www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 17:1127-1133(2017) DOI: 10.4194/1303-2712-v17_6_06

RESEARCH PAPER

5S rDNA Sequence Shows Differences between Diploid and Triploid Prussian Carp *Carassius gibelio* (Teleostei, Cyprinidae)

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Received 04 January 2017
Accepted 28 February 2017

Abstract

The Prussian carp may occur as diploid (2n=100) and/or triploid (3n=150) individuals, co-existing in many natural populations. The simultaneous occurrence of individuals with different ploidy makes the taxonomy of this species unclear. Additionally, the taxonomic status of *C. gibelio* has become even more enigmatic due to its hybridizing with other non-indigenous cyprinids. Since the variation within 5S rDNA can serve as a suitable marker for molecular identification of the fish ploidy, the main aim of present study was to compare this rDNA sequence between Prussian carp individuals with different ploidy levels: diploids (2n =100) and triploids (3n=150-160). PCR amplification of 5S rDNA generated two bands of approximately 340 and 470 bp in length (in both diploids and triploids) and band 200 bp visible in some individuals. These results indicate the presence of at least two different classes of 5S rRNA gene. Analysis of their nucleotide composition revealed no differences within the class of 340 bp and several nucleotide differences within the class of 470 bp between diploid and triploid individuals. The 5S rDNA variability detected in this study indicates the potential usefulness of this sequence for the identification of diploid and triploid individuals of the Prussian carp.

Keywords: 5S rDNA, *Carassius*, diploids, interspecific hybridization, polyploids.

Introduction

The Prussian carp, *Carassius gibelio* (Bloch, 1782) is an alien species in the European ichthyofauna, commonly thought to have been introduced from eastern Asia for breeding purposes (Tóth, Várkonyi, Hidas, Edviné Meleg, & Váradi, 2005; Sakai, Iguchi, Yamazaki, Sideleva, & Goto, 2009). *C. gibelio* is the most abundant and widespread species of *Carassius auratus* complex in Europe (Kalous, Bohlen, Rylková, & Petrtýl, 2012; Rylková, & Kalous, 2013). *C. auratus* complex includes diploids and polyploids exhibiting asexual and sexual reproduction modes (Gui & Zhou, 2010; Bai, Liu, Li, & Yue, 2011; Jiang *et al*., 2013; Šimková, Hyršl, Halačka, & Vetešník, 2015).

A wide spectrum of ecological tolerance and extraordinary resistance to adverse environmental conditions enabled *C. gibelio* to successively and progressively spread to various water bodies (Kottelat & Freyhof, 2007; Grabowska, Kotusz, & Witkowski, 2010; Boroń *et al*., 2011). Moreover, this species frequently hybridizes with other cyprinids, especially with the crucian carp, *Carassius carassius* (Linnaeus, 1758), which is native in Europe as well as with *C. auratus* and common carp, *Cyprinus carpio* (Linnaeus, 1758) (Sayer *et al*., 2011; Mezhzerin, Kokodii, Kulish, Verlatii, & Fedorenko, 2012; Wouters, Janson, Lusková, & Olsén, 2012; Rylková & Kalous, 2013). These hybrids show high morphological similarity and reveal better adaptations to unfavourable conditions than their parental species. Furthermore, they exhibit food competition with other fish, both in natural (e.g. *C. carassius*) and farmed (e.g. *C. carpio*) populations (Szczerbowski, 2002). In consequence, the Prussian carp, as well as its hybrids can easily replace other fish species. It may have a negative impact on the European ichthyofauna – affecting the overall range, distribution and abundance of commercially and recreationally valuable fish (Richardson, Whoriskey, & Roy, 1995; Witkowski & Grabowska, 2012).

Apart from arising of *C. gibelio* hybrids, the simultaneous occurrence of diploid (2n=100) males and females and triploid (3n=150-160) females in its many populations (Boroń *et al*., 2011; Rylková & Kalous, 2013; Kang *et al*., 2014) makes the identification of this species more unclear, especially as some *C. gibelio* populations remain unisexual, composed of triploid females (Jiang *et al*., 2013).

In higher eukaryotes, the sequence of 5S rDNA is organized in repetitive units, which consist of coding a conservative sequence with 120 base pairs and non-coding flanking sequence NTS. NTSs show extensive length and structure variation (Vierna, Wehner, Höner zu Siederdissen, Martinez – Lage, & Marz, 2013; Rebordinos, Cross, & Merlo, 2013; Qin, Wang, Wang, Liu, & Liu, 2015). Additionally, some differences involving gene expression between somatic- and oocyte-type of 5S rRNA have also been reported (Komiya, Hasegawa, & Takemura, 1986).

To date, the structural and functional organization of the 5S rRNA genes has been studied in fungi (Cihlar & Sypherd, 1980), plants (Negi, Rajagopal, Chauhan, Cronn, & Lakshmikumaran, 2002), animals, including fish (Martins & Wasko, 2004). In some fish species, different classes of this gene have been described. Each of the different-sized 5S rDNA classes vary in length, nucleotide changes and chromosome location (Martins, 2006; Messias *et al*., 2003; Pinhal *et al*., 2008; Campo, Machado-Schiaffino, Horreo, & Garcia-Vazquez, 2009). Most fish species are characterized by the occurrence of two 5S rDNA arrays with a different NTS (Martins & Galetti Jr., 2001; He *et al*., 2012; Qin, Wang, Wang, Liu, & Liu, 2015). Variations within NTS have often been used as species-specific molecular markers (Wang *et al*., 2014; Han, Yen, Chen, & Tseng, 2015) and also as a good tool for identification of individuals with different ploidy level (He *et al*., 2012; Qin, Wang, Wang, Liu, & Liu, 2015).

In the current study, a comparative analysis of nucleotide sequence and molecular organization of 5S rDNA in diploids (2n =100) and triploids (3n=150-160) of the Prussian carp were carried out to indicate the impact of ploidy on the 5S rDNA. Moreover, the possibility of using the NTS variations as a potential ploidy-linked molecular marker of *C. gibelio* were verified.

Materials and Methods

Sampling

In total, 35 individuals, including 31 diploid individuals 2n = 100 (15 females, 16 males) and 4 females 3n=150-160 of Prussian carp were collected from the Siemianówka Dam Reservoir (Poland) (52°55'N, 23°48'E). The fish were identified by the following morphological traits: silvery-brown body colour, strongly-serrated last unbranched (hard) ray in the anal and dorsal fin, concave or straight free edge of the dorsal fin and black peritoneum (Kottelat & Freyhof, 2007). Other morphological features, such as the number of gill rakers and the number of scales in a lateral line were also verified (not presented here). The sex of all specimens was determined according to the histology of gonads. All specimens were analysed cytogenetically in order to determine their ploidy level and the karyotype (not presented here; see Boroń *et al*., 2011 for details). Chromosome slides were made from kidney cells using conventional hypotonic treatment and methanol-acetic fixation following the standard air-drying technique as shown in detail by Boroń *et al*. (2011). Fin clips of each individual were preserved in 96% ethanol and stored at -20% for further DNA extraction.

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from the fin clips using the Chelex 100 method as described by Walsh, Metzger and Higuchi (2013) with some modifications (Kirtiklis, Boroń, Ptasznik, Lusková, & Lusk, 2011). Briefly, tiny fragments (1 x 2 mm) of fin clips were placed in an Eppendorf tube containing 500µl of 10% Chelex 100 solution (Biorad, USA) and Proteinase K (A&A Biotechnology, Poland) and incubated at 55°C for 3 hours with periodical shaking. After centrifugation at 2000 rpm for 10 minutes, about 200 µl of the supernatant was transferred into a new tube and stored at - 20°C.

A set of primers: 5S-1 (5'-TAC GCC CGA TCT CGT CCG ATC – 3') and 5S-2 (5' - CAG GCT GGT ATG GCC GTA AGC – 3') by Komiya and Takemura (1979) was used for amplification of the 5S rDNA. PCR amplifications were performed in 50 µl of reaction mixtures containing 10 µl of 10x PCR buffer with Mg^{2+}, 100 µM of each dNTP (A&A Biotechnology, Poland), 10 µM of each forward and reverse primers, 1.25 U of Run DNA Polymerase (A&A Biotechnology, Poland), 2 µl of DNA template, and dH_2O using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, USA). The PCR cycling conditions were as followed: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds and primer extension at 72°C for 30 seconds and a final extension at 72°C for 7 minutes.

Amplified products were visualized by electrophoresis in an ethidium bromide-stained 1.5% agarose gel (Sigma-Aldrich, USA). All amplicons were purified from the gel using Gel-Out Concentrator kit (A&A Biotechnology, Poland) and then sequenced on both strands using a commercial service (Genomed, Poland).

Sequence Alignment and Evaluation, Phylogenetic Analysis

All sequences were analysed using BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999) and then subjected to BLASTn analysis (Altschul *et al*., 1997). The obtained sequences were deposited in the GenBank database under the following accession numbers: KU359472 (340 bp fragment of diploid), KU359473 (340 bp fragment of triploid), KU359474 (470 bp fragment of diploid), KU359475 (470 bp

fragment of triploid). Multiple alignment of the analysed sequences was performed using Clustal W (Thompson, Higgins, & Gibson, 1994). Phylogenetic analysis was carried out using MEGA 6.0 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) by the neighbour-joining method with 1,000 bootstrap replicates. The reference sequences from the GenBank (NCBI) were used as followed: *C. auratus gibelio* (DQ659269), *C. auratus* (DQ659275), *C. auratus langsdorfii* (AB007784), *C. cuvieri* (KR706447), *Cyprinus carpio* (GU188692), *Danio rerio* (AF213517), *Salmo salar* (S73106), *Pagrus pagrus* (JN903267), *Leporinus elongatus* (AF284729), *Cobitis elongatoides* (HQ456228), *Sabanejewia aurata* (HQ456229), *Oreochromis urolepis* (GU075925), *Lepidorhombus boscii* (GQ247749), *Xenopus borealis* (V01425), *Anolis carolinensis* (FJ158977), *Gallus gallus* (AF419701), *Rattus norvegicus* (X83748), *Homo sapiens* (X71802).

Results

Amplification of 5S rDNA generated two major bands of approximately 340 and 470 bp in lengths (both in all diploid and triploid individuals) and one band 200 bp with lower intensity visible only in some diploid and triploid individuals (Figure 1). Although Figures 1a and 1b presented electrophoretic patterns of individuals which generated all three amplicons, in this study we focused on two of them: 340 and 470 bp.

DNA sequencing yielded data on the exact nucleotide sequence of 5S rDNA. Comparative analysis of the 5S rDNA fragment revealed no differences within the 340 bp class between diploid and triploid individuals of *C. gibelio* (Figure 2). However, several nucleotide differences – as a deletion (position 240) and substitutions (positions 197-254) – within the 470 bp class between diploids and triploids of *C. gibelio* were revealed (Figure 3). No sex-linked differences regarding the length and nucleotide composition of the 5S rDNA sequence among diploids were detected.

Phylogenetic analysis showed that all the species from the genus *Carassius*, apart from *C. cuvieri*, formed one group on the tree. A neighbour-joining analysis with a 97% and 80% bootstrapping value (respectively) based on 340 bp and 470 bp classes of 5S rRNA gene showed that *C. gibelio* had a closer relationship with *C. auratus* and *C. auratus langsdorfii* than *C. cuvieri*. Sequences 340 bp and 470 bp of 5S rRNA gene of *C. gibelio* formed two separated clades within the group of the genus *Carassius* (Figure 4).

Discussion

This is the first report concerning the 5S rDNA structure of diploid and triploid individuals of Prussian carp from the natural environment of the European population. To date, there is only data obtained from the Chinese farmed population of triploid (3n=162) *C. gibelio* (formerly *C. auratus gibelio)*, where the occurrence of two classes of the 5S rDNA sequences with lengths of 340 bp and 209 bp were detected. The first of these fragments has been sequenced and published in the GenBank (Zhu, Ma, & Gui, 2006). The latter 5S rDNA gene class, with an approximate length of 200 bp shown in *C. gibelio* in the present study, has also been reported in *C. auratus*, but the authors did not provide any discussion involving this rDNA fragment (He *et al*., 2012, Zhang *et al*., 2015).

Different classes of the 5S rRNA gene, identified in many fish species, varied by length, several nucleotide mutations or chromosomal location (Messias *et al*., 2003; Pinhal *et al*., 2008; Campo, Machado-Schiaffino, Horreo, & Garcia-Vazquez, 2009; He *et al*., 2012, 2013). Multi-class type 5S rDNA are considered an ancestral character that originated early in the history of vertebrates (Frederiksen, Cao, Lomholt, Levan, & Hallenberg, 1997). However, not all ancient groups of fish exhibit different types of minor rDNA (Messias *et al*., 2003). The presence of at least the two different classes (340 and 470 bp) of 5S rRNA gene in the Prussian carp may suggest that its genome retained some ancestral

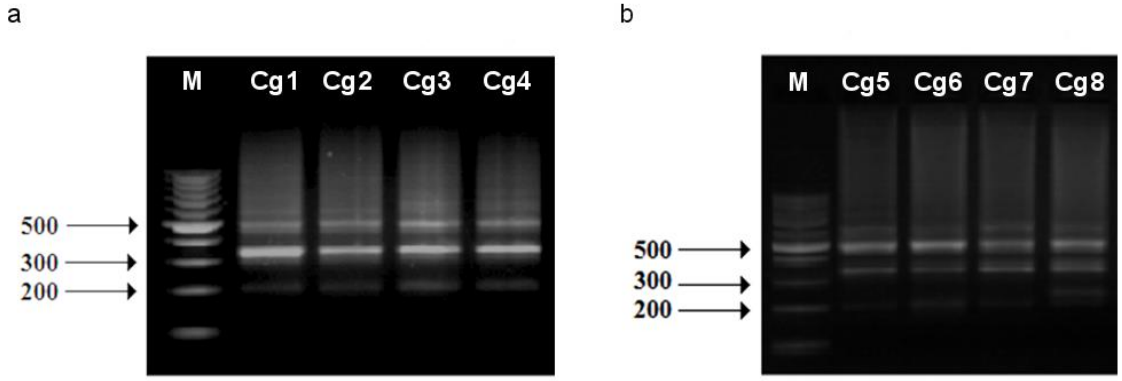

Figure 1. Electrophoresis of the 5S rDNA amplicons in a 1.5% agarose gel: M – weight DNA marker, (a) Cg1-Cg4 – diploid individuals of *C. gibelio*, (b) Cg5-Cg8 – triploid individuals of *C. gibelio*.

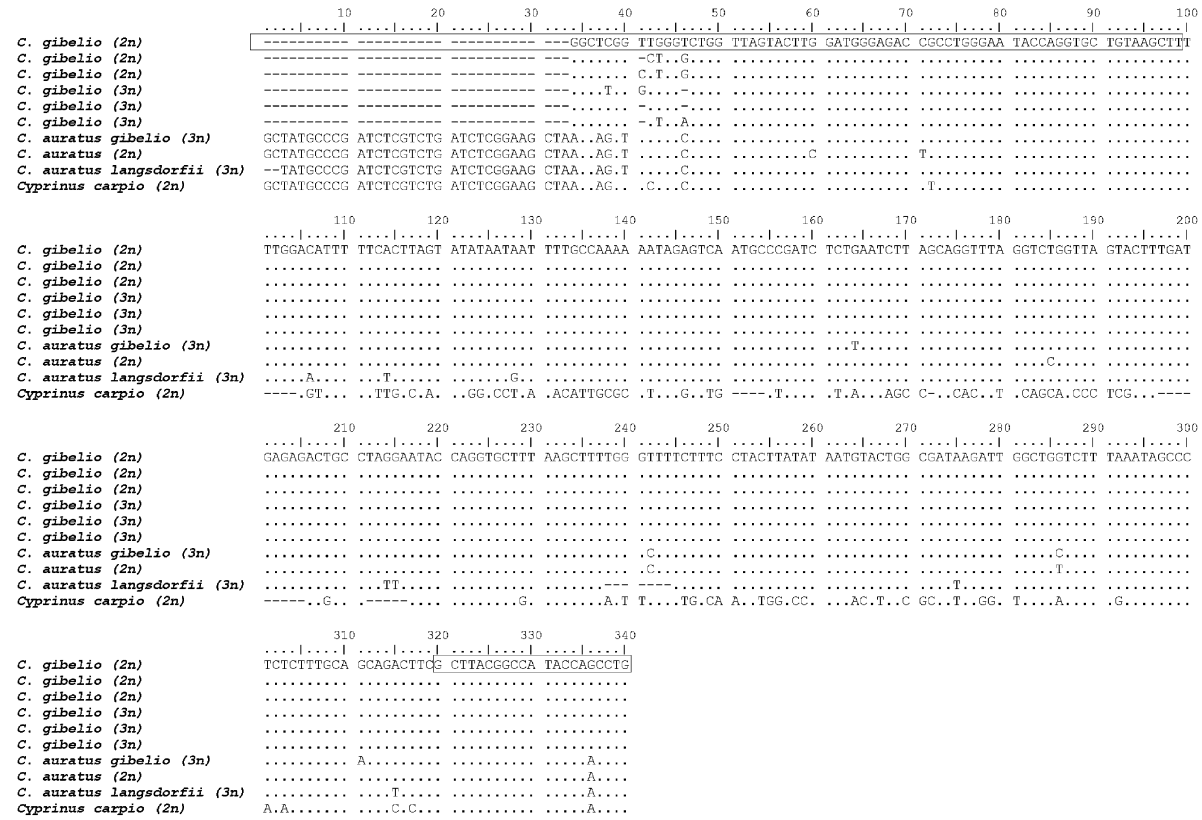

Figure 2. Nucleotide sequence alignment of 5S rRNA gene in class 340 bp of *C. gibelio*, *C. auratus gibelio* (DQ659269), *C. auratus* (DQ659275), *C. auratus langsdorfii* (AB007784) and *Cyprinus carpio* (GU188692).

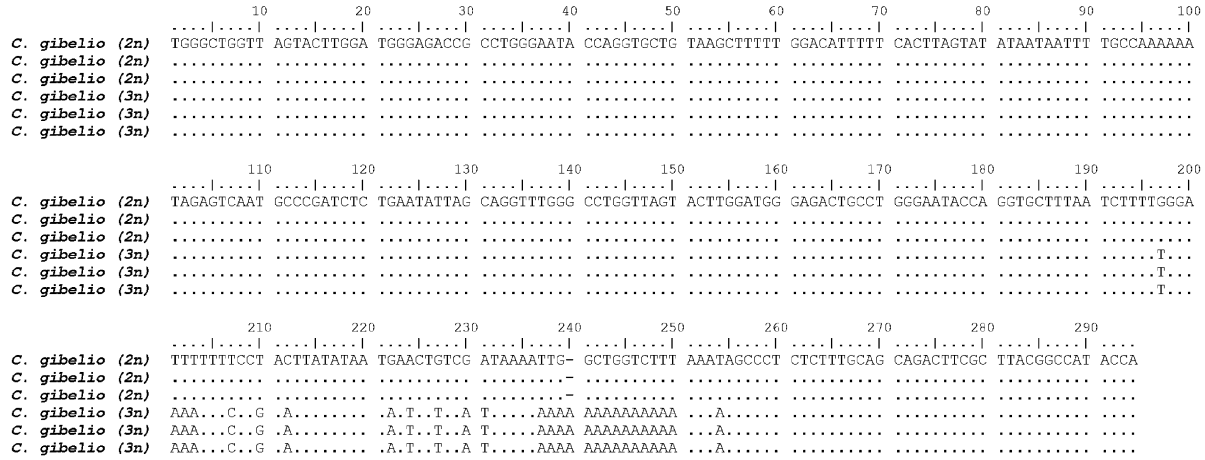

Figure 3. Nucleotide sequence alignment of 5S rRNA gene in class 470 bp in diploid and triploid individuals of *C. gibelio*. Dots indicate sequence identity and hyphens represent deletions.

characteristics.

The NTS region of the 5S rDNA seems to be subject to rapid evolution, which makes it important for tracing recent evolutionary events (Rebordinos, Cross, & Merlo, 2013). This part of the rDNA exhibited nucleotide variation, including insertion and deletions, observed between both diploids and triploids of the Prussian carp (Zhu, Ma, & Gui, 2006; present study). Such type of nucleotide variation has been observed in hybrid offspring of grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844) and blunt snout bream, *Megalobrama amblycephala* (Yih, 1955) (He *et al*., 2013) as well as in diploid, triploid and tetraploid hybrids of the red crucian carp, *Carassius auratus* red var. and topmouth culter, *Erythroculter ilishaeformis* (Bleeker, 1871) (He *et al*., 2012). Some published data has indicated the influence of polyploidy on the organization and

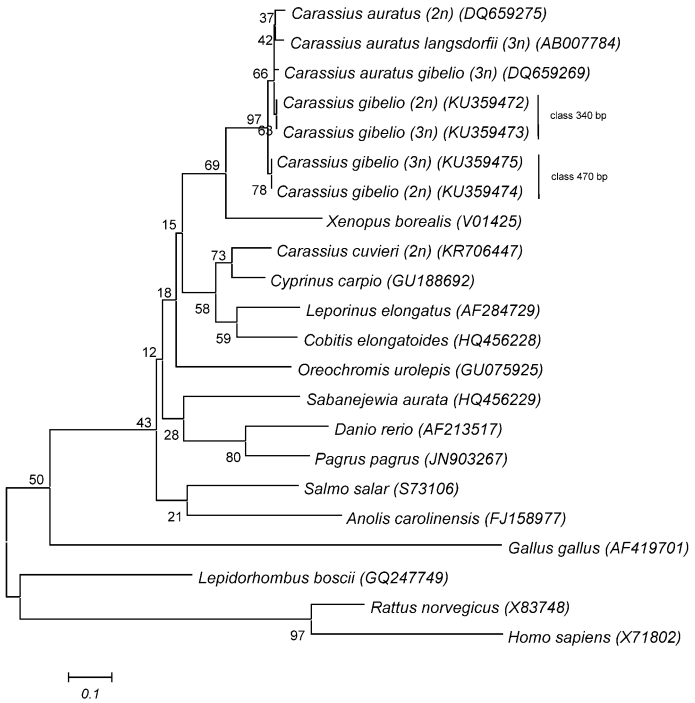

Figure 4. Phylogenetic tree of 5S rDNA sequences (classes 340 bp and 470 bp) from *C. gibelio* and several reference sequences from the GenBank (NCBI): *C. auratus gibelio* (former species name of *C. gibelio*) (DQ659269), *C. auratus* (DQ659275), *C. auratus langsdorfii* (AB007784), *C. cuvieri* (KR706447), *Cyprinus carpio* (GU188692), *Danio rerio* (AF213517), *Salmo salar* (S73106), *Pagrus pagrus* (JN903267), *Leporinus elongatus* (AF284729), *Cobitis elongatoides* (HQ456228), *Sabanejewia aurata* (HQ456229), *Oreochromis urolepis* (GU075925), *Lepidorhombus boscii* (GQ247749), *Xenopus borealis* (V01425), *Anolis carolinensis* (FJ158977), *Gallus gallus* (AF419701), *Rattus norvegicus* (X83748), *Homo sapiens* (X71802). The bootstrap analysis was replicated 1000 times. The number shown in each branch indicates the bootstrap values. The scale bar indicates the nucleotide diversity between sequences. 2n – diploid; 3n – triploid.

evolution of the 5S rDNA in teleost fish, including nucleotide variations within the NTS region (He *et al*., 2013; Qin, Wang, Wang, Liu, & Liu, 2015). In turn, most nucleotide mutations in NTS are considered to be neutral or almost neutral (Messias *et al*., 2003) and they can be easily fixed in the genome. Thus, nucleotide data involving this rDNA region may reflect a certain ploidy level of an individual.

In conclusion, the present study provides new information concerning the structure and nucleotide variation of the 5S rDNA in *C. gibelio* individuals with different ploidy levels. This data widens the molecular characterization of Prussian carp and also helps to better understand some molecular changes within ribosomal DNA in fish, possibly reflecting their polyploid origin. As our findings support the ploidy identification of *C. gibelio*, it may be a useful tool in any studies concerning the population structure of Prussian carp, for aquaculture purposes or biodiversity conservation of ichthyofauna.

Acknowledgements

The study was financed by a grant nos. 12.610.006-300 and 12.620.020-300 of the University of Warmia and Mazury in Olsztyn.

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