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ORIGINAL RESEARCH ARTICLE

First occurrence of thinlip grey mullet, *Liza ramada* (Risso, 1827) in the Odra River estuary (NW Poland): genetic identification

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Summary The presence of exotic fish species in the Baltic Sea and its tributaries poses a serious threat for native ichthyofauna, mainly due to the spread of new pathogens. As the accurate identification of species is essential for an effective assessment of changes related to the appearance of non-native species in an aquatic environment, in this paper we tested the usefulness of biometrics and molecular markers in identifying a specimen from the Mugilidae family found in the Odra estuary. The results demonstrated that unambiguous identification of the specimen using biometric features was impossible due to high morphological similarities shared by grey mullets. Unambiguous identification was possible only due to molecular markers, e.g. rhodopsin gene, which helped to identify the collected fish specimen as *Liza ramada* (Risso, 1827), the first specimen of this species found in the Odra River estuary. The presence of an *L. ramada* specimen in the Odra River – which could signal the expansion of non-native species into wider ranges – may be linked to climate change or human activity.

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1. Introduction

According to diverse estimates, the number of fish species in the seas surrounding Europe fluctuates between 40,000 and 48,000, constituting 15% of the estimated 230,000 marine species (Costello and Wilson, 2011). Based on the variety of available data (published papers, reports, grey literature, unpublished data), the Baltic Sea including Kattegat is host to at least 6065 species, including about 200 fish species (Ojaveer et al., 2010). The number of Baltic marine fish species gradually decreases eastwards from the Danish straits towards the Gulf of Finland (Nellen and Thiel, 1996), as for numerous fishes the conditions of the Baltic Sea are unfavorable, especially the salinity and temperature (Thiel et al., 1996). However, in recent years both rare and exotic species, for example the European seabass (*Dicentrarchus labrax*), the tub gurnard (*Chelidonichthys lucernus*), the Ballan wrasse (*Labrus bergylta*), the saithe (*Pollachius virens*) and the swordfish (*Xiphias gladius*) have been recorded in the southern Baltic and its estuaries (Keszka and Raczyński, 2002; Keszka et al., 2003; Krzykawski et al., 2001). Some species often occur unexpectedly in new regions following expansion of their natural range (Mohra, 1988; Nehring, 2002). One of these is the thicklip grey mullet (*Chelon labrosus* (Risso 1827)) which lives in the North Atlantic.

The Mugilidae family consists of more than 72 species from 17 fish genera with a worldwide distribution (Nelson, 2006). In the seas surrounding Europe, the Mugilidae family consists of 6 native and 1 exotic species in which only *Oedalechilus labeo* does not thrive in freshwater inland conditions (Kottelat and Freyhof, 2007). To date, *C. labrosus* has been the only Mugilidae species to be found in Polish marine areas (Czerniejewski et al., 2008; Grygiel, 2009). There are also two other Mugilidae species, i.e. *Liza aurata* and *Liza ramada*, that may potentially enter Polish sea waters from adjacent areas, e.g. North Sea (Winkler et al., 2000). Generally, grey mullets are easily recognized from other Acanthopterygii fishes by the two widely separated small dorsal fins (anterior fin with 4 slender spines and a soft-rayed posterior fin). While within the Mugilidae family it is cumbersome to identify grey mullets due to the high morphological similarities shared between the species (Menezes et al., 2010). Identification of the Mugilidae species occurring in European waters on the basis of external characteristics depends mainly on the combination of several measurable characters, the appearance of scales on the head or the number of scale rows around the caudal peduncle. It cannot be based on the body shape or color, which are very similar for all Mugilidae, especially for juvenile specimens (Kottelat and Freyhof, 2007). The main purpose of this study was to characterize and identify a Mugilidae specimen caught in Polish waters for the first time. Additionally, the aim was to compare the usefulness of biometric and molecular data applied in this study.

2. Material and methods

The study was carried out on a fish specimen caught with a fyke net during summer season in the Szczecin Lagoon near the town of Stepnica, GPS coordinates: 53°39'3.21"N,

14°36'39.34"E. The fish was measured with an electronic caliper and its mass was determined with the use of an Axis 3000 scale. Biometric data for 32 measurable and 7 countable characters was collected according to the methodology published by Brylińska (2000). The age of the fish was determined following the guidelines for fish age determination published by Glamuzina et al. (2007). The sex of the collected specimen was assessed based on gonad inspection.

2.1. Molecular analysis of RH1 and COI sequences

A fin clip of the grey mullet was excised and DNA extraction was performed with the use of a peqGOLD Tissue DNA Kit (PEQaLAB Biotechnologie). Purity and concentration of DNA eluates were assessed on a 1.5% agarose gel with the use of a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Genetic identification of the collected specimen was carried out based on the amplification of rhodopsin (RH1) and subunit I of cytochrome oxidase (COI) regions. The reactions were based on the primers Rod-F2W + Rod-R4n (Sevilla et al., 2007) and FishF2_t1 + FishR2_t1 (Ivanova et al., 2007) according to methodological guidelines provided by the respective authors. The results of each PCR were verified by separating the analyzed samples in 1.5% agarose gel, and then each PCR product was sequenced bidirectionally according to Sanger's method (Genomed, Poland). The results of sequence analysis were then analyzed with the following software: BLAST, MEGA5 and BioEdit (Altschul et al., 1990; Hall, 1999; Tamura et al., 2007).

3. Results

The Mugilidae fish specimen caught in the Szczecin Lagoon was a 5-year old male, characterized by the taxonomical formulae D₁ IV, D₂ I7, A III9, V I5, P14, I.I.41. The body is spindle-shaped and elongated, with two short dorsal fins; the head is strongly flattened. Morphometric characteristics of the thinlip grey mullet are presented in Fig. 1, as well as the detailed measurements are available as supplementary material (S1).



Figure 1 Thinlip grey mullet, *Liza ramada* caught in the Odra River, lateral view.

Table 1 Genetic distances between sequences based on Kimura's two-parameter model (K2P).

Liza_ramada (this_study)										
Liza_ramada	0.002									
Liza_ramada(2)	0.002	0.000								
Liza_ramada(3)	0.002	0.000	0.000							
Chelon_labrosus	0.004	0.007	0.007	0.007						
Chelon_labrosus(2)	0.004	0.007	0.007	0.007	0.000					
Chelon_labrosus(3)	0.004	0.007	0.007	0.007	0.000	0.000				
Liza_aurata	0.007	0.009	0.009	0.009	0.007	0.007	0.007			
Liza_aurata(2)	0.007	0.009	0.009	0.009	0.007	0.007	0.007	0.000		
Liza_aurata(3)	0.007	0.009	0.009	0.009	0.007	0.007	0.007	0.000	0.000	
Liza_saliens	0.007	0.009	0.009	0.009	0.002	0.002	0.002	0.009	0.009	0.009

3.1. Genetic identification of *Liza ramada*

Sequences obtained in this study were compared with records deposited in GenBank, resulting in a COI sequence similar with a record submitted for a thinlip grey mullet sample collected in Italy (EU392240). Screening of the GenBank sequence database resources with the RH1 sequence did not reveal entirely homological records, therefore the RH1 sequence was submitted and may be accessed as KM435345. Sequence analysis through the GenBank search for the COI sequence enabled correct assignment of the collected specimen as *L. ramada*. Comparison of the RH1 sequence derived in this study with those submitted in GenBank for *L. ramada* (JX298797.1, EU224158.1, EU224157.1) and related species, such as *Liza saliens* (Y18670.1), *L. aurata* (EF439127.1, Y18671.1, KF017144.1) or *C. labrosus* (DQ197837.1, Y18669.1, JX298796.1), revealed that this sequence may be used as a genetic marker. Genetic diversity among the aforementioned sequences ranged from 0.0% within the group of *L. ramada* sequences, up to 0.9% between *L. ramada* and *L. saliens* records (Table 1). Distances calculated between species pairs showed that the smallest differences (0.4%) existed between *L. ramada* and *C. labrosus*, whereas the highest between *L. ramada* specimen and *L. aurata* or *L. saliens* amounted to 0.7%.

4. Discussion

Correct assignment of Mugilidae species into one of the following genera: *Liza*, *Chelon* or *Mugil*, has been widely studied due to the significant morphological and genetic resemblances (Fraga et al., 2007; Semina et al., 2007). Results obtained by Heras et al. (2009) have shown that it is necessary to carry out additional genetic studies to provide reliable genetic markers for the species phylogeny description. In this study we analyzed the usefulness of two genetic markers, with only RH1 allowing the correct classification of the found *L. ramada* specimen. The selection of an appropriate molecular marker is particularly important for the Mugilidae family, consisting of a large number of species (Chauhan and Rajiv, 2010; Polyakova et al., 2013). Imsiridou et al. (2007) embarked on the task of genetic identification of six Mugilidae representatives (*M. cephalus*, *M. soiyu*, *C. labrosus*, *L. aurata*, *L. saliens* and *L. ramada*). For this purpose, they used the

5S rDNA marker, which provided interesting facts about the phylogeny of the Mugilidae family. According to the authors *L. ramada* and *L. aurata* are clustered together but *L. ramada* and *C. labrosus* differ significantly. In the present study, where the main aim was to identify a mullet specimen, the latter group had the highest similarity. Phylogenetic relationships among Mugilidae species are very complex and investigating them through carefully selected molecular markers is necessary to obtain reliable results. The 0.2% difference between the sequence from the collected specimen and that deposited at GenBank for the species *L. ramada* (JX298797.1, EU224158.1, EU224157.1) results from a sequencing error, the presence of Y (T or C) from the IUPAC code in those sequences. The sequence obtained in this study is free of errors and was submitted to GenBank (KM435345), and may be successfully used for species assignment within the Mugilidae family. In our opinion, the present study delivers a reliable molecular marker which might be used through amplification and sequencing. The authors of this paper do not support the idea of the interchangeable use of the genus name *Liza* or *Chelon* (Semina et al., 2007). Based on analysis of RH1 pairwise genetic distances we found 0.4% differences between *L. ramada* and *C. labrosus* sequences (Table 1). The results indicate that the smallest genetic distance between *L. ramada* and *C. labrosus* are consistent with the results obtained by Heras et al. (2009).

The natural range of a given species is often dictated by its optimal environment. According to Kottelat and Freyhof (2007), distribution of *L. ramada* includes the eastern Atlantic: from the coasts of southern Norway to Morocco, the Mediterranean and the Black Sea and as a landlocked population in the Fratel Reservoir, Portugal (Fig. 2). However, this euryhaline species can exploit fresh water habitats and tolerate abrupt changes in water salinity (Cardona, 2006; Thomson, 1990). The occurrence of thinlip grey mullet in northern Europe is not well known (Jonsson and Jonsson, 2008). The presence of an *L. ramada* specimen in the Odra River – which could signal the expansion of non-native species into wider ranges – may be linked to climate change or human activity (transport of fish in ballast water). According to Selgado et al. (2004) juveniles of thinlip grey mullet may often feed in intertidal creeks and small brooks in river deltas showing fast increase of population density, as it was reported for the Neretva River (Glamuzina et al., 2007). Additionally, exceptional water exchange between the Baltic Sea and the North Sea can result in the temporary occurrence

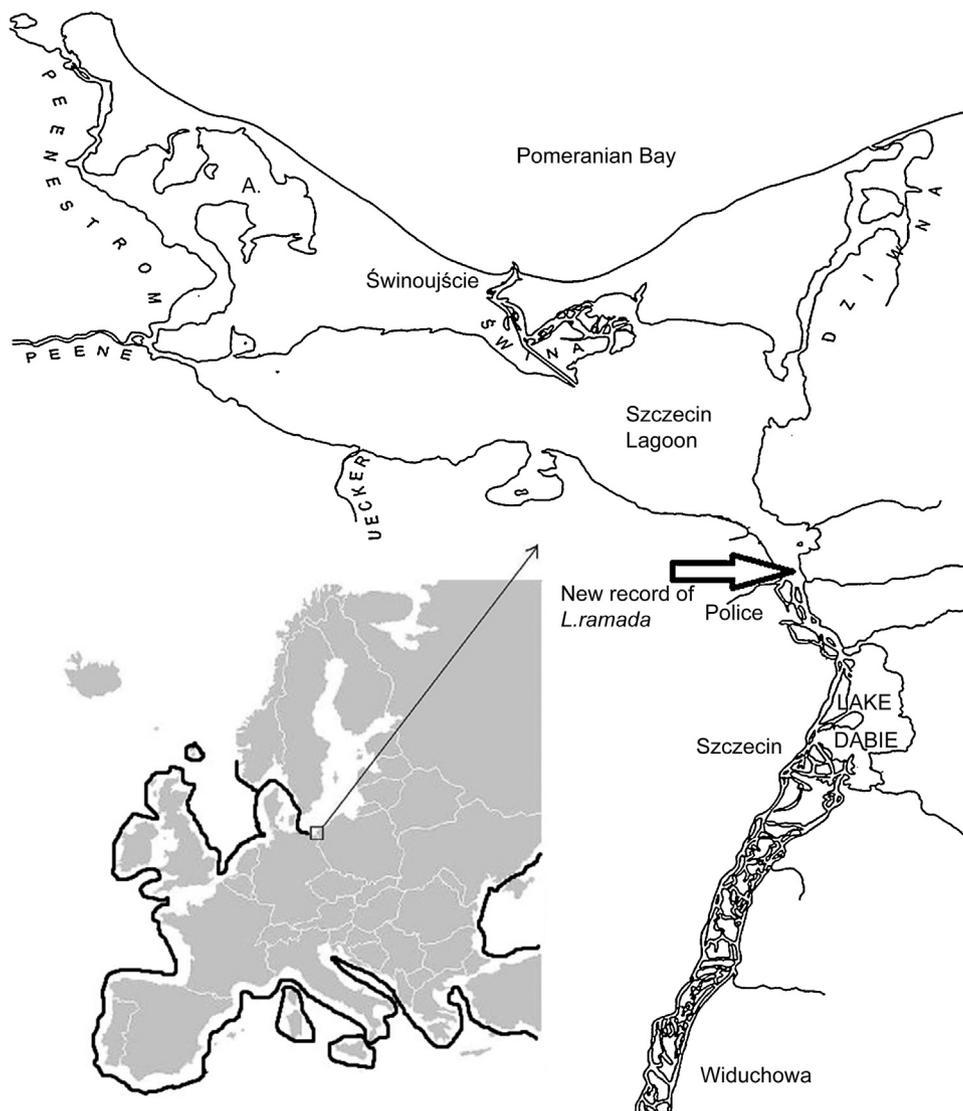


Figure 2 European distribution of *Liza ramada* including a new finding in the Odra River.

of species outside their normal ranges (Berge et al., 2005; Feistel et al., 2004).

In addition, the biology of European Mugilidae, including the alien species (especially the juveniles), prompts them to enter bays and lower reaches of rivers (Carvalho et al., 2007; Kottelat and Freyhof, 2007; Vieira, 1991). According to Gruszka (1999), the Odra River estuary belongs to those water bodies in the Baltic Sea area which are most exposed to immigration of alien species. Regardless of how the tested specimen reached the waters of NW Poland (Fig. 2), it is necessary to perform a detailed characteristic of the first recorded specimen, as it may constitute a serious threat to the biodiversity of the native fish fauna of the Odra River.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.oceano.2016.02.001](https://doi.org/10.1016/j.oceano.2016.02.001).

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