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Comparison of molecular and morphometric analysis in species discrimination of larvae among five cyprinids from the subfamily Leuciscinae: A tool for sustainable conservation of riverine ichthyofauna

Lech Kirtiklis[1], Katarzyna Palińska-Żarska[2], Sławomir Krejszeff[2], Krzysztof Kupren[3], Daniel Żarski[2], Dorota Fopp-Bayat[4] & Aleksandra Szabelska[1]

[1]*Department of Zoology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Poland; e-mail: leo@uwm.edu.pl*
[2]*Department of Lake and River Fisheries, Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn, Poland*
[3]*Department of Tourism, Recreation and Ecology, University of Warmia and Mazury in Olsztyn, Poland*
[4]*Department of Ichthyology, Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn, Poland*

Abstract: Fish species from the subfamily Leuciscinae are one of the most abundant and ecologically valuable among riverine cyprinids of Central Europe. In order to support their recruitment or properly manage the restocking operations, there is a need to recognize the recruitment success which relies on the necessity of proper species discrimination among the larvae of those species caught on the spawning and/or nursery grounds. In the present study a comparative analysis of the morphological and molecular (PCR-RFLP technique with two restriction endonucleases *HinfI* and *TaqI* based on mitochondrial DNA fragment encoding cytochrome *b*) techniques for identification of the species status among larvae of five cyprinid species, the ide (*Leuciscus idus*), common dace (*Leuciscus leuciscus*), asp (*Leuciscus aspius*), roach (*Rutilus rutilus*) and the European chub *(Squalius cephalus)* was performed. It was shown that the application of morphometric features solely could create many ambiguities in species discrimination. The use of applied molecular techniques was found to be a great support for the larvae identification, regardless the developmental stage of the fish. Moreover, this molecular method may also be used for the identification of the species even if the preserved fish (e.g., in ethanol), having usually deformed and/or shrunk body caused by preservation, are about to be investigated.

Key words: aquaculture; biodiversity; conservation evaluation; fish; *Leuciscus*; river; *Squalius*

Introduction

Human activities have caused significant changes in the natural environment, where one of the most affected ecosystems are the natural water bodies. This applies especially to the rivers where, apart from the pollution (Kruk & Penczak 2013), significant changes through the regulation and construction of dams have caused destruction of the number of valuable and specific habitats. This concerns, among others, a significant reduction of the spawning and nursery grounds for many fish species (Penczak & Kruk 2000; Paragamian & Hansen 2011; Humphries et al. 2013). Therefore, in recent years the necessity of active support towards recruitment of many rheophilic finfishes has occurred (Kamler & Wolnicki 2006; Kucharczyk et al. 2008; Targońska et al. 2008). This applies also to the riverine cyprinids, which are one of the most abundant and ecologically valuable part of riverine finfish fauna of Central Europe (Mann 1996).

The active support of recruitment may be conducted by rehabilitation of the habitats as well as by restocking operations (Braithwaite & Salvanes 2010; HELCOM 2011). However, in both cases the planned operations must be supported by very detailed and reliable evaluation of the ichthyofauna structure and the recruitment success. This is needed to determine, e.g., the suitability of the particular habitats for the nursery grounds as well as to plan the restocking priorities in particular parts of the rivers (Skinner et al. 2008). One of the most important parts of such activities is the evaluation of the recruitment success during the spawning season. To its end, the presence of the eggs on the spawning grounds or larvae at the nursery grounds were usually studied. The latter are of much more higher importance since the recruitment success is mostly determined during the larval stage when the larvae of many fish species are starting active predation on the zooplankton (Brander 2001; Burrow et al. 2011; Gleason & Burton 2012; Ko et al. 2013). Such sites are usually the ox-bow lakes, small tributaries or other specific parts of

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the riverine environment with relatively low water current and high abundance of the potential preys (Mann 1996; Lima & Araujo-Lima 2004; Penczak et al. 2004).

The ichthyofauna of riverine ecosystems is created by a number of cyprinid species, where one of the most numerous are the ide (*Leuciscus idus* L., 1758), common dace (*Leuciscus leuciscus* L., 1758), asp (*Leuciscus aspius*; formerly *Aspius aspius* L., 1758), roach (*Rutilus rutilus* L., 1758), and the European chub (*Squalius cephalus*; formerly *Leuciscus cephalus* L., 1758) (Kottelat & Freyhof 2007; Perea et al. 2010). Apart of the chub (spawning usually from May until July; see Mann 1996), all mentioned species are early spring spawners (spawning usually between March and May; see Mann 1996), very often spawning in a similar period during the year. In fact, the larvae of those species very often occur at the same time at the same location (Copp 1992). However, the larvae of all of those cyprinids are morphologically very similar, what may cause difficulties in a precise identification only on the basis of morphology or morphometry (Kupren et al. 2008, 2011; Kirtiklis et al. 2012). Especially, because the comparative data on the morphological features of larvae between the species are very scarce, this can significantly hinder the real structure of the fish species when being evaluated during the monitoring operations of the recruitment success at a particular locations. It must be emphasized that to date there is no data on comparative morphometric analysis aiming at fast, easy and impartial discrimination of particular cyprinid species.

Application of molecular examination into species identification is a kind of very efficient support increasing the success rate of a particular analysis. The use of nuclear (Kirtiklis et al. 2013) or mitochondrial (Chakraborty et al. 2006) parts of the genome is a common approach in fish species identification, including larvae (Paine et al. 2008).

The aim of the current study was to develop the molecular method of identification of five cyprinid species (roach, chub, ide, dace and asp) and to compare the effectiveness of the two methods (morphometric and molecular) in species discrimination of larvae of those species being at the same developmental stage.

Material and methods

Obtaining of larvae

The larvae were obtained by the standard controlled reproduction procedure (with the use of hormonal treatment, in both sexes, with preparation containing mammalian analogue of gonadoliberine and metoclopramide – a dopamine antagonist) of wild spawners as described by Krejszeff et al. (2009) for ide, Żarski et al. (2009) for dace, Targońska et al. (2010) for asp and Krejszeff et al. (2010) for the European chub. The larvae of roach were obtained by controlled fertilization of wild fish directly on the spawning ground and being ready to spawn at the moment of catching. The gametes of roach were collected manually and after fertilization, embryos were allowed to adhere to the substrate. In every case hand-stripped eggs from five females were mixed with the sperm (stripped into dry syringe prior to fertilization) from five males. The average body weight of the females of asp, roach, ide, dace and chub was 2300 ± 500 g, 260 ± 35 g, 650 ± 90 g, 153 ± 29 g and 310 ± 80 g, respectively. Eggs of ide, dace, asp and chub were incubated in Weiss jars (after removing the adhesive layer according to the method of Woynarovich & Woynarovich 1980) and eggs of the roach were incubated adhered to the substrate (branch of juniper). Temperature of incubation varied between 14 and 15 °C.

Rearing of larvae

At the moment of hatching, 0 day post hatch (DPH), larvae were transferred to a semi-closed recirculation aquaculture system (RAS) following slow acclimation to the temperature of 20 °C (at a rate below 1 °C per hour) and placed (each species separately) in 20 L glass tanks at the density of around 50 ind. L^{-1}. The water flow rate was 0.5 L per min. The rearing system was equipped with biological filtration, aeration and automatic temperature regulation (± 0.1 °C). The temperature in RAS was set at 20 °C and the photoperiod for the entire period of research was 24 h (24 L : 0 D). Ammonia content (measured every 3 days with the use of HI 83200 photometer, Hanna Instruments, Italy) for the entire period of rearing was below 0.01 mg L^{-1}. Oxygen content was measured twice a day (using oxygen meter Handy Polaris 2.0 OxyGuard, Denmark), and no decrease below 80% saturation was observed for the entire period of the experiment. Fish were not fed before sampling as they did not start exogenous feeding yet. Form among all the species studied below 2% of deformation rate was recorded only in the case of roach. Among the remaining species the deformation rate was below 10% in asp, dace and chub. The highest deformation rate (25%) was recorded in the case of ide. Until the sampling, in all the species mortality rate did not exceed 5%.

Morphometric analysis

Starting with the moment of hatching (0 DPH), every day 20 specimens of each species were randomly collected to check if the swim bladder inflation started. In order to do that, larvae were anesthetized in 2-phenoxyethanol solution at a concentration of 0.4 ml L^{-1} (Sigma-Aldrich, Germany) and after that they were photographed under a stereoscopic microscope (Zeiss, Discovery V.20) at different magnification levels, with the software AxioVs40 V 4.8.2.0 (Carl Zeiss MicroImaging GmbH, Germany). This software was also used to carry out measurements (± 0.1 mm). Anesthetized larvae on which the measurements were performed were not returned to the rearing tanks.

When swim bladder inflation was observed (stage: LS1 according to Kupren et al. 2016), 30 randomly chosen larvae with inflated swim bladder, from each species were collected, anesthetized (as described above) and the following basic parameters were measured: total length (TL), standard length (SL), head length (HL), trunk length (TRL), tail length (T1L), tail height (T1H), head height (HH) and eye diameter (ED). All measurements were taken along lines parallel or perpendicular to the horizontal axis of the body (Gisbert 1999; Kupren et al. 2015b) (Fig. 1). According to measured parameters the following relations were calculated (relative parameters): SL/TL, HL/TL, TRL/TL, T1L/TL, T1H/TL, HH/TL, ED/TL. The inflation of the swim bladder was chosen at the moment for sampling the larvae since this is the earliest possible stage allowing easily and undoubtedly identification, without specific measurements and/or skills needed. Dead and abnormal specimens (presence of malformations) were excluded from the analysis.

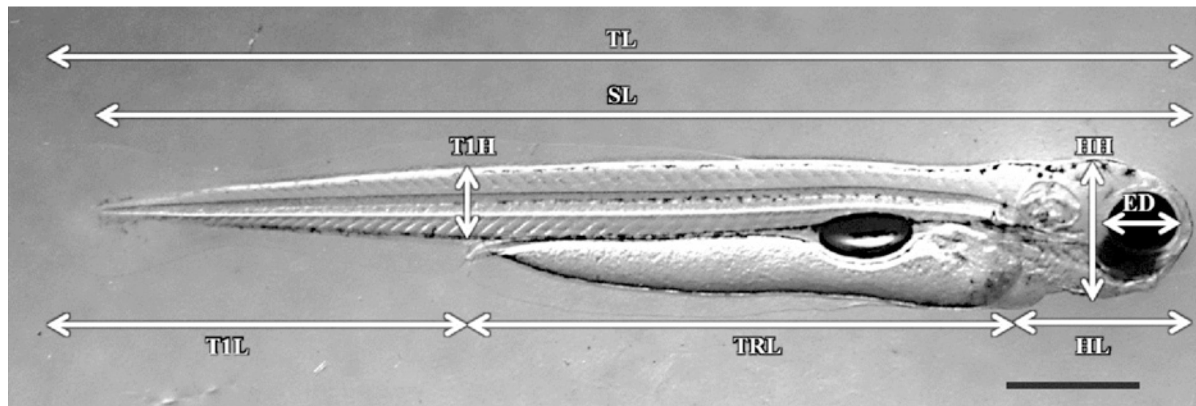

Fig. 1. Larva of asp, *Leuciscus aspius*, with swim bladder filled. Abbreviations: TL – total length, SL – standard length, T1L – tail length, TRL – truncus length, HL – head length, HH – head height, T1H – tail height, ED – eye diameter. Scale 1 mm.

Data analysis
The statistical differences were analysed with one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test and Kruskal-Wallis test at the significance level below 5% ($P < 0.05$). The statistical analysis was performed with STATISTICA (data analysis software system) version 10.

Molecular analysis
Sampling
Fin clips (caudal part) from at least 20 larvae of each species (separately collected) were used as a material for the investigation. Tissue samples were preserved in a 96% ethanol and stored at –20 °C.

DNA extraction
Total DNA was extracted from the fin clips using the method described by Walsh et al. (1991) with some modifications (Kirtiklis et al. 2011). DNA concentration and purity were checked both on a 1.5% agarose electrophoresis gel (Sigma-Aldrich, USA) containing 1 μg ml^{-1} of ethidium bromide (Sigma-Aldrich, USA) and by spectrophotometric method using BioPhotometer 6131 (Eppendorf, Germany). Obtained DNA was stored at –20 °C for further analysis.

PCR amplification and sequencing
Amplification of cytochrome *b* gene coding fragment of 664 bp length was conducted using PCR primers H15891 (5'-GTTTGATCCCGTTTCGTGTA-3') and L15267 (5'-AATGACTTGAAGAACCACCGT-3') described by Briolay et al. (1998). PCR reaction was performed in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, USA) with the following conditions: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and the final extension at 72 °C for 3 min. The reaction mixture (50 μl) contained as follows: 25 μl of GoTaq Colorless Master Mix (Promega, USA), 100 ng of DNA template, 10 pmol of each primer and dH_2O up to 50 μl. PCR product size was determined on a 1.5% agarose gel (Sigma-Aldrich, USA) containing 1 μg ml^{-1} of ethidium bromide (Sigma-Aldrich, USA), using DNA Marker 100-1000 as a molecular weight marker (A&A Biotechnology, Poland).

PCR products were purified using the GeneElute PCR Clean-Up Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction, and then two samples of each species were sequenced by an ABI 3730 DNA analyser (Applied Biosystems, USA) using BDT V3.1 RR-100, capillary length 360 mm, POP-7 Polymer (Live Technologies, USA). Obtained sequences were analysed using BioEdit software ver. 7.2.5 (Hall 1999), and then confirmed by the Basic Local Alignment Search Tool (BLAST) (Zhang et al. 2000). The sequences were deposited in the GenBank database (NCBI) under following numbers: KR025545, KR025546 (*Leuciscus idus*), KR025547, KR025548 (*Leuciscus leuciscus*), KR025549, KR025550 (*Leuciscus aspius*), KR025541, KR025542 (*Rutilus rutilus*) and KR025543, KR025544 (*Squalius cephalus*).

RFLP
Two restriction endonucleases *HinfI* and *TaqI* (Fermentas, Lithuania) were chosen based on test digestions and then used for RFLP analysis of amplified DNA fragment encoding cytochrome *b*. Enzymatic digestion was carried out according to the manufacturer's protocol. Briefly, the reaction was conducted in 31 μl mixture containing 400 ng of PCR product, 2 μl of 10X buffer (specific for proper endonuclease), 10 U of restriction endonuclease, 17 μl of dH_2O at 37 °C and 65 °C for *HinfI* and *TaqI*, respectively. A total of 10 μl of reactant mixture was electrophoretically separated in a 1.5% agarose gel (Sigma-Aldrich, USA), then visualized and analysed under UV light using the UVIsave Gel Documentation System (UVItec, UK).

Results

Morphometric analysis
Depending on the species larvae hatched (50% of hatched individuals) in the range 4–7 days after fertilization. The moment when the swim bladder was formed was more synchronous and took place (50% specimens at this stage of development) between 4 and 5 day after hatching.

The results of morphometric analysis showed, that it was possible to distinguish some of the studied species with the method undertaken. In general, the larvae of asp were possible to distinguish by higher ($P < 0.05$) values of most of the basic parameters, except HL, T1H and ED (Table 1). The lowest values ($P < 0.05$) of TL, SL, TRL and T1L were recorded for roach, allowing discrimination of this species according to those parameters. On the base of the used morphometric parameters it was impossible to distinguish ide and dace ($P > 0.05$) and in many cases also chub (Table 1). The only

Table 1. Results (mean ± SD) of morphological analysis of larvae of five cyprinid species at the moment of swim bladder inflation.

Parameter	*Leuciscus idus*	*Leuciscus leuciscus*	*Leuciscus aspius*	*Rutilus rutilus*	*Squalius cephalus*
	Basic measurements (mm)				
TL	8.50 ± 0.40^b	8.44 ± 0.39^b	9.55 ± 0.16^a	7.45 ± 0.23^d	8.06 ± 0.21^c
SL	8.09 ± 0.39^b	8.04 ± 0.36^b	9.15 ± 0.17^a	7.11 ± 0.21^d	7.67 ± 0.22^c
HL	1.65 ± 0.14^a	1.56 ± 0.14^a	1.37 ± 0.09^a	1.27 ± 0.09^a	1.59 ± 0.09^a
TRL	3.76 ± 0.14^b	3.66 ± 0.15^b	4.68 ± 0.14^a	3.38 ± 0.19^d	3.55 ± 0.11^c
T1L	3.07 ± 0.16^a	3.21 ± 0.17^a	3.11 ± 0.11^a	2.46 ± 0.10^b	2.92 ± 0.15^a
T1H	0.56 ± 0.04^a	0.53 ± 0.05^a	0.61 ± 0.03^a	0.50 ± 0.02^a	0.54 ± 0.03^a
HH	0.97 ± 0.06^b	0.93 ± 0.08^b	1.16 ± 0.10^a	0.94 ± 0.07^b	0.87 ± 0.05^c
ED	0.58 ± 0.05^a	0.55 ± 0.04^a	0.57 ± 0.03^a	0.50 ± 0.03^a	0.51 ± 0.05^a
	Relative parameters (% TL)				
SL/TL	95.12 ± 0.95^a	95.33 ± 0.90^a	95.78 ± 0.83^a	95.43 ± 1.16^a	95.23 ± 0.63^a
HL/TL	19.43 ± 0.95^{ab}	18.49 ± 1.02^b	14.30 ± 1.02^a	17.01 ± 0.84^b	19.80 ± 1.18^b
TRL/TL	44.27 ± 0.84^b	43.34 ± 1.30^c	48.96 ± 1.06^a	45.38 ± 1.94^{bc}	44.02 ± 1.40^{bc}
T1L/TL	36.16 ± 0.68^a	38.05 ± 0.88^b	32.51 ± 0.96^a	33.05 ± 1.30^b	36.24 ± 1.26^b
T1H/TL	6.63 ± 0.30^b	6.32 ± 0.47^a	6.41 ± 0.30^a	6.74 ± 0.32^{ab}	6.71 ± 0.30^{ab}
HH/TL	11.38 ± 0.35^a	11.04 ± 0.57^b	12.16 ± 0.97^a	12.63 ± 0.95^{ab}	10.81 ± 0.50^b
ED/TL	6.77 ± 0.44^b	6.48 ± 0.33^{ab}	6.00 ± 0.26^a	6.75 ± 0.34^b	6.26 ± 0.47^a

Abbreviations: TL – total length, SL – standard length, T1L – tail length, TRL – truncus length, HL – head length, HH – head height, T1H – tail height, ED – eye diameter. Data in rows marked with different letter superscript were statistically different ($P < 0.05$).

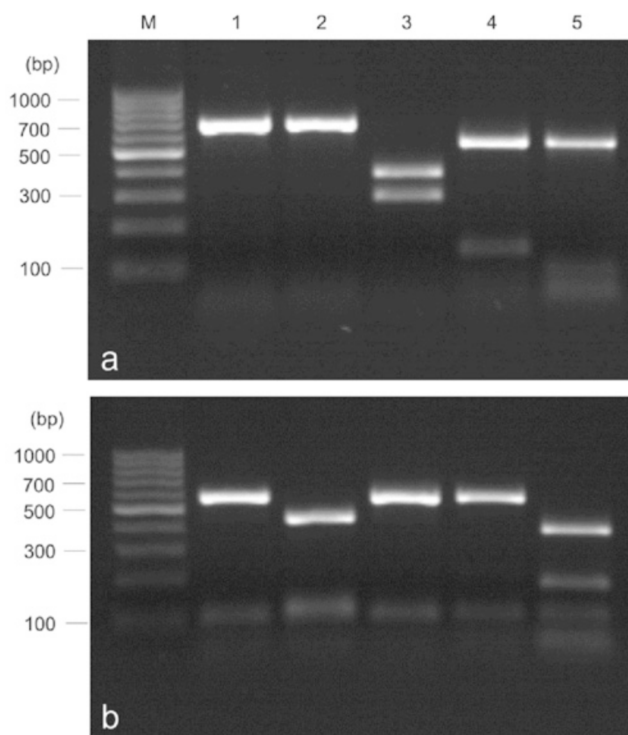

Fig. 2. RFLP profiles of cytochrome *b* based on *HinfI* (a) and *TagI* (b) in ide (1), common dace (2), asp (3), roach (4) and the European chub (5); M – molecular weight marker.

parameter allowing distinguishing chub was HD, which was the lowest ($P < 0.05$) in this species.

As considering the relative parameters there were no clear differences between dace and chub as well as between roach and dace. The only clear differences were found for TRL/TL, where asp was clearly distinguishable from the other species ($P < 0.05$). Also, distinguishing the remaining species required comparison all of the parameters together with other species, in order to be sure with species identification, since only one variable usually differentiated the particular species (see Table 1).

Molecular analysis

The applied DNA extraction method allowed obtaining an optimal quantity of template from each larva of *L. idus, L. leuciscus, L. aspius, R. rutilus and S. cephalus.* Cytochrome *b* DNA fragment of 664bp length was a result of PCR amplification from each species. RFLP analysis using *HinfI* and *TaqI* endonucleases allowed determine species-specific restriction patterns for each fish species (Fig. 2). The first step of this analysis based on *HinfI* divided all investigated fish species into four groups and showed no digestion effect in ide and common dace. Two different pairs of bands were visible in RFLP patterns of asp and roach, and three bands in the European chub. Two different restriction patterns in ide and common dace, equal two band-patterns in asp and roach, and three bands in the European chub were detected in the second step of analysis based on the use of *TaqI* endonuclease (see Table 2 for details). Finally, two-steps of RFLP analysis allowed obtaining species-specific restriction patterns of each investigated fish species being on the larval stage.

Discussion

In the present study a comparative analysis of the morphometry of the larvae of five freshwater riverine cyprinids, inhabiting similar niches during the larval phase, was performed for the first time.

The taxonomy of the fish was based on morphological features of adult specimens (Strauss & Bond 1990). It stem from the fact that larvae morphology is constantly changing since they undergo significant

Table 2. Restriction patterns for cytochrome *b* gene fragment of the larvae of five fish species from the subfamily Leucisciane; values in base pair (bp).

Endonuclease	*Leuciscus idus*	*Leuciscus leuciscus*	*Leuciscus aspius*	*Rutilus rutilus*	*Squalius cephalus*
HinfI	664	664	247/417	135/529	55/80/529
TaqI	115/549	110/120/434	115/549	115/549	20/110/160/374

metamorphosis prior to the adult stage (Nikolioudakis et al. 2010; Kupren et al. 2014a, b). Additionally, many fish species, especially belonging to closely related taxa, are morphologically very similar at the early life stages what has led to misidentification of the species creating serious problems in species discrimination (e.g., Snyder et al. 2005; Kupren et al. 2008; Ko et al. 2013). The problem becomes more evident when larvae at different stages of development are compared what is often associated to the variety of relative growth patterns observed, even among the closely related species (Kováč 1994; Gisbert et al. 2014; Kupren et al. 2015a, b, 2016). Together with the commonly known environmental impact on the fish larvae growth and morphology (Gozlan et al. 1999; Koumoundouros et al. 1999) the taxonomic identification of the larvae of different species obtained from the possibly sampled batch may be very difficult and ambiguous.

Data presented in this study clearly suggest that it is difficult to undoubtedly distinguish a single species by the morphometric assessment. Actually, only one relative parameter (TRL/TL) could be found which allowed undoubtedly distinguish asp from the other species studied. However, among the remaining species, even though the eggs were incubated and larvae were grown until very precisely determined developmental stage, under strictly controlled conditions, a particular species could only be identified by the comparison with the other species (see Table 1).

Additionally, it has to be taken into the consideration that in practice the species identification is very rarely based on living fish (as it was done in the present study) and usually preserved (in e.g. ethanol) larvae are subjected to analysis (Santos et al. 2009; Gómez et al. 2014). As the preservation techniques almost always alter the body shape (usually by shrinkage of the tissues) (Moku et al. 2004; Santos et al. 2009; Gómez et al. 2014), the morphometric analysis may become even more difficult. Therefore, the species identification on the basis of only morphometric analysis could be really applicable if the detailed developmental table is created for each species and the living (or shortly after death) fish larvae are available. However, even then the developmental table should consider data based on several rearing trials (in several different environmental conditions mimic the natural environmental preference and tolerance) and throughout the entire larval period or at least when the characteristic feature for particular species would become evident (e.g., barbs, colouration, mouth shape etc.).

Taking into account all weaknesses of the morphological analysis mentioned above, it is indispensable to apply another tool for proper species identification among fish larvae. In recent years the molecular techniques have started to be involved for that purpose (Gleason & Burton 2012; Ko et al. 2013). At present, there are some techniques based on DNA analysis which are able to support fish species discrimination among different taxa.

Until now, only few species belonging to the subfamily Leuciscinae have been distinguished by methods linked to microsatellite DNA markers (Barinova et al. 2004) and nuclear PCR-RFLP markers (Kirtiklis et al. 2012, 2013). However, the mitochondrial part of genome is most promising source of new molecular markers for that purpose. A lot of different sequences of mitochondrial genes or their fragments are often used for phylogenetic study in many fish groups (e.g., Briolay et al. 1998; Yokoyama & Goto 2005; Perea et al. 2010; Abe et al. 2014). Moreover, barcoding, which is a useful method in species discrimination among many animal taxa, including fishes, is also based on analysis of mitochondrial DNA (mtDNA) sequence encoding cytochrome oxidase I (COI) (Gleason & Burton 2012; Geiger et al. 2014; Knebelsberger et al. 2015; Kress et al. 2015). Considering the high usefulness of mtDNA mentioned above, we proposed in present study a new approach as a successful support for cyprinid larvae discrimination using gene encoding cytochrome *b*. It must be emphasized that our method, oppositely to commonly used molecular techniques based on DNA sequence analysis, is much simpler, less time-consuming and cheaper than others. Moreover, it may also be used in case of fish specimens being on other developmental stages than larvae.

In conclusion, we would like to emphasize that applying of morphological species identification supported by the proposed molecular method brings very useful, cheap and efficient tools for species discrimination among larvae of the subfamily Leuciscinae. Moreover, this method may also be used for verification of the taxonomic status of numerous larvae specimens collected in the past, regardless the means of preservation (e.g., dried, frozen, immersed in ethanol). It means that collected larvae have not to be identified directly after sampling, what may be a useful solution for the people who prepared hundreds of specimens in a short period of time. On the other hand, we have to be aware that particularly long period of storing and/or some aggressive preservation techniques may cause any DNA damages and provide some limitation of the method presented in this study. Therefore, we recommend perform a wider testing of this method. Additionally, it is worth mentioning that our method seems to be an inter-

disciplinary approach, combining traditional biological analysis and molecular technique, which may have significant contribution to the environmental studies considering the rehabilitation of the freshwater ecosystems.

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References

Abe K.T., Mariguela T.C., Avelino G.S., Foresti F. & Oliveira C. 2014. Systematic and historical biogeography of the Bryconidae (Ostariophysi: Characiformes) suggesting a new rearrangement of its genera and an old origin of Mesoamerican ichthyofaunal. BMC Evol. Biol. **14:** 152. DOI: 10.1186/1471-2148-14-152.

Barinova A., Yadrenkina E., Nakajima M. & Taniguchi M. 2004. Identification and characterization of microsatellite DNA markers developed in ide *Leuciscus idus* and Siberian roach *Rutilus rutilus*. Mol. Ecol. Notes **4 (1):** 86–88. DOI: 10.1046/j.1471-8286.2003.00577.x

Braithwaite V.A. & Salvanes A.G.V. 2010. Aquaculture and restocking: implications for conservation and welfare. Anim. Welfare **19 (2):** 139–149.

Brander K. 2001. Modelling the timing of plankton production and its effect on recruitment of cod (*Gadus morhua*). ICES J. Mar. Sci. **58 (5):** 962–966. DOI: 10.1006/jmsc.2001.1086

Briolay J., Galtier N., Brito R. M. & Bouvet Y. 1998. Molecular phylogeny of Cyprinidae inferred from cytochrome *b*DNA sequences. Mol. Phylogenet. Evol. **9 (1):** 100–108. DOI: 10.1006/mpev.1997.0441

Burrow J.F., Horwood J.W. & Pitchford J.W. 2011. The importance of variable timing and abundance of prey for fish larval recruitment. J. Plankton Res. **33 (8):** 1153–1162. DOI: 10.1093/plankt/fbr015

Chakraborty A., Aranishi F. & Iwatsuki Y. 2006. Genetic differences among three species of the genus *Trichiurus* (Perciformes: Trichiuridae) based on mitochondrial DNA analysis. Ichthyol. Res. **53 (1):** 93–96. DOI: 10.1007/s10228-005-0313-3

Copp G.H. 1992. Comparative microhabitat use of cyprinid larvae and juveniles in a lotic floodplain channel. Environ. Biol. Fish. **33 (1):** 181–193. DOI: 10.1007/BF00002563

Geiger M.F., Herder F., Monaghan M.T. & Almada V., Barbieri R., Bariche M., Berrebi P., Bohlen J., Casal-Lopez M., Delmastro G.B., Denys G.P., Dettai A., Doadrio I., Kalogianni E., Kärst H., Kottelat M., Kovačić M., Laporte M., Lorenzoni M., Marčić Z., Özuluğ M., Perdices A., Perea S., Persat H., Porcelotti S., Puzzi C., Robalo J., Šanda R., Schneider M., Šlechtová V., Stoumboudi M., Walter S. & Freyhof J. 2014. Spatial heterogeneity in the Mediteranean Biodiversity Hotspot affects barcoding accuracy of its freshwater fishes. Mol. Ecol. Resour. **14 (6):** 1210–1221. DOI: 10.1111/1755-0998.12257

Gisbert E. 1999. Early development and allometric growth patterns in Siberian sturgeon and their ecological significance. J. Fish Biol. **54 (4):** 852–862. DOI: 10.1111/j.1095-8649.1999.tb02037.x

Gisbert E., Asgari R., Rafiee Gh., Agh N., Eagderi S., Eshaghzadeh H. & Alcaraz C. 2014. Early development and allometric growth patterns of beluga *Huso huso* (Linnaeus, 1758). J. Appl. Ichthyol. **30 (6):** 1264–1272. DOI: 10.1111/jai.12617

Gleason L.U. & Burton R.S. 2012. High-throughput molecular identification of fish eggs using multiplex suspension bead arrays. Mol. Ecol. Resour. **12 (1):** 57–66. DOI: 10.1111/j.1755-0998.2011.03059.x

Gómez M.I., Sánchez S. & Fuentes C.M. 2014. Shrinkage of *Prochilodus lineatus* (Valenciennes, 1847) larvae preserved in either ethyl-alcohol or formalin in relation to their developmental stage and feeding condition. J. Appl. Ichthyol. **30 (1):** 140–144. DOI: 10.1111/jai.12308

Gozlan R.E., Copp G.H. & Tourenq J.N. 1999. Comparison of growth plasticity in the laboratory and field, and implications for the onset of juvenile development in sofie, *Chondrostoma toxostoma*. Environ. Biol. Fish. **56:** 153–165. DOI: 10.1007/978-94-017-3678-7_11

Hall T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:** 95–98.

HELCOM 2011 Salmon and Sea Trout Populations and Rivers in the Baltic Sea – HELCOM assessment of salmon (*Salmo salar*) and sea trout (*Salmo trutta*) populations and habitats in rivers flowing to the Baltic Sea. B. S. E. P. 126A. Helsinki Commission, Baltic Marine Environment Protection Commission, 79 pp.

Humphries P., Richardson A., Wilson G. & Ellison T. 2013. River regulation and recruitment in a protracted-spawning riverine fish. Ecol. Appl. **23 (1):** 208–225. DOI: 10.1890/11-2255.1

Kamler E. & Wolnicki J. 2006. The biological background for the production of stocking material of 11 European rheophilic cyprinids. A review. Arch. Hydrobiol. (Suppl.) **158 (4):** 667–687. DOI: 10.1127/lr/16/2006/667

Kirtiklis L., Boroń A., Ptasznik P., Luskova V. & Lusk S. 2011. Molecular differentiation of three loach species (Pisces, Cobitidae) based on the nuclear 5S rDNA marker. Folia biol. – Krakow **59 (3-4):** 141–145. DOI: 10.3409/fb59_3-4.141-145

Kirtiklis L., Grzymkowska M. & Boroń A. 2013. Characterization of three species from the subfamily Leuciscinae (Pisces, Cyprinidae) using the nuclear ITS-1 rDNA spacer. Folia Biol. – Krakow **61 (1-2):** 53–57. DOI: 10.3409/fb61_1-2.53

Kirtiklis L., Żarski D., Krejszeff S. & Kupren K. 2012. Molecular technique support for the species identification of larvae between the ide *Leuciscus idus* and the common dace *Leuciscus leuciscus* (Teleostei, Cypriniformes), pp. 33–36. In: Kucharczyk D. (eds), Domestication in Finfish Aquaculture, ElSet, 23.–25.10.2012 Olsztyn and Mrągowo, 124 pp. ISBN: 978-8362863-31-0

Knebelsberger T., Dunz A.R., Neumann D. & Geiger M.F. 2015. Molecular diversity of Germany's freshwater fishes and lampreys assessed by DNA barcoding. Mol. Ecol. Resour. **15:** 562–572. DOI: 10.1111/1755-0998.12322

Ko H-L., Wang Y-T., Chiu T-S., Lee M-A., Leu M-Y., Chang K-Z., Chen W-Y. & Shao K-T. 2013. Evaluating the accuracy of morphological identification of larval fishes by applying DNA barcoding. PLoS One **8 (1):** e53451. DOI: 10.1371/journal.pone.0053451

Kottelat M. & Freyhof J. 2007. Handbook of European Freshwater Fishes. Kottelat, Cornol and Freyhof, Berlin, 646 pp. ISBN: 978-2-8399-0298-4

Koumoundouros G., Divanach P. & Kentouri M. 1999. Ontogeny and allometric plasticity of *Dentex dentex* (Osteichthyes: sparidae) in rearing conditions. Mar. Biol. **135 (3):** 561–572. DOI: 10.1007/s002270050657

Kováč V. 1994. Early ontogeny of three *Gymnocephalus* species (Pisces: Percidae): reflections on the evolution of the genus. Environ. Biol. Fish. **40 (3):** 241–253. DOI: 10.1007/BF00002511

Krejszeff S., Targońska K., Żarski D. & Kucharczyk D. 2009. Domestication affects spawning of the ide (*Leuciscus idus*)-preliminary study. Aquaculture **295 (1-2):** 145–147. DOI: 10.1016/j.aquaculture.2009.06.032

Krejszeff S., Targońska K., Zarski D. & Kucharczyk D. 2010. Artificial reproduction of two different spawn-forms of the chub. Reprod. Biol. **10 (1):** 67–74. DOI: 10.1016/S1642-431X(12)60038-2

Kress W.J., Garcia-Robledo C., Uriarte M. & Erickson D.L. 2015. DNA barcodes for ecology, evolution, and conservation. Trends Ecol. Evol. **30 (1):** 25–35. DOI: 10.1016/j.tree.2014.10.008

Kruk A. & Penczak T. 2013. Natural regeneration of fish assemblages in the Pilica River after a reduction of point-source pollution. River Res.Appl. **29:** 502–511. DOI: 10.1002/rra.2562

Kucharczyk D., Targońska K., Żarski D., Kujawa R. & Mamcarz A. 2008. A review of the reproduction biotechnology for fish from the genus *Leuciscus*. Arch. Pol. Fish. **16 (4):** 319–340. DOI: 10.2478/s10086-008-0021-5,

Kupren K., Mamcarz A. & Kucharczyk D. 2011. Effect of variable and constant thermal conditions on embryonic and early larval development of fish from the genus *Leuciscus* (Cyprinidae, Teleostei). Czech J. Anim. Sci. **56 (2):** 70–80.

Kupren K., Mamcarz A., Kucharczyk D. & Prusińska M. 2008. Changes in morphometric parameters in selected early stages of three fish species from the genus *Leuciscus* (Teleostei, Cyprinidae). Arch. Pol. Fish. **16 (4):** 421–436. DOI: 10.2478/s10086-008-0029-x

Kupren K., Nowosad J., Żarski D., Targońska K., Hakuć-Błażowska A. & Kucharczyk D. 2015a. Early development and allometric growth in laboratory-reared European Chub *Leuciscus cephalus* (Linnaeus, 1758). Turk. J. Fish. Aquat. Sci. **15:** 385–392. DOI: 10.4194/1303-2712-v15_2_24

Kupren K., Prusińska M., Żarski D., Krejszeff S. & Kucharczyk D. 2014a. Early development and allometric growth in *Nannacara anomala* Regan, 1905 (Perciformes: Cichlidae) under laboratory conditions. Neotrop. Ichthyol. **12 (3):** 659–665. DOI: 10.1590/1982-0224-20130104.

Kupren K., Rams I., Żarski D. & Kucharczyk D. 2016. Early development and allometric growth patterns of rheophilic cyprinid common dace *Leuciscus leuciscus* (Cyprinidae: Leuciscinae). Ichthyol. Res. **63 (3):** 382–390. DOI: 10.1007/s10228-016-0508-9

Kupren K., Trąbska I., Żarski D., Krejszeff S., Palińska-Żarska K. & Kucharczyk D. 2014b. Early development and allometric growth patterns in burbot *Lota lota* L. Aquacult. Int. **22 (1):** 29–39. DOI: 10.1007/s10499-013-9680-3

Kupren K., Żarski D. & Kucharczyk D. 2015b. Early development and allometric growth patterns in ide *Leuciscus idus* (Linnaeus 1758). J. Appl. Ichthyol. **31 (3):** 509–517. DOI: 10.1111/jai.12747

Lima A.C. & Araujo-Lima C.A.R.M. 2004. The distributions of larval and juvenile fishes in Amazonian rivers of different nutrient status. Freshwater Biol. **49 (6):** 787–800. DOI: 10.1111/j.1365-2427.2004.01228.x

Mann R.H.K. 1996. Environmental requirements of European non-salmonid fish in rivers. Hydrobiologia **323 (3):** 223–235. DOI: 10.1007/BF00007848

Moku M., Mori K. & Watanabe Y. 2004. Shrinkage in the body length of myctophid fish (*Diaphus* slender-type spp.) larvae with various preservatives. Copeia **2004 (3):** 647–651. DOI: 10.1643/CI-03-113R

Nikolioudakis N., Koumoundouros G., Kiparissis S. & Somarakis S. 2010. Defining length-at-metamorphosis in fishes: a multi-character approach. Mar. Biol. **157 (5):** 991–1001. DOI: 10.1007/s00227-009-1379-7

Paine M.A., McDowell J.R. & Graves J.E. 2008. Specific identification using COI sequence analysis of scombrid larvae collected off the Kona coast of Hawaii Island. Ichthyol. Res. **55 (1):** 7–16. DOI: 10.1007/s10228-007-0003-4

Paragamian V.L. & Hansen M.J. 2011. Stocking for rehabilitation of burbot in the Kootenai River, Idaho, USA and British Columbia, Canada. J. Appl. Ichthyol. **27 (s1):** 22–26. DOI: 10.1111/j.1439-0426.2011.01839.x

Penczak T., Galicka W., Głowacki Ł., Koszaliński H., Kruk A., Zięba G., Kostrzewa J. & Marszał, L. 2004. Fish assemblage changes relative to environmental factors and time in the Warta River, Poland, and its oxbow lakes. J. Fish Biol. **64 (2):** 483–501. DOI: 10.1111/j.0022-1112.2004.00316.x

Penczak T. & Kruk A. 2000. Threatened obligatory riverine fishes in human-modified Polish rivers. Ecol. Freshwater Fish**9 (1-2):** 109–117. DOI: 10.1034/j.1600-0633.2000.90113.x

Perea S., Bohme M., Zupancic P, Freyhof J., Sanda R., Ozulug M., Abdoli A. & Doadrio I. 2010. Phylogenetic relationships and biogeographical patterns in circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. BMC Evol. Biol. **10:** 265. DOI: 10.1186/1471-2148-10-265

Santos J.N.S., Araújo F.G. & Silva D.S. 2009. Length correction for early-juvenile Brazilian herring *Sardinella janeiro* (Eigenmann, 1894) after preservation in formalin, ethanol and freezing. Neotrop. Ichthyol. **7 (1):** 87–92. DOI: 10.1590/S1679-62252009000100011

Skinner K., Shields D. & Harrison S. 2008. Measures of Success: Uncertainty and Defining the Outcomes of River Restoration Schemes, Chapter 10, pp. 187–208. DOI: 10.1002/978047086 7082.ch10. In: Darby S. & Sear D. (eds), River Restoration: Managing the Uncertainty in Restoring Physical Habitat, John Wiley & Sons, Ltd., Chichester, UK, 328 pp. ISBN: 978-0-470-86706-8

Snyder D.E., Bestgen K.R. & Seal S.C. 2005. Native cypriniform fish larvae of the Gila River Basin – morphological descriptions, comparisons, and computer-interactive keys. Final report of Colorado State University Larval Fish Laboratory to U.S. Department of the Interior Bureau of Reclamation in Phoenix, Arizona, 180 pp. http://www.usbr.gov/lc/phoenix/biology/azfish/pdf/Snydere2005GilaGuFnlRptr.pdf (accessed 06.04.2016).

Strauss R.E. & Bond C.E. 1990. Taxonomic methods: morphology, Chapter 4, pp. 109–140. In: P. Moyle P. & Schreck C. (eds), Methods for Fish Biology, American Fisheries Society, Bethesda, 684 pp. ISBN: 091323558X, 9780913235584

Targońska K., Kucharczyk D., Kujawa R., Mamcarz A. & Żarski D. 2010. Controlled reproduction of asp, *Aspius aspius* (L.) using luteinizing hormone releasing hormone (LHRH) analogues with dopamine inhibitors. Aquaculture **306 (1-4):** 407–410. DOI: 10.1016/j.aquaculture.2010.05.027

Targońska K., Żarski D. & Kucharczyk D. 2008. A review of the artificial reproduction of asp, *Aspius aspius* (L.) and nase, *Chondrostoma nasus* (L.). Arch. Pol. Fish. **16:** 341–354. DOI: 10.2478/s10086-008-0022-4

Walsh P.S., Metzger D.A. & Higuchi R. 1991. Chelex 100 as a medium for sample extraction of DNA for PCR-based typing from forensic material. BioTechniques **10 (4):** 506–513. DOI: 10.2144/000113897

Woynarovich E. & Woynarovich A. 1980 Modified technology for elimination of stickiness of common carp *Cyprinus carpio* eggs. Aquacult. Hung. **2:** 19–21.

Yokoyama R. & Goto A. 2005. Evolutionary history of freshwater sculpins, genus *Cottus* (Teleostei; Cottidae) and related taxa, as inferred from mitochondrial DNA phylogeny. Mol. Phyl. Evol. **36 (3):** 654–668. DOI: 10.1016/j.ympev.2005.06.004

Zhang Z., Schwartz S., Wagner L. & Miller W. 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. **7 (1-2):** 203–214. DOI: 10.1089/10665270050081478

Żarski D., Kucharczyk D., Targońska K., Jamróz M., Krejszeff S. & Mamcarz A. 2009. Application of Ovopel, Ovaprim and their combination in artificial reproduction of two rheophilic cyprinid fishes. Pol. J. Natur. Sci. **24 (4):** 235–244. DOI: 10.2478/v10020-009-0022-x

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