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THE UNCOMMON CRYPTOBENTHIC *MILLERIGOBIUS MACROCEPHALUS* (ACTINOPTERYGII: GOBIIDAE) ALONG THE COAST OF SALENTO, WITH A REVISION OF ITS DISTRIBUTION IN ITALIAN WATERS

RIASSUNTO

Il gobide criptobentonico *Millerigobius macrocephalus* (Kolombatovic, 1891) viene segnalato per due località lungo la costa salentina (Mar Grande di Taranto e Gallipoli). Ambedue le segnalazioni sono supportate da dati genetici e, oltre a confermare la presenza di *M. macrocephalus* lungo la costa salentina, rappresentano la prima segnalazione della specie per il Mar Ionio settentrionale. Questo risultato è particolarmente rilevante, dato che l'unica altra segnalazione per la costa salentina, relativa al lago di Acquatina, potrebbe essere erronea; di fatto, recenti studi hanno evidenziato la presenza nel lago di Acquatina di una specie superficialmente simile, *Zebrus pallaoroi*, cui le segnalazioni storiche potrebbero riferirsi. Un confronto tra le sequenze di citocromo c ossidasi subunità I ottenute in questo studio e quelle presenti in banca dati mette in evidenza una divergenza lievemente inferiore al 3% tra gli individui salentini e greci e gli individui turchi, suggerendo la possibilità che *M. macrocephalus* possa rappresentare un complesso di specie, al pari di altri gobidi mediterranei. Infine, la distribuzione della specie nel Mar Mediterraneo è rivista sulla base della letteratura disponibile, con particolare attenzione alle segnalazioni lungo la costa italiana. Questa analisi ha messo in evidenza diverse segnalazioni trascurate dalla letteratura e permette di confermare la presenza della specie in diverse località del Mar Ionio e del

Mar Tirreno, suggerendo che *M. macrocephalus* possa essere più comune e diffuso di quanto al momento si ritiene.

ABSTRACT

New records of the cryptobenthic goby *Millerigobius macrocephalus* (Kolombatovic, 1891) are reported for two localities in Puglia (Mar Grande of Taranto and Gallipoli), confirming the occurrence of this species along the coast of Salento. All records were checked by DNA barcoding and represent the first occurrences of this species in the northern Ionian Sea. Molecular data highlight a divergence between individuals from Salento and Greece and individuals from Türkiye, suggesting that *M. macrocephalus* might represent a species complex, and deserve further studies. Literature dealing with this species is critically re-examined, allowing to identify three records for Italian waters that were overlooked by recent literature.

INTRODUCTION

Gobiidae represent by far the most species-rich fish family in the Mediterranean Sea (Kovačić & Patzner, 2011) and their diversity, distribution and ecology in the basin are still incompletely known. This is especially true for the so-called cryptobenthic species, representing the smallest Mediterranean fishes. Cryptobenthic gobies are overall scarcely known because of their small size, usually not exceeding 30-40 mm of total length, and their preferential habitats, usually coralligenous outcrops, circalittoral hard and mixed bottoms and rhodolites beds. Commercial and recreational fisheries only sporadically sample these species; as a consequence, new records and range extensions are rather frequent (Ballesta *et al.*, 1998; Patzner, 2007; Colombo & Langeneck, 2013; Schembri & Kovačić, 2019), and the description of new species is not a sporadic occurrence either (Kovačić *et al.*, 2016a; Kovačić *et al.*, 2016b; Engin & Innal, 2017; Engin & Seyhan, 2017; Engin *et al.*, 2018; Kovačić *et al.*, 2018; Schliewen *et al.*, 2019). While some species are characterised by a rather distinctive appearance that makes identification through underwater photographs feasible (Patzner, 2007; Colombo & Langeneck, 2013), the identification of some species can be taken with reasonable certainty only when based on a careful examination of the individuals and/or on DNA barcodes, and given the sporadic collection of these species with traditional sampling methods, this increases the uncertainties surrounding their distribution.

Millerigobius macrocephalus (Kolombatovic, 1891) is the only species currently assigned to the genus *Millerigobius*, and represents a Mediterranean endemite, showing an apparently fragmented and scattered distribution that ranges at least from the Balearic Islands to the Levant Sea (Miller, 1977; Ramos-Esplá & Pérez-Ruzafa, 1987); it is considered an established introduced species in the northernmost part of the Black Sea (Boltačëv *et al.*, 2010), and at least one landlocked population, thriving in a oligohaline karstic environment, is known (Vanhove *et al.*, 2011). The uneven distribution of this species is most likely an artefact due to its similarity with the more frequent *Zebrus zebrus* (Risso, 1827), and the scarcity of detailed taxonomic studies for a large part of the basin. In Italian waters *M. macrocephalus* has been reported firstly for Orbetello Lagoon, in the Northern Tyrrhenian Sea (Gandolfi *et al.*, 1977), and then found in Acquatina Pond (Lumare *et al.*, 2009; Prato *et al.*, 2009). These publications were overlooked by subsequent literature, as Giacobbe *et al*. (2016) explicitly considered their record for Cape Peloro Lagoons and Marinello Lakes (Strait of Messina) as the first for Italian waters. The current note has the purpose to check the occurrence of *M. macrocephalus* along the coast of Puglia, extending the distribution of this species to the Ionian Sea, and to discuss the distribution of this species in Italian waters.

MATERIALS AND METHODS

Individuals of *Millerigobius macrocephalus* were sampled along the coast of Salento between 2019 and 2023; all individuals were examined and photographed alive, and subsequently fixed in 96% ethanol. The preserved individual was further examined to check the diagnostic features of the head pores and canals and measured with a calliper. Moreover, the identification was confirmed through DNA barcoding. Total genomic DNA was extracted from a small fin clip using the GenElute™ Mammalian Genomic DNA Miniprep Kit distributed by Sigma-Aldrich, following the manufacturer's instructions. A fragment of the mitochondrial gene coding for the subunit I of the cytochrome c oxidase (COI) was amplified using the primer pair COI-FishF1 (5′- TCAACCAACCACAAAGACATTGGCAC-3′) and COI-FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward *et al.*, 2005). Polymerase chain reactions (PCR) amplification were carried out in 20 µL solutions using 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer, 1 U of DreamTaq DNA polymerase (Thermo Scientific), and ~2.5 ng of template DNA. The PCR profile was set as follows: initial denaturing step at 94 °C for 3 min; 34 cycles of denaturing at 94 °C for 45 s, annealing at 50 °C for 1 min, and extending ay 72 °C for 1 min, and a final extending step at 72 °C for 7 min. A negative control was included in each reaction. Amplification success was checked by electrophoresis on agarose gel, and amplified fragments were sent to Macrogen Europe for sequencing.

The obtained sequences were compared with sequences of *M. macrocephalus*, *Z. zebrus* and the recently described *Zebrus pallaoroi* Kovačić, Sanda, Čekovská, Soukupová & Vukić, 2021 downloaded from GenBank and BOLD. Sequences were aligned with ClustalX 2.1 (Larkin *et al*. 2007), and alignments were edited in BIOEDIT version 7.2.5 (Hall 1999). Pairwise K2P distances (Kimura, 1980) were calculated using the software MEGA 7 (Kumar *et al*., 2016). The program jModelTest v. 2.1.6 (Guindon & Gascuel 2003; Darriba *et al*. 2012), based on the hierarchical likelihood ratio test, was used to assess the best model of evolution for the sequences under the Akaike Information Criterion (AIC) (Akaike 1974); the best fitting model of evolution was GTR + I + G, with I= 0.00, G= 0.12, and the following frequencies for each nucleotide base: f_A= 0.219; f_C= 0.300; f_G= 0.195; f_T= 0.286. An unrooted maximum likelihood tree (Guindon & Gascuel, 2003) was built using the software MEGA 7.

RESULTS

Millerigobius macrocephalus (Kolombatovic, 1891) (Figure 1)
Material examined. Mar Piccolo di Taranto (40.43605° N; 17.24177° E), 20 March 2019, 12 m depth, on mud with shell fragments under fish farming cages: one individual, sex undetermined. Gallipoli, port (40.05693° N; 17.98094° E), 28 April 2023, 1 m depth, on fouling assemblages among serpulids and photophilous algae: one individual, sex undetermined.

Description. Body short, slightly compressed laterally in the posterior region. Head large, slightly depressed, with large eye extending above head profile. Posterior edge of the jaws ending approximately below mideye. Snout short, blunt and wide. Anterior nostril tubular, reaching upper lip, posterior nostril tubular, short. Meristic and biometric characters reported in Table 1. Body covered in ctenoid scales, absent from head, predorsal area and breast, ventral region covered with cycloid scales. Morphometric and meristic characters reported in Table 1.

Head with anterior and preopercular canals, with pores σ, λ, κ, ω, α, β, ρ and γ, δ, ε, respectively. Rows of sensory papillae: suborbital row *a* absent. Seven transverse suborbital rows of sensory papillae. Four transverse suborbital rows before longitudinal row *b*, one below row *b*. Transverse suborbital rows *2* and *3* more distant from orbit, other suborbital rows (*1, 4, 5, 6, 7*) beginning close to the orbit. Anterior edge of row *b* at the level of the posterior edge of the eye. Suborbital row *d* divided, with gap below transverse suborbital row *3*. Two interorbital rows.

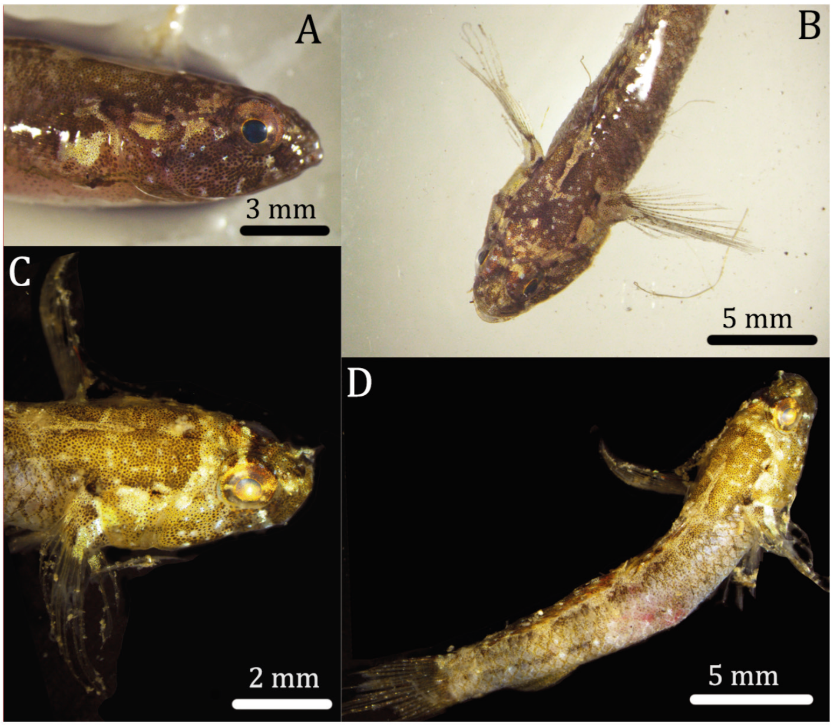

Figure 1 – Individuals of *Millerigobius macrocephalus* sampled along the Salento Peninsula. A) *M. macrocephalus* sampled off Taranto, close-up of the head; B) *M. macrocephalus* sampled off Taranto, anterior part of the body; C) *M. macrocephalus* sampled off Gallipoli, close-up of the head; D) *M. macrocephalus* sampled off Gallipoli, entire specimen.

Table 1 – Biometric and meristic measurements of the *Millerigobius macrocephalus* specimens collected along the Salento coastline.

Biometric parameters	Taranto	Gallipoli
Total length (TL)	25.6 mm	21.1 mm
Standard length (SL)	21.0 mm	16.8 mm
Opercular head length	7.0 mm	5.4 mm
Orbital diameter	1.4 mm	1.5 mm
First dorsal fin length	2.5 mm	2.3 mm
Second dorsal fin length	12.6 mm	8.1 mm
Anal fin length	7.8 mm	6.3 mm
Ventral fin length	5.0 mm	3.4 mm
Pectoral fin length	5.2 mm	4.1 mm
Greatest depth	3.8 mm	3.3 mm
Pectoral breadth	3.9 mm	2.5 mm
Meristic measurements		
First dorsal fin rays	VI	VI
Second dorsal fin rays	I/10	I/11
Pectoral fin rays	15	15
Ventral fin rays	I/5 + I/5	I/5 + I/5
Anal fin rays	I/9	I/9
Caudal fin branched rays	16	16
Caudal fin articulated rays	17	17
Scales on lateral line	30	29
GenBank accession number	OR735516	OR735517

Live colour chocolate brown, with some small turquoise spots on the dorsal part of the head, on the cheek and along the external edge of the eye. A few thin, broken and scarcely distinct pale transverse bands on the body. Characteristic yellowish pattern on head and anterior part of the body: a straight trait between the eyes, a crescent-shaped, jagged blotch just after the eyes, a coarsely rectangular blotch on each operculum, with a dark spot at each upper edge. A large, irregular blotch at the basis of each pectoral fin, with a dark anterior edge on the upper part. Basis of the first dorsal fin pale, yellowish. Fins yellowish, with poorly defined brownish spots forming irregular longitudinal (dorsal and anal fins) or transverse (caudal and pectoral fins) bands. Ventral fins whitish. Preserved colour in ethanol greyish, with well-defined pattern of pale blotches in the anterior part of the body and several small dark spots along the lateral line.

Molecular characterisation. The COI sequences obtained from these individuals (GenBank accession numbers: OR735516-OR735517) showed a good correspondence with sequences deposited as HQ232434 (99.32% identity) and KY176529 and MZ723165 (97.31% identity), all corresponding to *M. macrocephalus*. COI sequence data therefore confirm the identification based on morphological characters. The two species of the genus *Zebrus* showed a sequence identity around 85%, confirming the closeness between *Millerigobius* and *Zebrus*, but were rather distant. Finally, while all sequences downloaded from GenBank were correctly assigned and did not show any trace of bad quality, the majority of the sequences available on BOLD, assigned to either *Z. zebrus* or *M. macrocephalus*, showed the presence of alignment gaps and were excluded from the phylogenetic reconstruction.

The phylogenetic tree obtained (Fig. 2) showed a topology consistent with previous phylogenetic reconstructions. All specimens of *M. macrocephalus* were retrieved as a highly supported clade. The two specimens from Salento clustered together with a sequence from the freshwater karstic Lake Vouliagmeni (with distances spanning from 0.2 to 0.4% between individuals), and are clearly separated from two sequences, both corresponding to individuals sampled in Türkiye (with distances spanning from 2.6 to 2.9%). By comparison, intraspecific distances within *Z. pallaoroi* ranged from 0.2 to 0.5%, while interspecific distances between *Z. pallaoroi* and *Z. zebrus* ranged from 3.3 to 3.5%.

DISCUSSION

Millerigobius macrocephalus is a scarcely reported species, mostly because of its small size and the similarity with the allegedly more frequent *Zebrus zebrus*, that shows a rather similar morphology and colour pattern (Gandolfi

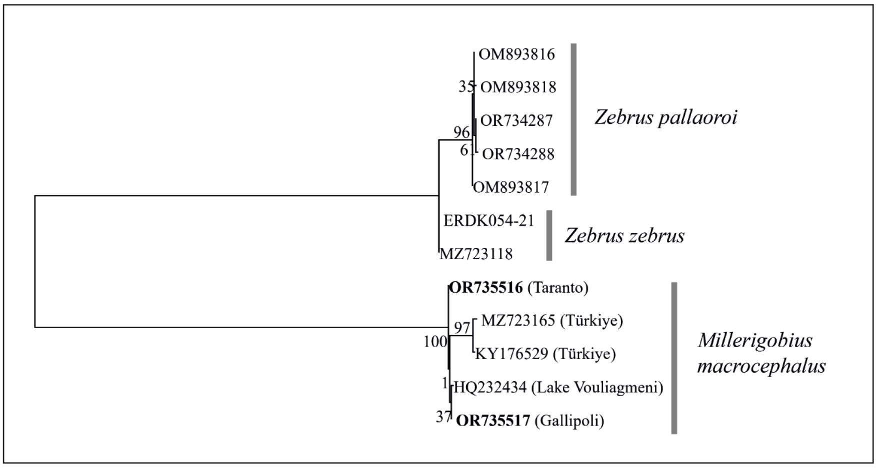

Figure 2 – Maximum likelihood phylogenetic tree based on COI sequences; sequences of *M. macrocephalus* specimens from Salento in bold.

et al., 1977; Miller, 1977). It has been already suggested that *M. macrocephalus* might actually be not rare at all; as a matter of fact, Trkov *et al.* (2019) identified this species as the most frequent cryptobenthic Gobiidae species in Slovenian coastal waters, and abundant populations were reported in several Mediterranean localities (Gandolfi *et al.*, 1977; Bouchereau & Guelorget, 1999; Bouchereau, 2002; Giacobbe *et al.*, 2016). Several papers report single records, or records of a few specimens, for several areas of the Mediterranean Sea. However, there are inconsistencies in the distributional data between different papers; in particular, the first report outside the Adriatic Sea has been consistently overlooked by all papers dealing with this species, and recent papers overlook the record for the Balearic Islands by Ahnelt & Patzner (1996). Here we provide an updated distributional map, encompassing all published records of the species, together with the literature upon which the distributional points are based (Figure 3). The distribution of this species ranges from the western Mediterranean Sea (Ramos-Esplá & Pérez-Ruzafa, 1987) to the Levant Sea (Miller, 1977), although the majority of the records are scattered along the Northern Mediterranean coastline. However, the scarce number of detailed studies on Gobiidae diversity and ecology along the Northern African coast might account for the apparent absence of this species from this area; indeed, several recent works highlighted a previously overlooked diversity of this family even in well-known geographical areas (Kovačić *et al.*, 2011; Kovačić *et al.*, 2013). The first record of the species from Italian waters is referred to Orbetello Lagoon, where this species was found with an abundant population in one of the channels connecting the lagoon to the sea (Gandolfi *et al.*, 1977); despite being, together with Miller's

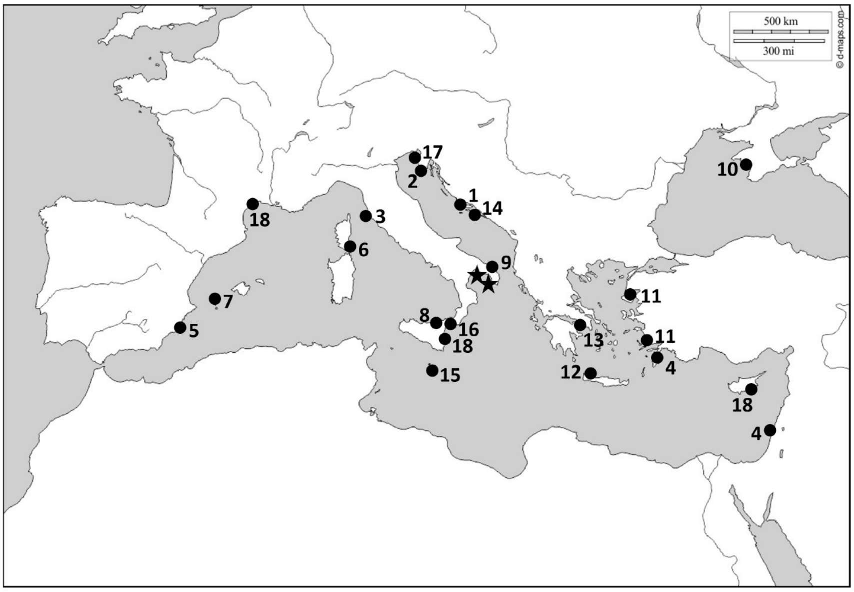

Figure 3 – Updated distribution of *Millerigobius macrocephalus* in the Mediterranean Sea, with references for the records. Legend: 1 – Kolombatovic, 1891; 2 – Bath, 1973; 3 – Gandolfi et al., 1977; 4 – Miller, 1977; 5 – Ramos-Esplá & Pérez-Ruzafa, 1987; 6 – Bouchereau & Tomasini, 1989; 7 – Ahnelt & Patzner, 1996; 8 – Costa et al., 1996; 9 – Lumare et al., 2009, Prato et al., 2009; 10 – Boltačëv et al., 2010; 11 – Bogorodsky et al., 2010; 12 – Kovačić et al., 2011; 13 – Vanhove et al., 2011; 14 – Kovačić et al., 2012; 15 – Kovačić et al., 2013; 16 – Giacobbe et al., 2016; 17 – Trkov et al., 2019; 18 – Kovačić et al., 2021; stars present records.

(1977) observations, the first record outside the Adriatic Sea, this paper was long overlooked, and Giacobbe *et al*. (2016) considered their observation for the brackish-water lagoon system of the Strait of Messina as the first record of the species in Italian waters. Interestingly, there is an additional, overlooked record for the southern Tyrrhenian Sea, in Oliveri-Tindari lagoon. The Gobiidae population reported for this area was initially identified as *Buenia affinis* Iljin, 1930 (Costa *et al*., 1996), but this identification was already challenged by the authors on the basis of discrepancies in the meristics, and later corrected as *M. macrocephalus* by Costa (2004) himself, albeit in a non-peer reviewed publication. The iconography in Costa *et al*. (1996) is not compatible with *B. affinis* (see Kovačić, 2002), and shows instead the typical features of *M. macrocephalus*. Further records extended the distribution of this species for Acquatina Lagoon (Adriatic Sea), where this species was reported as a bycatch of trap nets (Lumare *et al*., 2009; Prato *et al*., 2009); however, sampling activities carried out in this last locality highlighted the occurrence of the superficially similar *Zebrus pallaoroi* in the same area of the pond where *M. macrocephalus* was found by Lumare *et al*. (2009) (Langeneck & Putignano, *in accepted*). For this reason, the records of *M. macrocephalus* for Acquatina Pond remain currently unconfirmed. Lastly, Kovačić *et al*. (2021) reported the species from Aci Trezza, Ionian Sea, without further details on the ecology. Based on the data available in literature, the distribution of *M. macrocephalus* is probably fragmented and restricted to suitable environments, but most likely encompasses the whole Italian coastline, and the apparent rarity is most likely due to the scarcity of detailed studies on shallow-water gobies, and to misidentification with species of the genus *Zebrus*. Gandolfi *et al*. (1977) pointed out discrepancies in the descriptions of this latter species in the works by Spartà (1948) and Bini (1969), suggesting that these records should be possibly referred to *M. macrocephalus*.

The ecology of this species seems to be quite different from that of other cryptobenthic gobies. Although some authors reported *M. macrocephalus* on vegetated hard bottoms at shallow depths (0-10 m) (Ahnelt & Patzner, 1996; Trkov *et al*., 2019), and juveniles can be found hidden among sea urchin spines (Ahnelt & Patzner, 1996), several records refer to transitional environments. Although the majority of such environments are represented by coastal ponds with a limited freshwater inflow, and a tendency towards hyperhaline conditions in summer months (Gandolfi *et al*., 1977; Ramos-Esplá & Pérez-Ruzafa, 1987; Costa *et al*., 1996; Giacobbe *et al*., 2016), this species seems to be able to tolerate stable oligohaline conditions (Vanhove *et al*., 2011), and this tolerance explains how this species was able to establish reproductive populations in the northern Black Sea, which is characterised by relatively low salinities (Boltačëv *et al*., 2010). Interestingly, while several records of *M. macrocephalus* on hard bottoms refer to single observations, all records for brackish environments refer to large populations, suggesting that coastal ponds might represent a typical habitat for this species (Gandolfi *et al*., 1977). The specimen from Taranto was sampled in a fully marine environment, but the high organic enrichment due to the presence of fishing cages causes this habitat to be rather similar to typical brackish water ones. The scarcity of studies on cryptobenthic fish species in brackish-water environments, and more specifically coastal ponds and lagoons, might contribute to explain why tolerant species appear to be less widespread and more rare than they actually are (Langeneck, 2013; Tiralongo *et al*., 2016).

An interesting, and somehow unexpected, result of this study is represented by the relatively high genetic distance retrieved between *M. macrocephalus* from different geographical areas. The distance retrieved between the two groups of *M. macrocephalus* is slightly lower than both that detected between *Z. zebrus* and *Z. pallaoroi*, and the 3% threshold identified by Hebert *et al*. (2003) for interspecific distances. However, the majority of studies focusing on DNA barcoding currently stress the importance of a barcoding gap over the fixed threshold proposed by Hebert *et al*. (2003) (Čandek & Kuntner, 2015), and interspecific distances lower than the 3% have already been reported in other Gobiidae (Knebelsberger & Thiel, 2014; Victor, 2014). The interpretation of this genetic divergence is currently unclear; it might represent a clue of a strong geographical structuring of the species between different parts of the basin (Stefanni & Thorley, 2003; Mejri *et al*., 2011), a possible case of incipient speciation (Victor, 2014) or, similarly to what observed in the genus *Zebrus*, a case of cryptic or pseudocryptic species, potentially occurring in sympatry. To disentangle this issue, more complete data covering a wider part of the distributional range of this species are needed.

Cryptobenthic fish species are for a large part endemic to the Mediterranean Sea, and they are considered as very vulnerable to climate changes, facing a significant risk of extinction (Ben Rais Lasram *et al*., 2010); however, a precise evaluation of their conservation status is hindered by the scarcity of observations and on the uncertainties surrounding their ecology and distribution, which are mostly due to difficulties related to sampling techniques. Underwater sampling by snorkelling and scuba-diving, collecting the fishes with a hand net after anaesthetisation with Quinaldine appears the best technique for the study of cryptobenthic fish species (Trkov *et al*., 2019); however, this technique is only suitable for works aimed at the study of these taxa. Instead, the majority of cryptobenthic species are seldom caught with commercial fishing gear, and even in these rare cases they are usually in too bad conditions to be successfully studied. Conversely, the current record is based on the serendipitous sampling of a *M. macrocephalus* individual through a Van Veen grab; small gobies are often found among macrozoobenthic taxa, both on soft and hard bottoms (Frouin, 2000; Tempesti *et al*., 2020; pers. obs.), but as they represent an irrelevant fraction of the sampled individuals, they are usually not identified at the species level. It is advisable that cryptobenthic fish species obtained through sampling techniques employed for macrozoobenthic species are carefully treated and kept, as they might add information on the actual distribution and ecology of these species.

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