

First record of a landlocked population of marine *Millerigobius macrocephalus* (Perciformes: Gobiidae): Observations from a unique spring-fed karstic lake (Lake Vouliagmeni, Greece) and phylogenetic positioning

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Abstract

A hitherto undescribed population of gobies in the Greek brackish karstic Lake Vouliagmeni was identified as *Millerigobius macrocephalus*. Molecular phylogenetic analysis using partial mitochondrial ribosomal DNA sequences situates this species as a sister taxon to *Zebrus zebrus*, thus confirming earlier morphological work. Morphological variation as compared to previous studies on this species is discussed. This is the first report of *M. macrocephalus* occurring inland and under oligohaline conditions, underlining that our knowledge on diversity and distribution of the European gobiid fauna is still far from complete. The occurrence of this rarely observed fish species might enhance the conservational importance of the lake under study.

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1. Introduction

The goby family (Teleostei, Gobiidae) is perhaps the most speciose fish family worldwide, of which currently around 1950 species have been described (Miller, 1986; Nelson, 2006). A considerable portion of this was only recently discovered (180 during the last decade, only counting marine ones; Eschmeyer et al., 2010). Gobies display a wide habitat and behavioural range, and include freshwater, brackish and marine representatives (Miller, 1986; Kottelat and Freyhof, 2007). They are sometimes put forward as a characteristic

case of adaptive radiation (Rüber et al., 2003; Taylor and Hellberg, 2005; Larmuseau et al., 2010) and have many endemic species in the Mediterranean basin (Penzo et al., 1998; Huyse et al., 2004; Kovačić and Patzner, 2011).

Lake Vouliagmeni (25 km south-east of Athens, Greece) is a karstic, spring-fed brackish lake, created about 2000 years ago after the collapse of a large cavern near the rocky coastline below Mount Hymettos massif (Papapetrou-Zamanis, 1969). The surface waters of this unique collapse structure basin cover about 4000 m² but are rather deep (approximate average depth 9 m, maximum 13 m) (Gontikaki et al., 2003). Although the water body has been referred to as a “tectonic lagoon” (Chintiroglou et al., 2008) there is no evidence of the isolation of a paralic stretch of marine water by tectonic changes, a key criterion of this lagoon type

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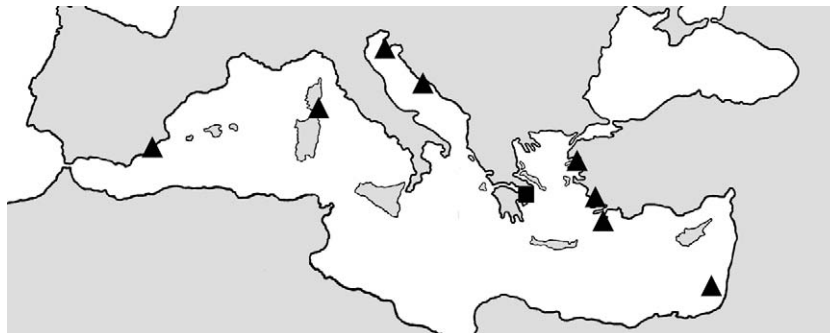


Fig. 1. Map of the Mediterranean showing the known marine records of *Millerigobius macrocephalus* (▲) and the new record from Lake Vouliagmeni (■).

(Guelorget and Perthuisot, 1992; Tagliapietra et al., 2009). Although this small lagoon-like basin is situated less than 100 m from the sea, there is no marine surface-water connection, no notable tidal influence, and only underground water-movements through deep fissures where there is a mixing of geothermally warmed sea water and freshwater spring flow. This almost landlocked water body maintains year-round remarkably stable brackish conditions (salinity of 14.5–17), a high water temperature (18–28 °C) and a stable pH (about 7) and conductivity (ca. 25 mS/cm). The northern part of the lake has a steep rocky shore, whereas its shallower southern side is characterised by gravel–sand substrates (Doumenc et al., 1987; Chintiroglou et al., 2000, 2008). Only two fish species have so far been recorded from Lake Vouliagmeni, namely *Anguilla anguilla* (Linnaeus, 1758) and the introduced *Poecilia latipinna* (Lesueur, 1821) (previously mentioned as *P. sphenops* Valenciennes, 1846) (Economou et al., 2007). The lagoon hosts at least one endemic species, the anemone *Paranemonia vouliagmeniensis* Doumenc, England and Chintiroglou, 1987, as well as other species of taxonomic interest (Doumenc et al., 1987; Chintiroglou et al., 2004). This lake and its surrounding limestone landscape are designated as a Natura 2000 protected area; while the lake's thermal spring water, rich in hydrogen sulphide, has been used as a recreational spa for more than a century (Chrystomanos, 1889; Chintiroglou et al., 2008).

In addition to the poeciliid fish, representatives of a hitherto unidentified goby species make up most of the fish community in the lake. The gobies maintain rather high densities in the shallow parts of the lake, and are particularly noticeable on the less-vegetated sand–gravel and cobble substrates. Using morphological and molecular analyses, we identified them as belonging to *Millerigobius macrocephalus* (Kolombatović, 1891), a small marine goby distributed along the eastern and northern Mediterranean coasts from Spain to Israel (Fig. 1) (Miller, 1977; Bouchereau, 2002; Bogorodsky et al., 2010). This rarely recorded cryptobenthic species lives in shallow water (at depths of less than 4 m), and can sometimes occur under hyperhaline and high water temperature conditions (Ramos Espla and Pérez-Ruzafa, 1987; Bogorodsky et al., 2010). In this study, we discuss the morphological observations on the *M. macrocephalus* population in Lake

Vouliagmeni, and the sequence data retrieved from this fish. This discovery of *M. macrocephalus* in Lake Vouliagmeni presents the first known landlocked population of this species and is the first record of this species in oligohaline conditions.

2. Materials and methods

2.1. Morphology

Materials: two females, 31.3 + 7.6 mm and 28.6 + 7.5 mm, PMR VP2337, Lake Vouliagmeni, 16 February 2010; six males, 30.6 + 7.9 mm to 41.3 + 10.2 mm, PMR VP2338, Lake Vouliagmeni, 16 February 2010 (Fig. 2); collected by N. Koutsikos and S. Zogaris and deposited in the Prirodoslovni muzej Rijeka (PMR). **Methods:** meristic methods follow Miller (1988). Meristic abbreviations: A, anal fin; C, caudal fin; D, dorsal fins; P, pectoral fin; V, pelvic disc; LL, scales in lateral series; TR, scales in transverse series. Terminology of lateral-line system follows Sanzo (1911), Bath (1973) and Miller (1986). Presence of head canals and pores was checked on both sides of all specimens. Rows of sensory papillae were checked and counted on the left side of all specimens.

2.2. Extraction and amplification of mitochondrial DNA

Fishes were fixed and preserved in absolute ethanol. DNA of six specimens was extracted with the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) following the manu-



Fig. 2. *Millerigobius macrocephalus*, preserved specimen, male, 31.0 + 8.1 mm, PMR VP2238, Lake Vouliagmeni, 16 February 2010.

facturer's guidelines; DNA was eluted in 100 μ L. Polymerase Chain Reaction (PCR) was carried out using a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Halle, Belgium). The reaction volume contained 1x PCR buffer (Eurogentec, Seraing, Belgium), 200 μ M of dNTPs (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 2 mM of $MgCl_2$ (Eurogentec), 0.5 U of *Taq*-polymerase (Eurogentec), 0.8 μ M of each primer (Eurogentec), 1 μ L of template DNA, topped up with milli-Q water to a total volume of 25 μ L. Primers used for mitochondrial ribosomal DNA were 16SH 5'-CCGGTCTGAACTCAGATCACGT-3', 16SL 5'-CGCCTGTTTATCAAAAACAT-3' (Palumbi et al., 1991), 12SH 5'-TGACTGCAGAGGGTGACGGGGCGGTGTGT-3' and 12SL 5'-AAAAAGCTTCAAAGTGGGATTAGATACCCCACTAT-3' (Kocher et al., 1989). Following Ward et al. (2005), we used the primers FishF1 5'-TCAACCAACCACAAAGACATTGGCAC-3' and FishR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' to amplify a ca. 600 bp part of the mitochondrial cytochrome oxidase subunit I (cox1) gene, for barcoding purposes.

After an initial denaturation of 3 min at 95 °C, samples were subjected to 35 cycles of 30 s at 95 °C, 30 s at 52 °C (16S and cox1) or 64 °C (12S) and 50 s at 72 °C. After a final elongation of 7 min at 72 °C, samples were cooled to 4 °C. PCR products were purified using NucleoFast (Macherey-Nagel) according to the manufacturer's protocol. Sequencing was done using the same primers as above (only the forward primer in the case of cox1), with the Big Dye Terminator 3.1 kit (Applied Biosystems), applying a 1/8 dilution of the Big Dye Terminator sequencing protocol. Products were finally run on an ABI 3130 Genetic Analyser (Applied Biosystems).

2.3. Sequence alignment and phylogenetic analyses

In the six sequenced individuals, three different 16S haplotypes were found, either differing in one A/G substitution or one insertion. Focusing on the closest matches in a nucleotide BLAST (Altschul et al., 1997; Zhang et al., 2000) and a couple of their congeners, 12S and 16S rDNA sequences of other Atlantic or Mediterranean goby species were added from GenBank (Table 1). Sequences were aligned using ClustalX (Thompson et al., 1997; Larkin et al., 2007) using default parameters, and inspected by eye in MEGA v.4 (Tamura et al., 2007).

Following Huysse et al. (2004), the 12S and 16S rDNA fragments were treated as a single dataset. ModelTest v.3.06 (Posada and Crandall, 1998) was used to estimate the optimal model of molecular evolution. The TrN (Tamura and Nei, 1993) + I + Γ model was selected after hierarchical likelihood ratio tests (hLRTs) (proportion of invariable sites: 0.4; gamma shape parameter: 0.6). With the parameters resulting from ModelTest, a neighbour-joining (NJ) distance-based phylogeny reconstruction was applied using PAUP* v.4.01b

(Swofford, 2001) with the PaupUp interface (Calendini and Martin, 2005), assessing nodal support through 1000 bootstrap samples. Pairwise genetic distances were calculated according to the optimized model. Using 1000 replicates of nearest-neighbour interchange branch swapping under a heuristic search, a maximum parsimony (MP) search was carried out in the same program. Gaps were treated as missing data. Instead of the optimized model, a GTR + I + Γ model (as this is the next more complex implemented option) was applied in Bayesian inference (BI) of phylogeny, using MrBayes v.3 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Posterior probabilities were calculated over 1 000 000 generations (after which stationarity of the Markov chain had evidently been reached, indicated by a standard deviation of split frequencies of 0.008, and by a Potential Scale Reduction Factor (Gelman and Rubin, 1992) converging to 1), while sampling the Markov chain at a frequency of 100 generations. We discarded 25% of the samples as "burn-in".

Several Mediterranean gobiid genera, for which 12S rDNA sequences are currently unavailable, have not been included in the above-mentioned phylogeny reconstructions, while some of them deserve special attention regarding the clade under study (see e.g. Miller (1977) for the morphological affinities of *Chromogobius* de Buen, 1930 and *Thorogobius* Miller, 1969). Hence, we performed an additional analysis on a wider taxon list, using partial 16S rDNA sequences only (Table 1). The phylogeny was inferred in an identical way. ModelTest selected the HKY85 (Hasegawa et al., 1985) + I + Γ model with a transition/transversion ratio of 2.2, a proportion of invariable sites of 0.5 and a gamma shape parameter of 0.7. In the Bayesian analysis, an average standard deviation of split frequencies of 0.007 was reached after 2 000 000 generations.

The "sand gobies" *Economidichthys pygmaeus* and *Knipowitschia milleri*, and *Pseudaphya ferreri* (suggested to be nested within *Pomatoschistus* Gill, 1863 by Giovannotti et al. (2007)), were used for outgroup rooting. For the MP analysis, monophyly of the outgroup was enforced. Taxon and author names in this study follow Eschmeyer (2010). Sequences obtained in this study were deposited in GenBank under accession numbers HQ232430–HQ232434.

3. Results

3.1. Morphology

Diagnosis. The following minimum combination of morphological characters positively identifies the investigated specimens as *M. macrocephalus* among European freshwater and marine species of the Gobiidae family: (1) suborbital papillae without longitudinal row a; (2) anterior oculoscapular canal and preopercular canal present, posterior oculoscapular canal absent; (3) V disc complete; (4) interorbital rows present.

Table 1. Published goby sequences used in this study.

Species	GenBank accession number	Reference
12S and 16S rDNA		
<i>Gobius xanthocephalus</i> Heymer and Zander, 1992	AF491117	Rüber et al. (2003)
<i>Microgobius erectus</i> Ginsburg, 1938	AF491110	
<i>Vanneaugobius pruvoti</i> (Fage, 1938)	AF491120	
<i>V. canariensis</i> Van Tassel, Miller and Brito, 1988	AF491119	
<i>G. auratus</i> Risso, 1810	AF067254 and AF067267	Penzo et al. (1998)
<i>G. bucchichi</i> Steindachner, 1870	AF067255 and AF067268	
<i>G. niger</i> Linnaeus, 1758	AF067256 and AF067269	
<i>G. paganellus</i> Linnaeus, 1758	AF067258 and AF067271	
<i>Padogobius bonelli</i> (Bonaparte, 1846) ^a	AF067261 and AF067274	
<i>P. nigricans</i> (Canestrini, 1867)	AF067257 and AF067270	
<i>Zebrus zebrus</i> (Risso, 1827)	AF067266 and AF067279	
<i>Zosterisessor ophiocephalus</i> (Pallas, 1814)	AF067265 and AF067278	
<i>Aphia minuta</i> (Risso, 1810)	EF218623 and EF218638	Giovannotti et al. (2007)
<i>Buenia affinis</i> Iljin, 1930	EF218628 and EF218643	
<i>Crystallogobius linearis</i> (Düben, 1845)	EF218635 and EF218650	
<i>G. cobitis</i> Pallas, 1814	EF218629 and EF218644	
<i>G. cruentatus</i> Gmelin, 1789	EF218626 and EF218641	
<i>Lesueurigobius friesii</i> (Malm, 1874)	EF218624 and EF218639	
<i>L. suerii</i> (Risso, 1810)	EF218625 and EF218640	
<i>Pseudaphya ferreri</i> (de Buen and Fage, 1908)	EF218631 and EF218646	
<i>Economidichthys pygmaeus</i> (Holly, 1929)	GQ398131–2	Larmuseau et al. (2010)
<i>Knipowitschia milleri</i> (Ahnelt and Bianco, 1990)	GQ398133–7	
16S rDNA only		
<i>Chromogobius zebratus</i> (Kolombatović, 1891)	FJ460193	M.B. Yokes (direct submission)
<i>Deltentosteus collonianus</i> (Risso, 1820)	FJ517537	
<i>Oxyurichthys petersii</i> (Klunzinger, 1871)	EU364559	
<i>Thorogobius macrolepis</i> (Kolombatović, 1891)	FJ460194	
<i>Vanderhorstia mertensi</i> Klausewitz, 1974	FJ517536	

^a *P. martensii* is a synonym of this species.

Description. Body short, laterally compressed. Head large, slightly depressed. Eye extending above head profile. Angle of jaws ending about below mideye. Snout short and blunt. Anterior nostril tubular, long, reaching upper lip, without process from rim. Posterior nostril weakly erected. Branchiostegal membrane attached to entire side of isthmus, ending posteriorly below opercular edge.

Fins: D1 VI (in one specimen with deformed D1 just III); D2 I/9–11 (9:1, 10:6, 11:1); A I/9–10 (9:5, 10:3); C 15–17 branched rays (15:3, 16:2, 17:3), 17–18 articulated rays (17:7, 18:1); P 11–16 (11:1, 12:1, 13:3, 14:4, 15:4, 16:3); VI/5 + I/5. D1 rounded, of similar height as D2. C rounded. Three to four P uppermost rays moderately free at tips. V complete and rounded. V anterior transverse membrane narrowed, with straight edge and without lateral lobes. V not reaching anus posteriorly. In a few specimens, some of the fins (D1, D2, A, C, P) were deformed and reduced, influencing the shape and in D1 and P also the count of fin rays.

Body with ctenoid scales, LL 28–31 (both sides; in a male of standard length 32.6 mm, the right side was too damaged for count: 28:1, 29:7, 30:5, 31:2), TR 8–10 (both sides; in a male of standard length 32.6 mm, the right side was too damaged for count: 8:6, 9:7, 10:2). Head, pre-

dorsal area and breast naked. Belly covered with cycloid scales.

Head with anterior and preopercular canals, with pores σ , λ , κ , ω , α , β , ρ and γ , δ , ε , respectively (Fig. 3). However, some aberrations were present: male 41.3 + 10.2 mm had additional small pore near pore λ , female 31.3 + 7.6 mm was without pore δ and female 28.6 + 7.5 mm was without pore δ , pore ε was as open furrow, and there were no pores β and ρ on the right side of this specimen. Rows of sensory papillae (Fig. 3): (I) *preorbital*: snout with four rows in median preorbital series: longitudinal row r (3–5) converging rostrally to his pair. Upper row s^1 (2–4) transversal, below pore σ , lower transversal s^2 (2–3) present rostrally to anterior nostril, and s^3 longitudinal, above upper lip (1–3). Lateral series c in four parts: superior c^2 behind posterior and anterior nostrils as two rows, upper (2–4) and lower (3–4); middle transversal c^1 (2–4) below anterior nostril; inferior upper c_2 (4–6) and lower c_1 (1–5) as two rows between upper lip and row I , vertical to row I . (II) *suborbital*: No suborbital row a . Seven transverse rows of sensory papillae below eye (I : 7–11, 2: 4–9, 3: 5–9, 4: 7–14, 5: 8–22, 6i: 0–10, 6s: 6–12, 7: 1–4). Transverse suborbital rows 2 and 3 more distant from orbit, other suborbital rows (I , 4, 5, 6, 7) begin close to the

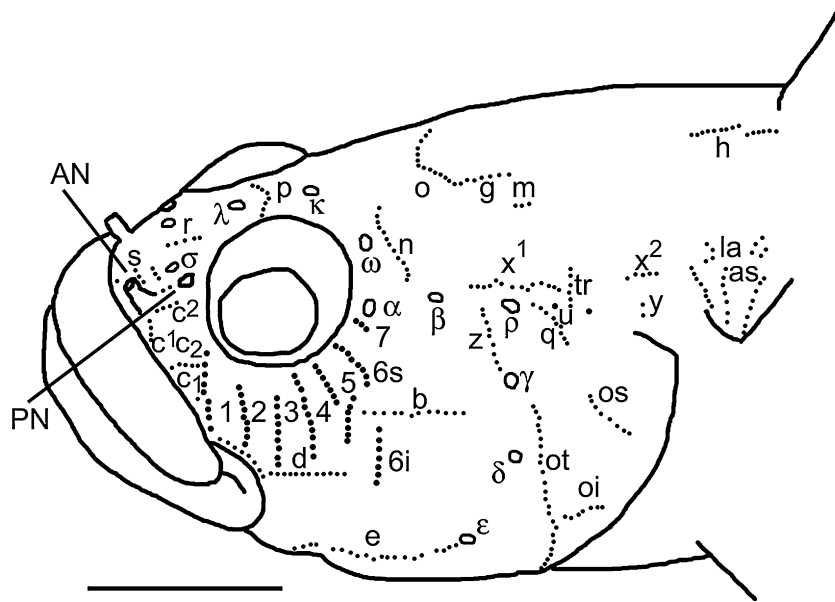


Fig. 3. *Millerigobius macrocephalus*, head lateral-line sensory papillae and canal pores, male, 31.0 + 8.1 mm, PMR VP2238, Lake Vouliagmeni, 16 February 2010. AN, PN, anterior and posterior nostrils; see other terminology in text. Scale = 3 mm.

orbit. Row 7 near pore *a*. Longitudinal row *b* (10–14) mostly continuous, ending anteriorly at row 5 or near it, and dividing row 6 in two parts. In two specimens longitudinal row *b* was not continuous but divided. Row 5 divided in two parts, only in one specimen in three parts. In one specimen the lower part of row 5 was positioned more backwards, below row *b*. Upper part of row 6 was missing in one specimen. Longitudinal row *d* (17–23) continuous. (III) *preoperculo-mandibular*: external row *e* (38–49) divided, with papillae of size as in suborbital rows and parallel internal row *i* (14–21) with larger papillae, also divided, both ending before frontal end of jaw. Mental row *f* longitudinal (4–5) near frontal end of row *e*. (IV) *oculoscapular*: Anterior longitudinal row x^1 (9–14) beginning between pores β and ρ , posterior longitudinal row x^2 (4–6) above transversal row *y* (2–6); transversal row *z* (6–10) with lower end above pore γ ; transversal row *q* (4–9) beginning behind pore ρ ; row *u* as one or two papillae below x^1 between row *q* and transversal row *tr* (2–6); additional single papilla was present in all specimens between rows *tr* and *y*. Transversal axillary rows as^1 (5–8), as^2 (4–8), as^3 (6–8) and longitudinal axillary rows la^1 (3–5) and la^2 (2–6) present. (V) *opercular*: transverse row *ot* (17–28); superior longitudinal row *os* (6–10); and inferior longitudinal row *oi* (5–7). (VI) *anterior dorsal*: transversal row *n* behind pore ω (9–15); transversal rows *o* (5–8) connected at dorsal midline (one specimen with visible gap between rows *o*); row *g* (5–9) ends anteriorly in the level of row *o* or slightly behind, posterior part of row *g* above the beginning of row *m* (2–5); longitudinal row *h* (8–25) present. (VII) *interorbital*: transversal interorbital row *p* (3–4) as two sister rows present between pores λ and κ .

Colour preserved (Fig. 2): Body greyish brown, darkness variable among specimens. Origin of P, isthmus, breast and

belly paler comparing to the rest of the body. Six to eight vertical dark brown bands, in frontal half intensive, backwards brighter and blurred, present along lateral side below dorsal fins. Reticulate pattern, formed by dark markings along the scale margins visible. Some specimens were likely infested with digenean metacercariae on skin and fins, and displayed irregular pigmentation. Head more or less uniformly pigmented. All fins pigmented. D1 with one bright longitudinal band and bright upper edge of fin at rays tips. D2 and C with irregularly scattered bright dots. Dark brown spot present on base of C. A, V and P more or less uniformly pigmented.

3.2. Sequences and phylogenetic analyses

The length of the amplified products was about 451 and 613 bp for 12S and 16S rDNA, respectively. Sequences were trimmed after addition of species retrieved from GenBank leaving a total of 819 bp in the combined dataset. The matrix contained 327 variable sites of which 243 sites were parsimony-informative (this amounts to 197 and 157 characters, respectively, for the 16S dataset).

Comparison with unpublished DNA data from *M. macrocephalus* positively identified our study specimens as this species (L. Rüber and A. Agorreta, in litt. 2010). All three phylogenetic reconstructions (Fig. 4) firmly cluster *M. macrocephalus* together with *Zebus zebus*, the zebra goby. The pairwise gamma-corrected distance between both species is about 13.5% for the combined 12S and 16S regions.

Both species are, with considerable statistical support, consistently placed in a clade including the representatives of the genera *Gobius* Linnaeus, 1758, *Vanneaugobius* Brownell, 1978, *Padogobius* Berg, 1932 and *Zosterisessor* Whitley,

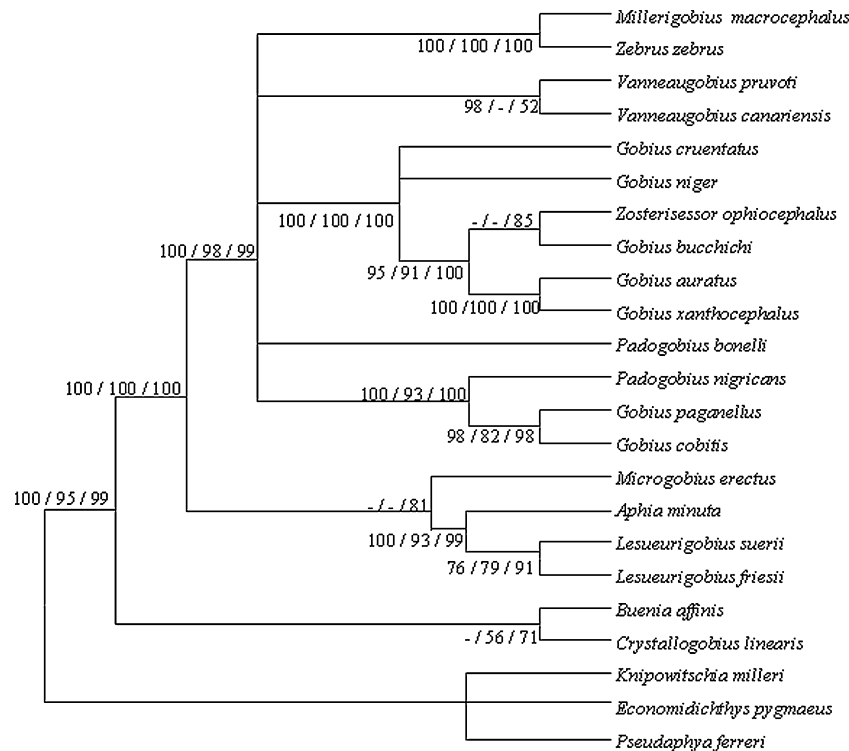


Fig. 4. Cladogram constructed for the 12S and 16S rDNA sequences (819 bp) of the gobies under study. Statistical support for each node is shown as follows: BI posterior probability/MP bootstrap/NJ bootstrap. Clades supported by a bootstrap lower than 50 and with a posterior probability below 75 were collapsed, creating a polytomy. *Microgobius erectus* appeared as a sister taxon to the clade containing *Gobius*, *Padogobius*, *Zosterisessor*, *Vanneaugobius*, *Zebrus* and *Millerigobius* under MP and BI (with a bootstrap support of 70 and a posterior probability of 87, respectively). *Zosterisessor ophiocephalus* is sister to *Gobius auratus* and *G. xanthocephalus* under MP (bootstrap support: 60).

1935 (Fig. 4). Phylogenetic analysis including more goby genera (Fig. 5) did not yield any close affinity of additional taxa to either *Millerigobius* or *Zebrus*.

Applying a nucleotide BLAST to the *cox1* partial sequence of *M. macrocephalus* indicated the American goby species *Barbulifer ceuthoecus* (Jordan and Gilbert, 1884) as the closest match (AF391353, from Thacker, 2003).

4. Discussion

The goby specimens under study were confirmed to belong to *Millerigobius macrocephalus* based on external morphological characters and genetic data of 12S and 16S rDNA. The morphology of Vouliagmeni *M. macrocephalus* matches well with the detailed species redescription (Bath, 1973), as well as with short morphological descriptions of various Mediterranean populations of this species (Miller, 1977; Ramos Espla and Pérez-Ruzafa, 1987; Bouchereau and Tomasini, 1989; Bogorodsky et al., 2010). The observed differences among these records, for instance in fin and scale meristics, are more likely the result of the small sample sizes used in these studies rather than reflecting real differences among various populations. The largest sample (Ramos Espla and Pérez-Ruzafa, 1987) shows the largest range of meristics among

samples. However, the Vouliagmeni population in addition shows deformation and reduction of fins, as well as aberrations of head lateral-line system in some specimens, which was not reported elsewhere for this species. The number of canal pores and the extension of rows of sensory papillae are usually stable characters within species (Miller, 1986). A high intraspecific variability of head lateral-line system characters was noted only in some small Mediterranean brackish and freshwater gobies phylogenetically distant from this species, but occurring in similar habitats as the Vouliagmeni *M. macrocephalus* (Ahnelt et al., 1995; Kovačić, 2005).

The closest representative of *M. macrocephalus*, according to the present genetic data, appears to be the marine goby *Zebrus zebrus*, thus corroborating the view of Miller (1977) who suggested a close affinity between both genera based on morphological data.

In our phylogenetic analysis, both species ended up in a cluster of *Gobius*-related goby species (Miller, 1986), most of them marine. However, some representatives of this clade have developed a freshwater lifestyle, as exemplified by the *Padogobius* species (Penzo et al., 1998; Huyse et al., 2004), while others, like *Zosterisessor ophiocephalus*, show high tolerance for oligohaline conditions (Miller, 1986). Hence, salinity tolerance does not seem to be strictly phylogenetically constrained among Mediterranean gobies to the

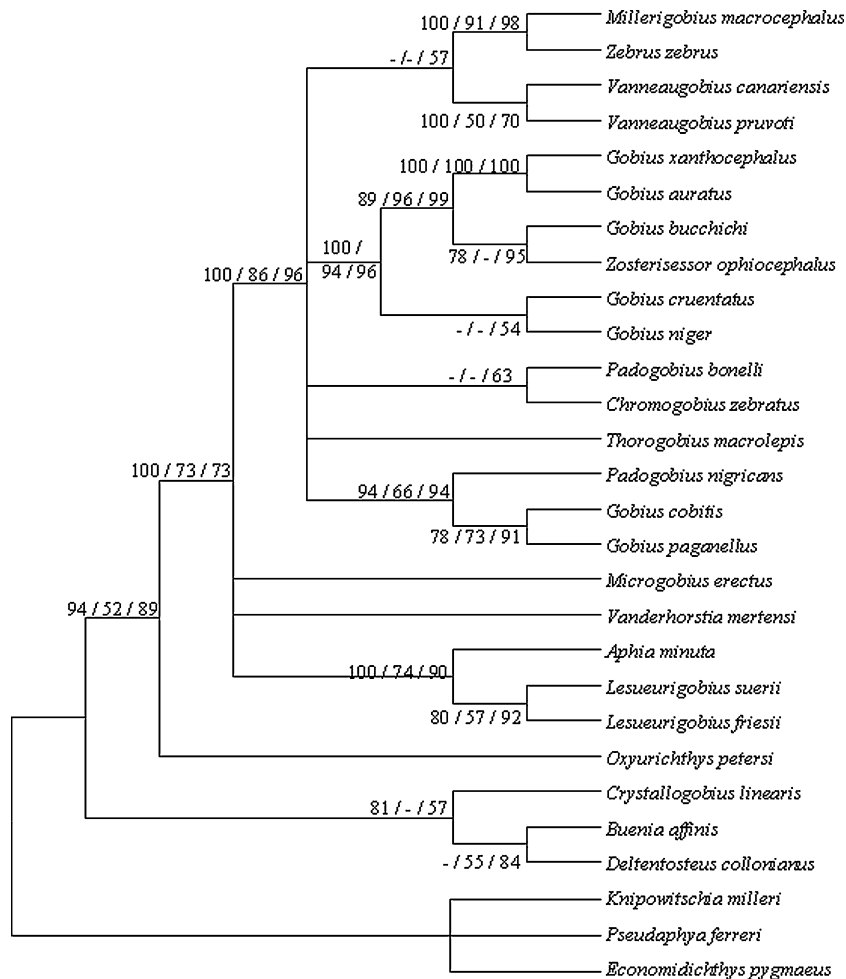


Fig. 5. Cladogram constructed for the 16S rDNA sequences (464 bp) of the gobies under study. Statistical support for each node is shown as follows: BI posterior probability/MP bootstrap/NJ bootstrap. Clades supported by a bootstrap lower than 50 and with a posterior probability below 75 were collapsed, creating a polytomy. *Zosterisessor ophiocephalus* is sister to *Gobius auratus* and *G. xanthocephalus*, and *G. niger* to a clade including the latter three species plus *G. bucchichi*, under MP (each bootstrap support: 57).

brackish and freshwater *Knipowitschia* and *Pomatoschistus* species. Nevertheless, *M. macrocephalus* was thus far only known as a marine goby, even tolerating hypersaline conditions. Though marine gobies have been observed to enter coastal lagoons in Greece (Nicolaidou et al., 2005; Economou et al., 2007), they normally restrict themselves to a narrow strip of the lagoon or river-mouth, influenced by the sea. The role of the level of marine influence in spatial distribution patterns of lagoonal fish communities is well-known (Mariani, 2001). We did not however observe an aggregated dispersion for Vouliagmeni *M. macrocephalus*. Furthermore, a true marine connection, apart from underground through calcareous rock fissures, is missing in this lake (Chintiroglou et al., 2008). Hence, the current *Millerigobius* population seems to be a truly landlocked one, tolerating oligohaline conditions. Apart from an unexpectedly high salinity tolerance range in this species, this observation mostly highlights our lack of knowledge on the biology and biogeography of the Mediterranean goby fauna. Indeed, with new records (e.g.

Van Neer et al., 1999; Stefanni, 2000; Mejri et al., 2009) and even descriptions (e.g. Kovačić and Šanda, 2007; Miller and Šanda, 2008; Miller, 2009) still regularly being published, it is clear that much remains to be discovered on this fish family, even in Europe. As molecular techniques have been shown to be a useful additional tool in goby identification (Larmuseau et al., 2008), efforts to barcode more goby species might perhaps be of assistance here. Only 22% of all goby species worldwide have been barcoded so far (Fish-BOL, 2010), and Europe is lagging even more behind as barcodes are available for no more than 10% of European goby species. Indeed, our barcoding results of *M. macrocephalus* show that data for meaningful comparisons are evidently lacking. The use of nuclear sequences in addition is recommended, as studying biodiversity based on the *cox1* gene only, or limiting molecular studies to mitochondrial sequences, can fail to capture the complexity of certain evolutionary events or even lead to misinterpretations. For example, the “true” species tree could differ from the mitochondrial gene tree due to introgression

(Rubinoff, 2006). Notwithstanding its shortcomings, DNA barcoding remains a widely advocated tool, and molecular work in general can add useful data to taxonomy and conservation research (Rubinoff et al., 2006; Ward et al., 2009).

Coastal wetlands and lagoons in general are sensitive to anthropogenic impact because of their natural environmental instability and their proximity to intensive anthropogenic lowland land-uses (Reizopoulou and Nicolaidou, 2004). In view of the exploitation of its waters by bathers and the urbanization of its wider karstic catchment, Lake Vouliagmeni is indeed vulnerable to environmental degradation (Gontikaki et al., 2003). The identification of a poorly known Mediterranean goby may bring added scientific attention to this unique coastal lotic system, which is of interest to biodiversity conservation. Over most of its range, *M. macrocephalus* is scarce and presumably rather cryptic, yet within this lake it is very common and easily apparent in the shallower waters. It is unknown how and when this species entered and became established in this almost land-locked basin. Unlike the alien *Poecilia latipinna* that maintains large populations in the lake since the mid 1960s, *M. macrocephalus* is not regularly held in captivity by amateur aquarists. Hence, it is unlikely that the species was intentionally introduced to the lake by man. Since no surface connection with the sea currently exists, and the likelihood of subterranean colonization is poorly known, other potential transport vectors should also be considered, e.g. passive dispersal of eggs by water birds (Figuerola and Green, 2002). Further work on evolutionary genetics of this and other gobiid populations may help unravel the history of this unique occurrence of a poorly studied fish.

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