restriction enzyme permitted the discrimination of these two species (Fig. 4).

In the past, fish species discrimination was based mainly on morphological features, while nowadays, there are many different molecular techniques used as support for species discrimination. Proper identification of some species from the subfamily Leuciscinae has been carried out using biochemical-genetic tools (MANARESI *et al*. 2001) and some DNA analysis. BARINOVA *et al*. (2004) applied microsatellite markers for this purpose. However, mtDNA fragments are mostly used for differentiating these closely related species (DURAND *et al*. 2002). Some differences in the genomes of the ide, the common dace and the European chub were detected also at the chromosome level. KIRTIKLIS *et al*. (2010) found characteristic patterns in the distribution of co-localized ribosomal DNA sequences which were specific for each of the studied species. Thus, proper identification within this fish group may be efficiently supported also by the cytogenetic method.

In addition to other methods, the present results provide an efficient and relatively simple tool for discriminating these three fishes. Such a method brings results in a rather short time and generates low costs. Moreover, it does not require killing the fish specimens because only a small amount of tissue for DNA extraction is needed. This identification technique may be successfully used as support for other species discrimination methods. We expect that this new methodological tool can be useful for people and institutions working for biodiversity conservation of natural ecosystems and for aquaculture purposes.

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