First record of Calma gobioophaga Calado and Urgorri, 2002 (Gastropoda: Nudibranchia) in the Mediterranean Sea

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First record of *Calma gobioophaga* Calado and Urgorri, 2002 (Gastropoda: Nudibranchia) in the Mediterranean Sea

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**Abstract**

Specimens of the nudibranch genus *Calma* were observed under boulders at two Croatian localities while feeding on gobiid eggs. Some ambiguous morphological features compared with the original descriptions of the known species of the genus, *C. glaucoides* and *C. gobioophaga* hampered easy identification. Genetic data (COI and 16S sequences) confirmed the distinction between the two species of the genus *Calma*, and allowed unambiguous identification of the Croatian specimens as *Calma gobioophaga*. This is the first record of this species for the Mediterranean and extends its distribution range remarkably. Finally, the eggs consumed by the Croatian *C. gobioophaga* specimens have been taxonomically identified by using the 12S rDNA marker as *Gobius cobitis*.

**Keywords:** DNA-Barcoding, Mollusca, nudibranchs, Aeolidida, *Calma*, new records, Mediterranean.

The genus *Calma* was introduced by Alder & Hancock (1855) to include the single species *Eolis glaucoides* Alder & Hancock, 1854, from the north-eastern Atlantic and the Mediterranean. The genus was subsequently included in the monotypic family Calmidae Iredale & O’Donoghue, 1923. Recently, a second species was described from Portugal and NW Spain, *Calma gobioophaga* Calado & Urgorri, 2002, based on morphological and ecological features such as the diameter and position of the eyes, the size of the propodial tentacles, the size of the metanefridium, body length and diet preference. *C. glaucoides* feeds on eggs of *Lepadogaster lepadogaster*, *L. purpurea*, *L. candollei*, *Parablennius gattorugine*, and *P. pilicornis*, while *C. gobioophaga* has been reported so far on spawn of *Gobius niger* only (Calado & Urgorri, 2001, 2002).

The geographical distribution of *C. glaucoides* ranges in the Atlantic from Norway, southern Great Britain and France, to Portugal and NW Spain. In the Mediterranean, it has been reported from the Gulf of Naples and the Mediterranean coastline of France. Cesari (1994) reported it from the Venice lagoon (Adriatic Sea, Italy) but it is unclear from his description which species was involved. The known distribution of *C. gobioophaga* was so far limited to the Atlantic Ocean, from Great Britain and France, to NW Spain and Portugal (Calado & Urgorri, 2002; Urgorri et al., 2011). Some specimens from the Atlantic, originally identified as *C. glaucoides* (Hecht, 1896; Farran, 1903; Thompson & Brown, 1984), seem to be *C. gobioophaga* after checking the relevant figures and description. However, some unchecked specimens from Cabo de Palos (Mediterranean, Spain) reported by Templado et al. (1987), which were collected on gobiid eggs, could represent a Mediterranean record. The known records are summarized in Figure 1 (see also Suppl. Table 1 only on electronic publication).

Recently, specimens of *Calma* sp. (c. 160 in total) were observed *in situ* at two different Croatian localities (Marina, 43°30’36” N, 016°07’42” E, 30 April 2012; Split, 43°30’50” N, 016°24’02” E, 14 April 2013), when feeding on spawn of *Gobius* sp. and laying their eggs under boulders at a depth of 0.1-0.5 m (Fig. 2). Samples were collected by hand. The specimens are held at the Department of Biology and Biotechnologies, “La Sapienza” University (BBCD, Rome, Italy), Museo Nacional de Ciencias Naturales (MNCN, Madrid, Spain) and California State...
Animals were found in remarkable aggregations (40-70 specimens under each boulder) at both localities. A similar concentration of calmids on a batch of fish eggs was already reported by Thompson & Brown (1984) in Great Britain. The specimens were quite abundant during April-May 2012 and their occurrence was confirmed in April 2013. The Croatian calmids had an average body length of 15 mm (max 20 mm); body colour: whitish or yellowish on the notum and a pale orange tinge in the head area and lower part of the rhinophores; the cerata set in up to 11 pairs of lateral groups, varying in number from one to four per group (generally 2-3), and very dark in colour (mostly dark brown or blackish); the gonadal units, easily visible dorsally and ventrally, vary in shape and position; the eyes are generally situated below the base of the rhinophores (at their lateral or rear border), sometimes just behind the rhinophores, rarely considerably behind them; a metanefridium variable in shape and length (starting from the second ceratal group and ending mostly at 9-10th, sometimes at 4-6th), and dark ramifications of the digestive gland (Fig. 3).

The diagnostic differences between the two known species of *Calma* reported by Calado & Urgorri (2002), based on Atlantic specimens, were the position and diameter of the eyes, the shape and length of the metanephridium, the diet, maximum body length and the shape and size of propodial tentacles. Based on the frequently long metanephridium and the diet consisting of *Gobius* sp. eggs, the Croatian specimens were more similar to...
Fig. 2: In situ pictures of *Calma gobioophaga* specimens from Croatia removed from water. (A – C) Specimens of *Calma gobioophaga* from Marina, 30 April 2012, on their egg-masses (A – B) and on eggs of *Gobius cobitis* (C); smallest specimens ca. 10 mm, largest specimens ca. 20 mm. (D – E) Eggs of *G. cobitis*, from Split, 10 May 2012.

Table 1. Genetic distances (COI in bold, and 16S) observed (p-distance, above diagonal) and estimated using the Kimura 2 parameter substitution model (below diagonal). Interspecific comparisons in shaded area.

<table>
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<th>Cc</th>
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Fig. 3: Specimens of *Calma gobioophaga* from Croatia. (A – B) Split, 14 April 2013, length: 16.5 mm (A), 3 mm (B); (C – G) Marina, 30 April 2012, length: 20.5 mm (C), 15.3 mm (D), 14.8 mm (E), 15.3 mm (F) 11 mm (G). Specimens not to scale. The white ribbon on the tail of specimen G is a part of spawn, laid in captivity.
C. gobioophaga. Nevertheless, the Croatian individuals of Calma displayed some apparently misleading morphological features, sometimes intermediate between Calma glaucoides and C. gobioophaga. Calado & Urgorri (2002) reported 10 mm as maximum length of Atlantic C. gobioophaga, while the Croatian specimens reached up to double the size (20 mm); the smallest collected juvenile Croatian specimen (c. 3 mm long: Fig. 3B) showed only 4 ceratal groups, with two cerata in each group, while in even smaller Atlantic specimens (about 2 mm long) 6 ceratal groups were shown, each one consisting of a single ceratum (Calado & Urgorri, 2002). Since the morphological examination was not conclusive, we tested their identity by using genetic markers (mitochondrial 16S rDNA and COI partial sequences). We also attempted to identify the fish eggs by using the 12S rDNA partial sequence since this marker has one of the largest taxonomic coverage for gobiiids, especially for the Mediterranean region.

Two specimens of C. glaucoides from Portugal (Arrábida Coast, Cozhadourou: 38°26’48” N; 09°02’24” W), two specimens of C. gobioophaga from its type locality in Portugal (Arrábida coast, Artflos: 38°30’24” N; 08°55’09” W) and two specimens of Calma sp. from Croatia (Marina) were used for the molecular phylogenetic analyses. Additional sequences of one C. glaucoides from Portugal and Calmella cavolini from Italy (as outgroup) were obtained from Genbank (see Supplementary Table 2 for full list of samples, localities, and voucher references). A piece of tissue was dissected from the foot for DNA extraction. Total genomic DNA was extracted using a standard proteinase K phenol/chloroform method with ethanol precipitation, as reported in Oliverio & Mariotti (2001) for all samples except two specimens of C. gobioophaga (MNCN-408, MNCN-409) for which the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA, 09/2001) was used.

Partial sequences of 16S and COI were amplified by polymerase chain reaction (PCR) using the primers: 16Sar-L and 16Sbr-H (Palumbi et al., 1991) for 16S rDNA, and LCO1490 and HCO2198 (Folmer et al., 1994) for COI (PCR profile: 5 min denaturation step at 94°C; 35 cycles of 94°C/30 s, 60°C/60 s, 72°C/60 s; 7 min final extension at 72°C). The gobidi eggs consumed by the slugs (Fig. 2D, E) were similarly processed for DNA extraction. A partial sequence of the mitochondrial 12S rDNA was amplified using the newly designed specific primers Gob12S+- 5’-CCCTAGAAAGCTTCATGGACA-3’ and Gob12S- 5’-CAAGTCTTGGTTTAAAGC-3’ (PCR profile: 5 min denaturation step at 95°C; 30 cycles of 95°C/60 s, 60°C/45 s, 72°C/75 s; 7 min final extension at 72°C). All amplicons were sequenced by the Genechron Centre of Sequencing, ENEA (La Casaccia, Rome, Italy) or the European Division of Macrogen Inc. (Amsterdam, The Netherlands), using the same PCR primers.

Phylogenetic relationships among the Calma sequenc-
tension (over 2,000 km in a straight line; over 3,000 km on a sea pathway). Therefore, C. gobioophaga is to be added to the Mediterranean fauna. Remarkably, when the morphological variation over a large geographic scale (including the Croatian population) is taken into consideration, some characters do not prove diagnostic (both species of the genus can attain over 20 mm in length, and have an equally variable metanephridium). As far as ecology is concerned, while C. gobioophaga was reported in the Atlantic as feeding on eggs of Gobius niger only, in Croatia we found it feeding on spawn of G. cobitis, a rocky intertidal goby with a large distribution (from the western English Channel to Morocco, and the Mediterranean Sea). Additionally, a single specimen of Calma gobioophaga was observed at Biograd, Croatia (16 November 2013) in 2 m depth, on eggs of a different gobid species, probably Gobius bucchichi Steindachner, 1870 (Alen Petani, pers. comm.). In Croatia, eggs of Parablennius and Lepadogaster laid under rocks were regularly observed, but never associated with Calma (C. glaucoides has never been found in Croatia, despite extensive fieldwork during the last 10 years by the first author). This would support the hypothesis that the diet of C. gobioophaga includes only gobiid eggs. It is worthy mentioning that in situ we observed C. gobioophaga individuals feeding on G. cobitis spawn at different stages of development, deposited under the same stones. Despite the different yolk content, which in turn confers a different colour to the developing egg masses, the chromatic pattern of the Croatian C. gobioophaga remained constant, with dark cerata and whitish-yellow body.

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