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Fish larvae DNA barcoding indicated the potential appearance of rare species: *Buenia massutii* Kovačić, Ordines, and Schliewen, 2017 in the Adriatic Sea

Jakov DULČIĆ[1], Ivana LEPEN PLEIĆ[1], Barbara ZORICA[1*], Ivana BUŠELIĆ[1], Marija ŠESTANOVIĆ[1] and Marcelo KOVAČIĆ[2]

[1] *Institute of Oceanography and Fisheries,* Šetalište *Ivana Meštrovića 63, 21000 Split, Croatia*

[2] *Natural History Museum Rijeka, Lorenzov prolaz 1, HR–51000 Rijeka, Croatia*

**Corresponding author: zorica@izor.hr*

By introduction of DNA barcodes in Adriatic larval fish identification possible presence of new Gobiidae - Buenia massutii Kovačić, Ordines, and Schliewen, 2017, was noticed. Till now, occurrence of this species was restricted only to the Western Mediterranean and to the neighbouring part of the Atlantic Ocean.

Key words: Adriatic Sea; larvae; *Buenia massutii*; COI gene; first record

INTRODUCTION

Two new *Buenia* species from the Balearic Islands were recently described, *Buenia massutii* KOVAČIĆ, ORDINES, and SCHLIEWEN, 2017 and *Buenia lombartei* KOVAČIĆ, ORDINES, and SCHLIEWEN, 2018, doubling the number of known species of the genus *Buenia* to four (KOVAČIĆ *et al*., 2017; KOVAČIĆ *et al*., 2018). *B. massutii* was later found at the deep continental shelf in the Alboran Sea and the Gulf of Cadiz, north-eastern Atlantic Ocean (ORDINES, KOVAČIĆ *et al.* 2019; ORDINES, RAMÍREZ-AMARO *et al.*, 2019), expanding the known species geographic distribution from the type locality to west all the way to the eastern coast of the Atlantic Ocean. *Buenia massutii* inhabits deeper continental shelf (50-125 m) (KOVAČIĆ *et al*., 2017, ORDINES, KOVAČIĆ *et al*., 2019); it is particularly abundant on beds of red algae, but also frequent on coarse sand bottoms, where it is often found with *Buenia affinis* (KOVAČIĆ *et al.* 2017; KOVAČIĆ *et al.*, 2018).

There is no data at all on biology of *B. massutii*, including any knowledge on early life history stages (eggs, larvae, postlarvae). In general, the early life-history stages of gobies are relatively poorly-known even though gobies are important constituents of coastal larval fish assemblages, which is reflected in the high density of goby species found in littoral bottoms (BORGES *et al*., 2011). The information on the early life-history of the Gobiidae is available for only about half of more than two hundreds genera and for about 1/10 of nearly 2000 recognized species (BORGES *et al*., 2011). Genus *Buenia* has been represented in the Adriatic Sea only by *Buenia affinis*.

The aim of the present paper is to report the species presence in the Adriatic Sea and the first finding of its larval stage.

MATERIALS AND METHODS

In July 2020, precisely from 08th to 29th, scientific survey was carried out with research vessel BIOS DVA along the eastern Adriatic Sea (Croatian fishing ground, Fig. 1). Throughout this survey ichthyoplankton samples were also collected (WP2 sampler - mouth opening, , 0.255 m²; mesh size, 0.200 mm) and preserved in 96% absolute ethanol for further ananlysis. Sea surface temperature and salinity were also measured at each station using a temperature probe.

In the laboratory, ichthyoplankton samples were separated and analysed under the stereomicroscope ZEISS SteREO Discovery.V12. Each collected larva was photographed by a camera coupled to Zeiss binocular microscope and a PC. By image analysis and processing that was carried out using ZEISS-software the total length of larvae was measured.

The larvae were then isolated and stored in absolute ethanol until genomic DNA (gDNA) extraction. gDNA from individual larva was isolated using standard proteinase K digestion followed by phenol:chlorophorm extraction protocol (TAGGART *et al*., 1992) and stored at -20°C until used. Quantity and quality of isolated gDNA were assessed using Nanophotometer (IMPLEN), at 260 and 280 nm. The partial fragment of cytochrome oxidase subunit I (COI) gene was amplified by PCR using primer pair FishF2/FishR2 (WARD *et al*., 2005). PCR was run in 25 µl reactions combining 0.125 µl of Hot-StarTaq DNA Polymerase (Qiagen) (5 u/µl), 2.5 µl of 10xPCR buffer, 1 µl of MgCl2 (25mM), 0.5 µl of dNTP (0.25 mM each), 0.5 µl of each primer (10 µM) and DNase/RNase free PCR water to a volume of 25 µl. PCR conditions were as follows: 15 min at 95°C, 35 cycles of 94°C for 45 s, 54°C for 45 s and 72°C for 1 min, with final extension at 72°C for 10 min. PCR products were visualized on 1% agarose gel under UV transilluminator. Products sequencing using

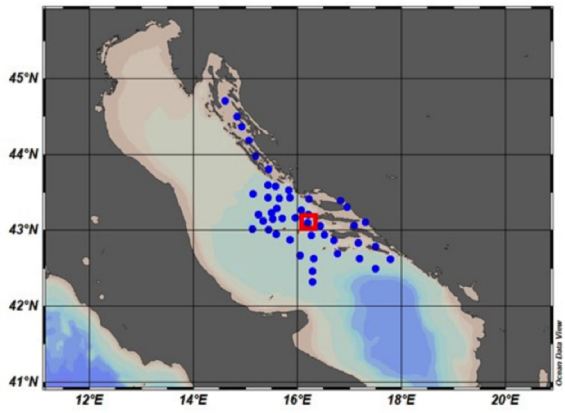

Fig. 1. Map of the eastern Adriatic sampling area cover by the scientific survey MEDITS (July, 2020) showing the sampling stations. N5, in red rectangle is station where larva of goby Buenia massutii*, was collected)*

FishF2 primers were performed by Macrogen Europe (Amsterdam, The Netherlands). The obtained sequence was analysed for similarity with other known vertebrate sequences using Blast Local Alignment Search Tool (BLAST) (ALTSCHUL *et al*., 1990). Using Multiple sequence alignment by ClustalW DNA sequence was further compared with COI sequences from other known Buenia species available in GenBank, i.e., *B. massutii* (Accession numbers of COI haplotypes are as follows: MK370063.1 , KY400545.1, KY400544.1, KY400537.1, KY400536.1, KY400538.1, KY400535.1, KY400531.1, KY400530.1, KY400533.1, KY400547.1, KY400543.1, KY400541.1, KY400540.1, KY400546.1, KY400542.1,

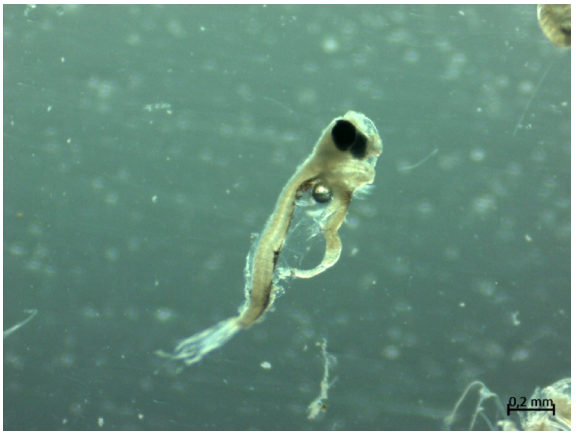

Fig. 2. Photo of the larva of goby Buenia massutii*, collected on 18th July 2020 in the area of central Adriatic (Station number 21: 43°05'55.1"N; 16°11'25.2"E at 18:05)*

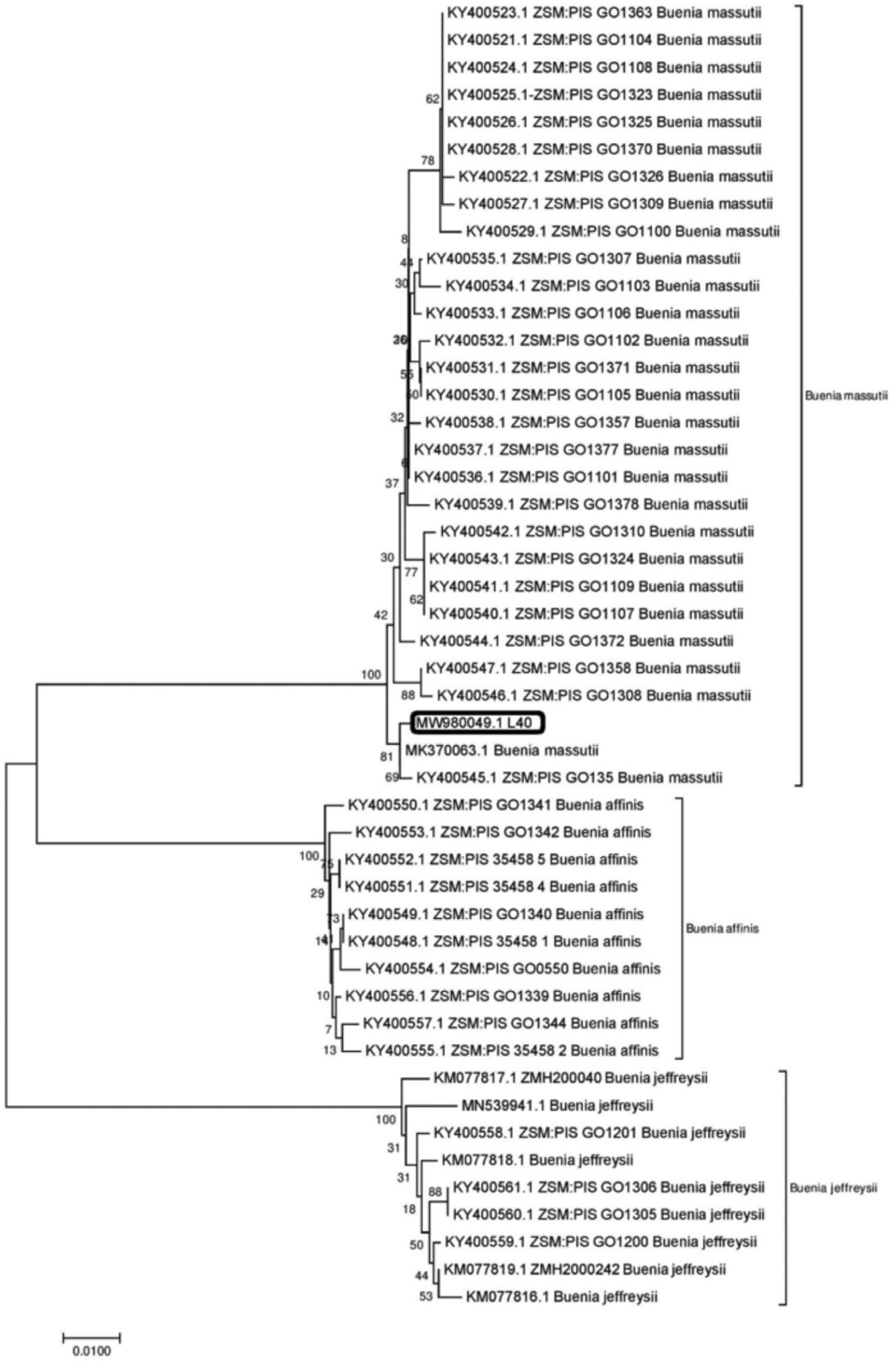

Fig. 3. Evolutionary relationships of taxa. Unrooted neighbour-joining tree based on the p-distance recovered from COI sequences. The sequences were aligned using CLUSTALW and the NJ tree was generated with MEGA 7. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The branches were validated by bootstrap analysis from 2000 repetitions and are shown next to the branches. Tip labels are composed of the species name followed by Museum collection number and the GenBank accession number.

KY400539.1, KY400528.1, KY400526.1, KY400525.1, KY400524.1, KY400527.1, KY400522.1, KY400534.1, KY400523.1, KY400521.1, KY400529.1, KY400532.1), , *B. affinis* (Accession numbers of COI haplotypes are as follows: KY400552.1, KY400551.1, KY400549.1, KY400556.1, KY400548.1, KY400553.1, KY400550.1, KY400557.1, KY400555.1, KY400554.1) and *Buenia jeffreysii* (Accession numbers of COI haplotypes are as follows: KY400558.1, KM077817.1, KY400559.1, KM077819.1, KM077818.1, KY400561.1, KY400560.1, KM077816.1, MN539941.1). Mean genetic distance (p-distance) between groups were estimated using MEGA 7 (KUMAR *et al*., 2016). Phylogenetic tree was generated also with MEGA 7 using the neighbor-joining method, p-distance and complete deletion of gaps. The branches were validated by bootstrap analysis from 2000 repetitions.

RESULTS

During the summer scientific cruise (8-29 July, 2020) on 18th July 2020 in the area of central Adriatic (Station number 21: 43°05'55.1''N; 16°11'25.2''E at 18:05; near the island of Vis) among collected fish larvae, one larva of Gobiidae was separated and stored (Figs. 1 and 2). The sampling depth was 100 m, although the sea bottom depth at station 21 was 111 m, and sea surface temperature and salinity noted by used probe was 22.2°C and 38.3, respectively. Total length of isolated larvae was 1.39 mm (Fig. 2).

The sequencing of the COI locus (521 base pairs) of gobiid larva revealed that specimen L40 most likely belongs to the *B. massutii* (KOVAČIĆ *et al*., 2017). The COI fragment of L40 (GenBank Accession MW980049.1) showed the lowest value of genetic distance (p-distance = 0.0074) when compared to known *B. massutii* samples from the Mediterranean Sea. This was in accordance to the low intraspecific p-distance of the three *Buenia* species: *B. massutii* (0.008), *B. affinis* (0.005) and *B. jeffreysii* (0.007). On the other hand, genetic distance (p-distance) between L40 and *B. jeffreysii* and *B. affinis* were significantly higher: 0.1379 and 0.118, respectively. This was also reflected in the phylogenetic tree analysis (Fig. 3), that grouped L40 together with other *B. massutii* haplotypes, branching away from other two *Buenia* species. Interestingly, L40 specimen features a transversion (substitution of one C→A) on the short COI-barcoding fragment which is not shared by any of 28 sequenced *B. massutii.* However, this transversion did not result in amino acid substitutions specimens.

DISCUSSION

This record represents the first potential record of this species in the Adriatic Sea, the third for the Mediterranean Sea and the first finding of species larval stage. Namely, despite the little genetic distance between L40 specimen and other known *B.massutii* haplotypes, as there is no earlier data of morphology of *B. massutii* larvae to which L40 larvae could be compared, we cannot exclude the possibility that discovered specimen in fact belong to undescribed species. Only similar case among European Gobiidae was reported by KOVAČIĆ *et al*. (2018) for the same genus, where new described *B. lombartei* shared very low genetic distance to known haplotypes of *B. jeffreysii*, but clearly differed by morphology, coloration, depth range, and disjunct and distant geographic distribution from the later species. Until morphological descriptions of other *B. massutii* larvae are available, we can only assume that collected L40 larvae belongs to the species *B. massutii*. Taking all the above into account, we can conclude that the collected larva belongs to *Buenia* species, much more likely being *B. massutii* individual than an undescribed *Buenia* species.

Our finding confirms the general life history pattern of benthic Gobiidae, with benthic eggs and planktonic larvae of 1 to 8 mm size, depending on species (BORGES *et al*., 2011). The most of the European gobiid species are shallow water species. While the larvae has been collected and described earlier for eurybathic European marine gobies extending over wide range of depths on continental shelf, this is the first col-

lected larva for European gobiid species which are restricted to the deeper continental shelf. However, the most of them have been, as well as European exclusively bathyal gobies, only recently described (BORGES *et al.,* 2011, KOVAČIĆ, 2020). The present record of possible *B. massuti* larva in the eastern Adriatic coast would expand the species known geographic distribution from the Balearic Islands as the most eastern species record to the Adriatic Sea. Two hypotheses could in that case be considered as explanation of the presence of this larvae. One would be that this larva come directly from ballast water discharges, since previous studies have demonstrated that fish larvae remain (especially from Gobiidae family) viable in ship tanks (WONHAM *et al*., 2000). This could suggest that the ballast water was the vector of transport. However, this hypothesis is very unlikely considering that the found larva was very young (oil globule still not absorbed, mouth still not functional, Fig. 2) and the sampling site is relatively far from the main commercial shipping routes. A second, much more likely, hypothesis is that the larva was transported after hatching by currents from the nearest still undetected settlement of adult population. Considering the general scheme of sea currents and circulation in the Adriatic Sea (ORLIĆ *et al*., 1992), it could be somewhere on the continental shelf of the Adriatic Sea or of the northeastern Ionian Sea (BiOS system, see CIVITARESE *et al*., 2010). Furthermore, sampling area where this larva was collected is known as an area of induced coastal upwelling (BERGAMASCO and GAČIĆ, 1996; GAČIĆ *et al*., 1997) in summer months (July-September). Hence, the presence of fish early life stages in this area is more than expected due to enrichment, food aggregations and physical processes (LAFUENTE *et al*., 2002) that in general increase survival of any larvae.

Unambiguous identification of fish eggs and larvae is an important tool for fish ecology and conservation. For instance, it may allow the detection of spawning areas, the monitoring of fish stocks affected by dams and improve fisheries management and conservation policies (VALDEZ-MORENO *et al*., 2010). DNA barcoding techniques proved to be very effective for large-scale biodiversity assessments and were already employed to identify marine mesozooplankton (STEFANNI et al., 2018) and fish larvae to a species level by comparing the queries with sequences from adult stage as reference library (WIBOWO *et al*., 2018; RAM *et al*., 2020). The DNA barcoding by AZMIR *et al*. (2017) showed that they correctly identified Gobiidae larvae from morphology to family level because of gobiid distinct morphology, contrary to other fish families. However, their results also showed that DNA barcoding is a better method for deeper taxonomic levels identification of larvae than morphology, if the robust sequence reference libraries exist. Although combining DNA barcoding with detailed morphological comparison is always highly recommended, present study confirmed once again that in the absence of the latter, DNA barcoding is useful technique that can be successfully applied not only on adults but also on fish early life stages as early stated by HUBERT *et al*. (2010), HUBERT *et al*. (2014) and AYALA *et al*. (2016). Implementation of DNA barcoding obviously improve the knowledge of local fish richness, as it was also confirmed within this study.

We expect that in the future, the more careful examination of trawl catches or the applications of beam trawls or of deep trimix diving would find the adult population of this species in the Adriatic Sea (see KOVAČIĆ and GLAVIČIĆ (2019)). Furthermore, these methods, combined with the larval sampling and identification, should continue the discoveries in the Adriatic Sea of circalittoral Mediterranean gobies, especially those recently described and still not found in the area (compare the gobiid species lists for the Mediterranean Sea in KOVAČIĆ (2020) and for the Adriatic Sea in DULČIĆ and KOVAČIĆ (2020)).

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Molekularnom analizom ribljih larvi utvrđena potencijalna prisutnost rijetke vrste: *Buenia massutii* Kovačić, Ordines i Schliewen, 2017. u Jadranskom moru

Jakov DULČIĆ[1], Ivana LEPEN PLEIĆ[1], Barbara ZORICA[1*], Ivana BUŠELIĆ[1], Marija ŠESTANOVIĆ[1] and Marcelo KOVAČIĆ[2]

**Kontakt e-pošta: zorica@izor.hr*

SAŽETAK

Larva nedavno opisane vrste *Buenia massutii* Kovačić, Ordines i Schliewen, 2017, s poznatom geografskom distribucijom ograničenom na zapadno Sredozemlje i susjedni dio Atlanskog oceana, je pronađena u Jadranskom moru. Naime, tijekom ihtioplanktonskog uzorkovanja je izolirana larva navedene vrste, čija je taksonomija utvrđena molekularnom analizom (DNA barcoding).

Ključne riječi: Jadransko more; larve; *Buenia massutii*; COI gen; prvi nalaz