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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. glaucooides* 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*.

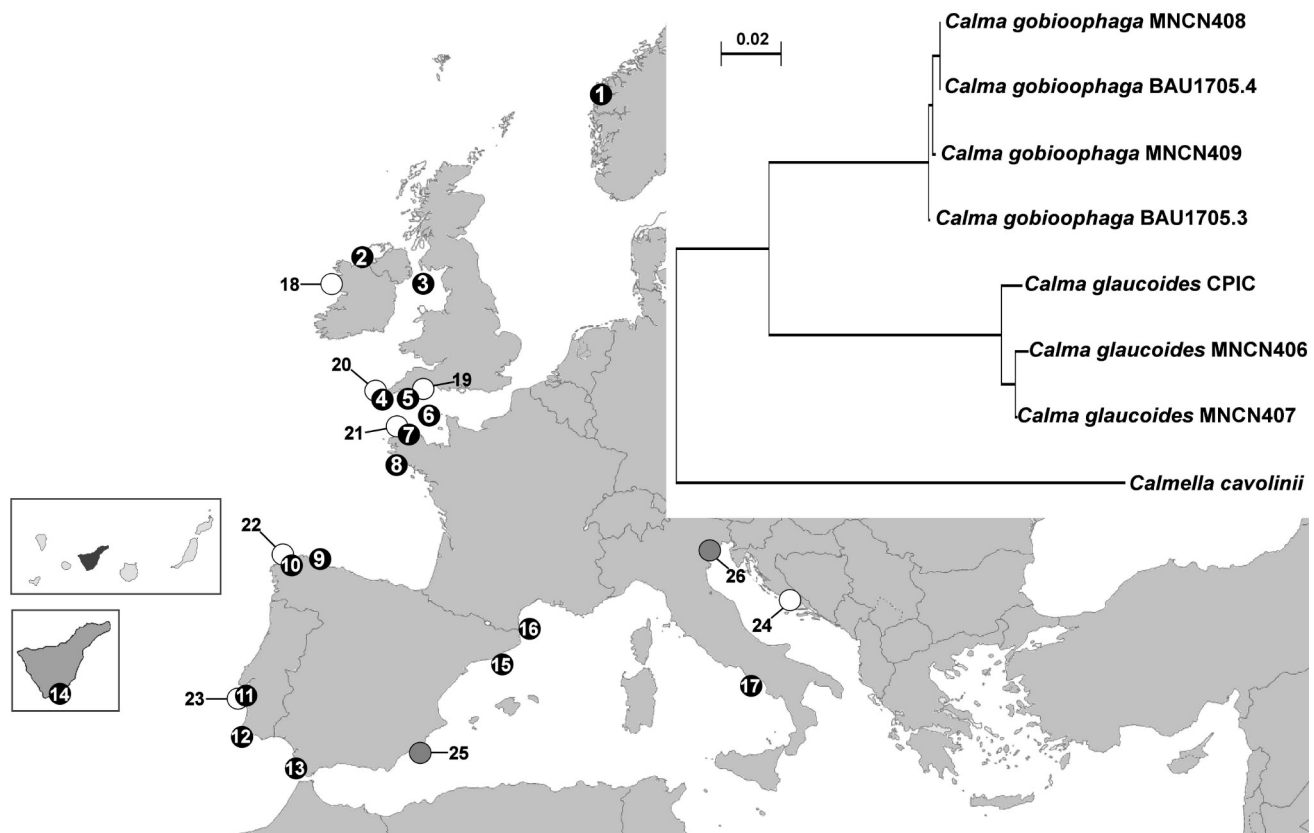
**Keywords:** DNA-Barcoding, Mollusca, nudibranchs, Aeolidida, *Calma*, new records, Mediterranean.

The genus *Calma* was introduced by Alder & Hancock (1855) to include the single species *Eolis glaucooides* 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. glaucooides* 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. glaucooides* 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. glaucooides* (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



**Fig. 1:** Map of the known records of *Calma* spp. (see Suppl. Table 1 for references of records, only on electronic publication). Black circles are checked *Calma glaucooides* records: 1) Florø (Norway); 2) Teelin, Co. Donegal (Eire); 3) Isle of Man (Irish Sea); 4) Penzance, Cornwall (UK); 5) Plymouth (Great Britain); 6) Herm Island, Guernsey (UK); 7) Roscoff (France); 8) Concarneau (France); 9) Burela, Galicia (Spain); 10) Ría de Ferrol, Galicia (Spain); 11) Arrábida coast (Portugal); 12) Sagres (Portugal); 13) Cabo Trafalgar, Andalusia (Spain); 14) El Médano, Tenerife, Canary Islands (Spain); 15) Mediterranean, Catalonia (Spain); 16) Baie de Peyrefite, Banyuls-sur-mer (France); 17) Gulf of Naples (Italy). White circles are checked *C. gobiophaga* records: 18) Co. Galway (Eire); 19) Hope Cove, S. Devon (UK); 20) Penzance, Cornwall (UK); 21) Roscoff (France); 22) A Cabana; Cambados; Leuseda - Galicia (Spain); 23) Arrábida coast (Portugal); 24) Split and Marina (Croatia). Grey circles are records of *Calma* sp. that could not be checked at the species level: 25) Cabo de Palos, Andalusia (Spain); 26) Venice lagoon (Italy). The inlets are the Canary Islands and the particular of Tenerife Is. The tree portrays the phylogenetic relationships among the assayed specimens, based on the combined (COI+16S) dataset, after Bayesian analysis ( $5 \times 10^6$  generations, and 25% burnin). The two main clades had 99-100% support in all recovered trees (NJ, ML and BI trees, on 16S, COI or combined COI+16S datasets); all other nodes had 85%-96% supports.

Polytechnic University Invertebrate Collection (CPIC, Pomona, USA) (Suppl. Table 2 only on electronic publication).

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 9-10<sup>th</sup>, sometimes at 4-6<sup>th</sup>), and dark ramifications of the digestive gland (Fig. 3).

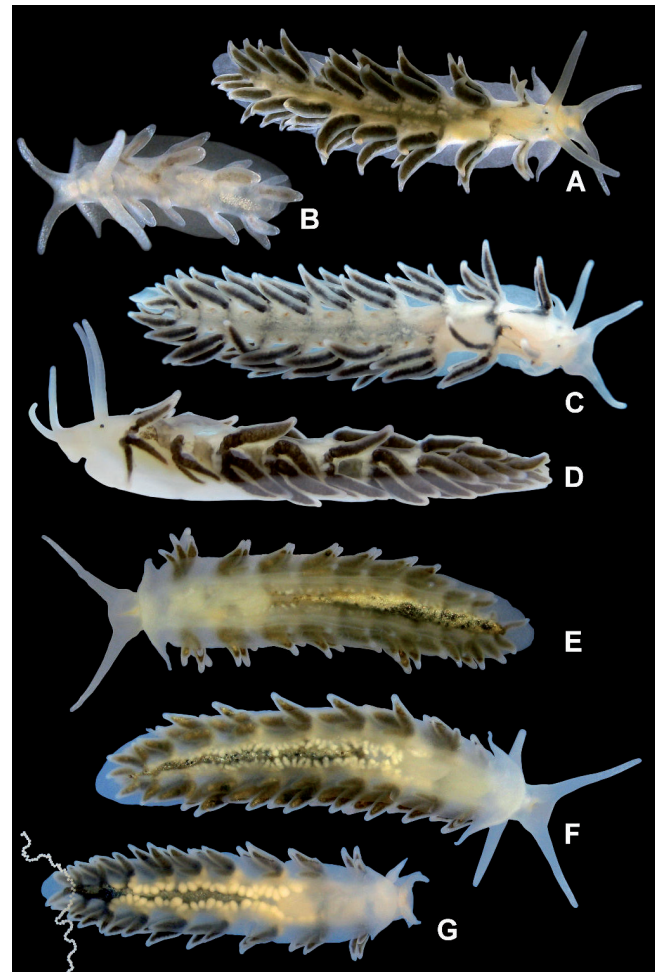
The diagnostic differences between the two known species of *Calma* reported by Calado & Urgorri (2002), based on Atlantic specimens, were the position and diameter of the eyes, the shape and length of the metanefridium, the diet, maximum body length and the shape and size of propodial tentacles. Based on the frequently long metanefridium and the diet consisting of *Gobius* sp. eggs, the Croatian specimens were more similar to

**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.

	Cc	CPIC	407	406	409	408	1705.3	1705.4
<i>Calmella cavolini</i> [Cc]	-	<b>0.214</b>	<b>0.213</b>	<b>0.217</b>	<b>0.194</b>	<b>0.196</b>	<b>0.193</b>	<b>0.196</b>
		0.126	0.126	0.128	0.139	0.139	0.139	0.139
<i>Calma glaucoides</i> [CPIC]	<b>0.254</b>	-	<b>0.014</b>	<b>0.012</b>	<b>0.125</b>	<b>0.128</b>	<b>0.125</b>	<b>0.128</b>
	0.139		0.002	0.002	0.025	0.025	0.023	0.023
<i>Calma glaucoides</i> [MNCN407]	<b>0.252</b>	<b>0.014</b>	-	<b>0.005</b>	<b>0.124</b>	<b>0.127</b>	<b>0.124</b>	<b>0.127</b>
	0.139	0.002		0.004	0.024	0.024	0.022	0.022
<i>Calma glaucoides</i> [MNCN406]	<b>0.259</b>	<b>0.012</b>	<b>0.005</b>	-	<b>0.127</b>	<b>0.130</b>	<b>0.127</b>	<b>0.130</b>
	0.142	0.002	0.004		0.028	0.028	0.026	0.026
<i>Calma gobioophaga</i> [MNCN409]	<b>0.226</b>	<b>0.140</b>	<b>0.138</b>	<b>0.142</b>	-	<b>0.003</b>	<b>0.003</b>	<b>0.003</b>
	0.156	0.026	0.024	0.029		0.000	0.002	0.002
<i>Calma gobioophaga</i> [MNCN408]	<b>0.228</b>	<b>0.144</b>	<b>0.142</b>	<b>0.146</b>	<b>0.003</b>	-	<b>0.003</b>	<b>0.000</b>
	0.156	0.026	0.024	0.029	0.000		0.002	0.002
<i>Calma gobioophaga</i> [BAU01705.3]	<b>0.224</b>	<b>0.140</b>	<b>0.138</b>	<b>0.142</b>	<b>0.003</b>	<b>0.003</b>	-	<b>0.003</b>
	0.156	0.024	0.022	0.026	0.002	0.002		0.000
<i>Calma gobioophaga</i> [BAU01705.4]	<b>0.228</b>	<b>0.144</b>	<b>0.142</b>	<b>0.146</b>	<b>0.003</b>	<b>0.000</b>	<b>0.003</b>	-
	0.156	0.024	0.022	0.026	0.002	0.002	0.000	



**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.



**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 *C. glaucooides* 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 gobiids, especially for the Mediterranean region.

Two specimens of *C. glaucooides* from Portugal (Arrábida Coast, Cozinhadouro: 38°26'48" N; 09°02'24" W), two specimens of *C. gobioophaga* from its type locality in Portugal (Arrábida coast, Arflor: 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. glaucooides* from Portugal and *Calmella cavolinii* 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 & Mariottini (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 rRNA, 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 gobiid 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'-CAAGTCCTTTGGGTTTTAAGC-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-

es were inferred by neighbour-joining (NJ) and maximum likelihood (ML) (both bootstrapped over 1000 replicates), using the MEGA 5.0 software package (Tamura *et al.*, 2011), and Bayesian Inference (BI) (with  $5 \times 10^6$  generations, and 25% burnin) by MrBayes 3.2.2 (Ronquist *et al.*, 2011) on single gene and combined datasets.

To define species, we used the criteria of divergence and reciprocal monophyly supported by independent genetic markers (Knowlton, 2000; Wheeler & Meier, 2000; Reid *et al.*, 2006; Malaquias & Reid, 2009). Since different groups of organisms may present distinct rates of evolution, the use of genetic threshold is difficult to apply (Hebert *et al.*, 2003; Williams *et al.*, 2003). However, Carmona *et al.* (2013), based on an integrative taxonomic revision of the nudibranch family Aeolidiidae, established a threshold of 5.5-16% between sister species (uncorrected *p*-distance for COI gene). We used these values as reference thresholds. The uncorrected *p* and the Kimura-2-parameters (*K2p*) genetic divergence among the COI sequences were computed using the MEGA 5.0 software package.

All trees recovered from the phylogenetic analyses showed the same topology. The tree of Figure 1 shows the resulting phylogenetic hypothesis based on the Bayesian analysis of the combined dataset. The specimens of *Calma* included in this study were split in two monophyletic groups with high support (99-100%). The specimens from Croatia were nested with the specimens of *C. gobioophaga* (minimum *p* and *K2p* distance 0.3%). The values of genetic divergence are reported in Table 1. The outgroup-ingroup *p*-distance for the COI sequences was 19.3-21.7% (*K2p* 22.4-25.9%); the *p*-distance between the COI sequences of the two *Calma* clades ranged from 12.4% to 13% (*K2p* 13.8-14.6%); the intraspecific COI *p*-distance was 0.5-1.4% in *C. glaucooides*, and 0-0.3% in *C. gobioophaga*. Thus, the molecular data confirmed the existence of two reciprocally monophyletic *Calma* clades. Specimens of the two clades from the same area (Arrabida coast, Portugal), showed very high genetic divergence, whilst specimens of *C. gobioophaga* from the type locality and those from Croatia showed a barely detectable genetic divergence. Therefore, on the one side we confirm on the basis of genetic data that *C. glaucooides* and *C. gobioophaga* are actually distinct species; on the other side, despite some morphological uncertainty, the genetic data allowed unambiguous identification of the Croatian specimens as *Calma gobioophaga*.

We also molecularly identified the fish eggs using 12S rDNA as genetic marker. The BLAST (Altschul *et al.*, 1990) search conducted for the 12S partial sequence from the gobiid egg sample, obtained the best score (99% homology) with the sequence EF218629 (Giovannotti *et al.*, 2007), from a specimen of *Gobius cobitis* from Ancona (Adriatic Sea, Italy).

This is the first confirmed Mediterranean record for this species and represents a very remarkable range ex-

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 gobiid species, probably *Gobius bucchichii* 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. glaucooides* 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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