Molecular phylogenies confirm the presence of two cryptic *Hemimycale* species in the Mediterranean and reveal the polyphyly of the genera *Crella* and *Hemimycale* (Demospongiae: Poecilosclerida)

Maria J Uriz Corresp. 1, Leire Garate 1, Gemma Agell 1

1 Department of Marine Ecology, Centre for Advanced Studies of Blanes (CEAB-CSIC), Blanes, Girona, Spain

**Background.** Sponges are particularly prone to hide cryptic species as their paradigmatic plasticity often favors species phenotypic convergence as a result of adaptation to similar habitat conditions. *Hemimycale* is a sponge genus (F. Hymedesmiidae, O. Poecilosclerida) with four formally described species, from which only *H. columella* had been recorded in the Atlanto-Mediterranean basin, on shallow to 80 m deep bottoms. Contrasting biological features between shallow and deep individuals of *H. columella* suggested larger genetic differences than those expected between sponge populations. To assess whether shallow and deep populations belonged indeed into different species, we performed a phylogenetic study of *H. columella* across the Mediterranean. We also included other *Hemimycale* and *Crella* species from the Red Sea, with the additional aim of clarifying the relationships of the genus *Hemimycale*. **Methods.** *Hemimycale columella* was sampled across the Mediterranean, and Adriatic Seas. *H. arabica* and *Crella cyathophora* were collected from the Red Sea and Pacific. From two to three specimens per species and locality were extracted, amplified for COI (M1-M6 partition), 18S rRNA, and 28S (D3-D5 partition) and sequenced. Sequences were aligned using Clustal W v.1.81. Phylogenetic trees were constructed under Neighbour Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) criteria as implemented in Geneious software 9.01. Moreover, spicules of the target species were observed through Scanning Electron microscope. **Results.** The several phylogenetic reconstructions retrieved both *Crella* and *Hemimycale* polyphyletic. Strong differences in COI sequences indicated that *Crella cyathophora* from the Red Sea might belong in a different genus, closer to *Hemimycale arabica* than to the Atlanto-Mediterranean *Crella* spp. Molecular and external morphological differences between *H. arabica* and the Atlanto-Mediterranean *Hemimycale* also suggest that *H. arabica* fit in a separate genus. On the other hand, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale.*
Moreover, what was known up to now as *H. columella*, is formed by two cryptic species with contrasting bathymetric distributions. Some small but consistent morphological differences allow species distinction. **Conclusions.** A new family (Hemimycalidae) including the genus *Hemimycale* and the two purported new genera receiving *Crella cyathophora* and *H. arabica*, might be proposed according our phylogenetic results. However, the inclusion of additional OTU’s appears convenient before taking definite taxonomical decisions. A new cryptic species (*Hemimycale mediterranea* sp. nov.) is described. Morphologically undifferentiated species with contrasting biological traits, as those here reported, confirm that unidentified cryptic species may confound ecological studies.
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Maria J. Uriz, Leire Garate, Gemma Agell

Centre d’Estudis Avançats de Blanes CEAB-CSIC
Access Cala St Francesc, 14. 17300 Blanes Girona

**Running title:** Cryptic Mediterranean *Hemimycale* spp.

**Abstract**

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**Methods.** *Hemimycale columella* was sampled across the Mediterranean, and Adriatic Seas. *H. arabica* and *Crella cyathophora* were collected from the Red Sea and Pacific. DNA was extracted from two to three representative specimens per species and locality and amplified for COI (M1-M6 partition), 18S rRNA, and 28S rRNA (D3-D5 partition) and sequenced. Sequences were aligned using Clustal W v.1.81. Phylogenetic trees were constructed under Neighbour Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) criteria as implemented in Geneious software 9.01. Moreover, spicules of the target species were observed through Scanning Electron microscopy.
Results. The several phylogenetic reconstructions retrieved both *Crella* and *Hemimycale* polyphyletic. Strong differences in COI sequences indicated that *Crella cyathophora* from the Red Sea might belong in a different genus, closer to *Hemimycale arabica* than to the Atlanto-Mediterranean *Crella* spp. Molecular and external morphological differences between *H. arabica* and the Atlanto-Mediterranean *Hemimycale* spp. also suggest that *H. arabica* fit in a separate genus. On the other hand, the Atlanto-Mediterranean *Crellicidae* appeared in 18S and 28S phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*. Moreover, what was known up to now as *H. columella*, is formed by two cryptic species with contrasting bathymetric distributions. Some small but consistent morphological differences allow species distinction.

Conclusions. A new family (Hemimycalidae) including the genus *Hemimycale* and the two purported new genera receiving *Crella cyathophora* and *H. arabica*, might be proposed according to our phylogenetic results. However, the inclusion of additional OTU’s appears convenient before taking definite taxonomical decisions. A new cryptic species (*Hemimycale mediterranea* sp. nov.) is described. Morphologically undifferentiated species with contrasting biological traits, as those here reported, confirm that unidentified cryptic species may confound ecological studies.

Introduction

The discovery of cryptic species is continuously improving our knowledge on real ecosystem biodiversity and functioning, which are intimated related (Frainer, McKie & Malmqvist, 2014). Unrecognized cryptic diversity may mask biological features such as divergent reproduction patterns, growth dynamics, and inter-species interactions, among others (Knowlton, 1993; Prada et al., 2014; de Meester et al., 2016; Loreau, 2004), which may confound conservation studies (Forsman et al., 2010) and obscure the introduction pathway of invasive species (Knapp et al., 2015).

Molecular tools help to confirm suspected hidden species. However, molecular based identifications alone do not solve the problem of species misidentification, in particular when the cryptic species have overlapping distributions (e.g. Knowlton & Jackson, 1994; Tarjuelo et al., 2001; De Caralt et al., 2002; Blanquer & Uriz, 2007, 2008; Pérez-Portela et al., 2007). In these cases, deep studies on their morphology, biology (e.g. life-history traits), and ecology (e.g.
growth dynamics) become crucial to understand the mechanisms underlying their coexistence (López-Legentil et al., 2005; Pérez-Portela et al., 2007; Blanquer, Uriz & Agell, 2008; Payo et al., 2013).

Sponges are sessile, aquatic filter-feeders that are widespread across oceans, depths, and ecosystems (Van Soest et al., 2012), with so far 8,789 accepted species inventoried in 2016 (Van Soest et al., 2016) and ca. 29,000 predicted to be discovered in the forthcoming years (Hooper & Lévi, 1994; Appeltans et al., 2012), many of which remain currently hidden among supposed widespread morpho-species (Uriz & Turon, 2012).

The poor dispersal capacities of sponges prevent in most cases gene flow among populations even at short geographical distances (Boury-Esnault et al. 1993; Uriz et al., 1998; Nichols & Barners 2005; Marinai et al. 2006; Uriz, Turon & Mariani 2008). Consequently, sponge populations become genetically structured (Boury-Esnault et al. 1993; Duran, Pascual & Turon, 2004; Blanquer, Uriz & Caujapé-Castells, 2009; Guardiola, Frotscher & Uriz, 2012, 2016), which favors speciation, while the sponge plasticity fosters phenotypic (morphological) convergence to similar habitats (Blanquer & Uriz, 2008).

Many cryptic, new sponge species have been discovered in the last decades thanks to the use of molecular markers (see Uriz & Turon, 2012 for a review until 2012, Knapp et al., 2015; de Paula et al., 2012). However, less often, molecularly discovered new species have also been described morphologically (but see Blanquer & Uriz, 2008; Cárdenas & Rapp, 2012; Reveillaud et al., 2011, 2012), which is necessary if phylogeny is aimed to translate into taxonomy, and the new species are wanted to be considered in ecological studies.

Sponge species can be both morphologically (e.g. Uriz & Turon, 2012) and, more rarely, molecularly (with the markers used) cryptic (Carella et al., 2016; Vargas et al., 2016) but show contrasting biological features. For instance, *Scopalina blanensis* (Blanquer & Uriz, 2008), which is sympatric with *Scopalina lophyropoda*, mainly grows in winter. Conversely, *S. lophyropoda* regresses in winter and grows principally in summer-autumn (Blanquer, Uriz & Agell, 2008), thus indicating temporal niche partition.

The Order Poecilosclerida (Porifera: Demospongiae) harbors the highest number of species within the Class Demospongiae (Systema Porifera) and it is far from being resolved from a phylogenetic point of view (Morrow et al., 2012; Thacker et al., 2013). Within Poecilosclerida, the Family Hymedesmiidae represents a hotchpotch where genera of dubious adscription have
been placed (Van Soest, 2002). As expected, this family appeared clearly polyphyletic in a molecular phylogeny of the so-called G4 clade based on 28S rRNA gene (Morrow et al., 2012).

Hymedesmiidae currently contains ten accepted genera among which, *Hemimycale* Burton, 1934 (Van Soest et al. 2016). The position of genus *Hemimycale*, which shares with *Hymedesmia*, and *Phorbas* (Hymedesmiidae) and with *Crella* (Crellidae) the so-called aerolate areas with an inhaling function, has changed from Hymeniacidonidae in Halichondrida (Lévi, 1973) to Hymedesmiidae in Poecilosclerida (Van Soest, 2002). More recently, in 18S phylogenies of Poecilosclerida, *Hemimycale columella* was retrieved within the Crellidae clade, although with low support (Redmond et al., 2013).

*Hemimycale* harbors only four formally described species (Van Soest et al. 2016): the type species *Hemimycale columella* (Bowerbank, 1874), from Northwestern Atlantic and Mediterranean, *Hemimycale rhodus* (Hentchel, 1929) from the North Sea, *Hemimycale arabica* Illan et al., 2004 from the Red Sea and *Hemimycale insularis* Moraes, 2011 from Brazil. However, the simple spicule complement of the genus, which only consists of strongyles with some occasional styles, may propitiate the existence of morphologically (based on the spicules) cryptic species.

*H. columella*, the type species of *Hemimycale*, is widely distributed across the Atlanto-Mediterranean basin, from shallow (ca. 10 m) to deep (ca. 60 m) waters (Uriz, Rossell & Martin 1992). Assays performed with eight microsatellite loci developed from deep specimens of *H. columella* (González-Ramos, Agell & Uriz, 2015) failed to amplify a high percentage of the assayed individuals from a shallow population, which suggested larger genetic differences than those expected between intra-species sponge populations.

Furthermore, the species life cycle has been monitored in a shallow Northwestern Mediterranean population of what was though to be *H. columella* (Pérez-Porro, González & Uriz, 2012), where all individuals disappeared after larval release in early November and new individuals arose the forthcoming year but on different rocky sites, which pointed to annual mortality and subsequent recruitment from sexually produced propagula (settling larvae). Conversely, during a study of deeper populations of *H. columella* (González-Ramos, Agell & Uriz, 2015), we recorded their survival for more than three years. Thus, shallow and deep populations of *H. columella* seemed to show contrasting life spans, which were thought to be a result of contrasting habitat characteristics. However, a two-year monitoring of two, some km
apart, populations (one deep and one shallow) and the main environmental factors at both
locations, confirmed their contrasting life span and growth traits, as well as proved no correlation
between biological features and environmental factors (authors unpublished data), which rather
pointed to population intrinsic (genetic) differences.

To assess whether these two population types with contrasting biological traits but
without clearly distinct morphological characters belonged or not to different species, we
performed a phylogenetic study of individuals considered as *H. columella* across the
Mediterranean, using three molecular (nuclear and mitochondrial) gene partitions. We
incorporated additional species to the analyses to gain knowledge on the relationships between
*Hemimycale* species and other genera of families Hymedesmiidae and Crellidae.

**Material and Methods**

**Sampling**

Fragments of what *a priori* was thought to be *Hemimycale columella* were collected by SCUBA
diving across the Northwestern, central and eastern Mediterranean, and Adriatic Sea, between 12
and 45 m of depth during several campaigns (Coconet, Benthomics, and MarSymbiOomics
projects) (Table 1). Moreover, fragments of *Hemimycale arabica* and *Crella cyathophora* from
the Red Sea (Dedalos and Ephistone) and Pacific (Bempton Islands) between 5 and 20 m depth
were also collected (Table 1). Individuals were photographed underwater before sampling.
Collected fragments were divided into two pieces, one of them was preserved in 100% ethanol
and, after three alcohol changes, kept at −20°C until DNA extraction; the other fragment was
fixed in 5% formalin in seawater and preserved in 70% ethanol as a voucher for morphological
and spicule studies. All vouchers have been deposited at the Sponge collection of the Centre
d’Estudis Avançats de Blanes (numbers CEAB.POR.GEN.001 to CEAB.POR.GEN.029).

**DNA extraction, amplification, and sequencing**

DNA extractions were performed on two to three specimens per species and locality (totaling 18
individuals). *Hemimycale* spp. were extracted with QIAmp DNA stool kit (Qiagen), while *Crella*
spp. were extracted with DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s
protocol. Standard primers were used for COI partitions M1-M6 (Folmer et al., 1994) and 18S
rRNA (1F and 1795R, from Medlin et al., 1988), and Porifera primers for the D3-D5 partition of
28S rRNA (Por28S-830F and Por28S-1520R, from Morrow et al., 2012). Different amplification protocols were performed for each gene (Table 2). COI (M1-M6 partition) amplifications were performed in a 50 µL volume reaction, containing 37.6 µL H₂O, 5 µL buffer KCL (BIORON), 2 µL BSA, 2 µL dNTP (Sigma), 1 µl of primers, 0.4 µL Taq (BIORON) and 1µL of genomic DNA. 18S rRNA amplifications were performed in a 50 µL volume reaction, containing 36.85 µL H₂O, 5 µL buffer (INVITROGEN), 0.75 µL MgCl (INVITROGEN), 1.2 µL DMSO (dimethyl sulfoxide), 1 µL BSA, 1.5 µL dNTP (Sigma), 1 µl of primers, 0.7 µL Taq (INVITROGEN) and 1 µL of genomic DNA. Finally, partition D3-D5 of 28S rRNA amplifications were performed in a 50 µL volume reaction, containing 36.85 µL H₂O, 5 µL buffer (INVITROGEN), 0.75 µL MgCl (INVITROGEN), 2 µL BSA, 2 µL dNTP (Sigma), 1 µl of primers, 0.4 µL Taq (INVITROGEN) and 1 µL of genomic DNA. PCR products were purified and sequenced in both directions using Applied Biosystems 3730 xl DNA analyzers in Macrogen, Korea.

Sequence alignment and phylogenetic reconstructions

Sequences of COI, 28S, and 18S were aligned using Clustal W v.1.81, once their poriferan origin was verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as implemented in Genieous 9.01 (Kearse et al., 2012). When sequences were identical only one sequence per locality and species was included in the phylogenetic trees. After alignment, ambiguous regions were determined with Gblocks v.091 b software (Castresana, 2000), which removes from 1% to 4 % of poorly aligned positions and divergent regions of an alignment of DNA. Representatives of family Hymedesmiidae (i.e genera Phorbas and Hymedesmia) and Crambeidae, (i.e. genera Crambe and Monanchora) were selected as outgroups. The inclusion of Crambeidae as an outgroup was decided because the species H. arabica had been reported to contain similar secondary metabolites (polycyclic guanidine alkaloids) to those of Crambe and Monanchora (Ilan et al. 2004)

JModelTest 0.1.1 (Posada, 2008) was used to determine the best-fitting evolutionary model for each dataset. The model GTR+I+G was used for both mitochondrial and nuclear genes. Phylogenetic trees were constructed under Neighbour Joining (NJ) (default parameters), Bayesian Inference (BI) and Maximum Likelihood (ML) using Geneious software 9.01 (Kearse et al., 2012). NJ generates unrooted minimum evolution trees (Gascuel & Steel, 2006). BI analyses were performed with MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003). Four Markov
Chains were run with one million generations sampled every 1000 generations. The chains converged significantly and the average standard deviation of split frequencies was less than 0.01 at the end of the run. Early tree generations were discarded by default (25%) until the probabilities reached a stable plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree. ML analyses were executed with PhyML v3.0 program (Guindon & Gascuel, 2003; Guindon et al., 2005). The robustness of the tree clades was determined by a nonparametric bootstrap resampling with 1000 replicates in PhyML. MrBayes and PhyML were downloaded by Genieous.

Incongruence Length Difference (ILD) test (PAUP 4.0b10) was run (Swofford, 2002) to verify sequence homogeneity or incongruence between the 18S rRNA and COI markers and the 18S and 28S rRNA markers. The ILD test indicated no significant conflict (p = 0.93 and p = 0.91, respectively) between the marker pairs to be concatenated. Thus, concatenated 18S-COI and 18S-28S rRNA datasets were constructed for the species with sequences available for both markers. The phylogeny on the three genes concatenated was not performed due to the few species/individuals for which the three genes were available.

Phenotypic characters

To assess whether molecular differences among the target populations and species (H. columella, senso latus, H. arabica, and C. cyathophora) were supported by morphological and spicule traits, the target species were observed both in situ and on recently collected samples. Moreover, spicules of all the species were observed through light and Scanning Electron Microscopes (SEM) after removing the sponge organic matter from small (3 mm³) pieces of each individual by boiling them in 85% Nitric acid in a Pyrex tube and then washed three times with distilled water and dehydrated with ethanol 96% (three changes). A drop of a spicule suspension in ethanol per individual was placed on 5 mm diameter stuffs, air dry, and gold-palladium metalized (Uriz, Turon & Mariani, 2008) in a Sputtering Quorum Q150RS. Observation was performed through a Hitachi M-3000 Scanning Electron Microscope at the Centre d’Estudis Avançats de Blanes.

Genus name: urn:lsid:zoobank.org:act:3601D851-F9D2-4364-83F5-7A466DC432F3, Species name: urn:lsid:zoobank.org:act:2FC4AC75-2378-4FFA-801A-2A3475BFDF31, Publication
Results

18S rRNA phylogeny
The resulting phylogeny using the 18S rRNA partition on 25 sequences (17 new) of 695 nt. (46 variable positions, from which 38 were parsimony informative) was congruent under BI, and ML and just differed in the position of *H. arabica* which appeared as a sister group of the remaining *Crella* spp and *Hemimycale* spp under NJ (Fig. S1). The representatives of the family Crambeidae (*Monanchora*) appeared as outgroups and the genus *Phorbas* was a sister group of the remaining species. In the BI, NJ, and ML trees, the genera *Hemimycale* and *Crella* appeared polyphyletic, with the Red Sea species *Hemimycale arabica* and *Crella cyathophora*, far away from the Atlanto-Mediterranean *Hemimycale* and *Crella* species. The Atlanto-Mediterranean *Crella* formed a well-supported clade (1/ 81/98, posterior probability/bootstrapping values), which was the sister group of the Atlanto-Mediterranean *Hemimycale* (1/97/98). Moreover, the deep *Hemimycale columella* clustered with an Atlantic sequence downloaded from the GeneBank (0.89/89/88) forming a separate clade from the also well-supported (1/97/98) group containing the shallow Mediterranean *Hemimycale*. No genetic differences for this partition were found among shallow individuals. In the BI and ML trees the two individuals of *H. arabica* appeared in unresolved positions while they formed a poorly supported (75%) clade in the tree under the NJ criterion (not shown).

28S rRNA (D3-D5) phylogeny
The 28S rRNA (D3-D5) dataset comprised 31 sequences (24 new) of 623 nt. (84 variable positions from which, 60 parsimony informed) The resulting phylogenies were congruent with the three clustering criteria and matched in most cases the phylogeny based on the 18S rRNA partition, although the supporting values of some clades were in some cases slightly lower (Fig. S2).

The three phylogenies retrieved the representatives of Family Crambeidae (*Monanchora* and *Crambe*) as outgroup. The monophyly of the in-group containing *Crella* spp. and
Hemimycale spp. was strongly supported under the BI, NJ, and ML criteria (1/100/100). The genus Phorbas was a sister group of the remaining species considered. Crella was polyphyletic, with C. cyathophora separated from the well-supported clade (1/100/100) encompassing the Atlanto-Mediterranean Crella. The latter appeared as a sister clade of a poorly supported group (0.7/77/70) harboring C. cyathophora and Hemimycale spp. The Hemimycale spp. group, although monophyletic, was poorly supported under the NJ and ML criteria (77/70) while the Atlanto-Mediterranean Hemimycale clade was well supported under the three clustering criteria (1/92/95).

The deep and shallow Mediterranean populations of Hemimycale formed two well-supported monophyletic groups (0.96/87/83 and 0.96/100/98, for deep and shallow individuals, respectively), the former containing the Atlantic sequence of H. columella. No genetic differences for this partition were retrieved for shallow individuals despite their spread distribution across the Mediterranean. The individuals of C. cyathophora from the Red Sea clustered with those from the Pacific collected between Australia and Nouvelle Caledonie (1/89/76).

COI phylogeny

The COI dataset included 21 sequences (15 new) of 535 nt. (169 variable positions, from which 149 parsimony informative).

The COI phylogeny, which was congruent under BI, NJ, and ML, also retrieved the representatives of Crambeidae as outgroups of the group formed by Crella, Phorbas, and Hemimycale. The genus Phorbas clustered with the Atlanto-Mediterranean Crella spp. (0.98/100/86) likely because we only included one individual/species of Phorbas (Fig. S3).

A clade containing Hemimycale spp. and C. cyathophora was well supported (0.94/94/80).

The Hemimycale clade was divided into two subclades corresponding to deep and shallow individuals. No genetic differences among shallow individuals were found. A sister, well supported group (1/100/94) contained C. cyathophora and H. arabica representatives with almost no genetic differences between them (Fig. S3).

Concatenated trees
The concatenated 18S+28S rRNA (Fig. 1) confirmed the outgroup position for the Crambeidae representative (Monanchora), the polyphyly of Crella with the Red Sea and Pacific species forming a separate clade (1/100/100) from the Atlanto-Mediterran Crella, which appeared in a non-resolved position. Hemimycale also appeared polyphyletic, but the position of H. arabica was unresolved. The Atlanto-Mediterranean Hemimycale clade was confirmed as well as its division into two subclades: one containing the deep Mediterranean individuals together with two Atlantic sequences of the species and the other one harboring the shallow Mediterranean individuals, which did not show any genetic difference across the Mediterranean and Adriatic Sea.

The concatenated 18S rRNA+COI (Fig. 2) tree contained only 13 sequences and no representative of Crambeidae could be included. The representatives of the Atlanto-Mediterranean Crella appeared as outgroups of the remaining target species, which formed two well-supported clades: one containing C. cyathophora and H. arabica representatives (1/100/100) and the other with the Atlanto-Mediterranean Hemimycale (1/100/100) divided in two monophyletic well-supported groups (deep and shallow individuals).

**Discussion**

The phylogenetic reconstructions performed with 18S, 28S rRNA and COI, as well as with concatenated genes (18S rRNA+COI and 18S +28S rRNA) support the polyphyly of Crella and Hemimycale, under the three clustering criteria used. As although Hemimycale was monophyletic with the 28S rRNA (D3-D5) marker, the clade was not statistically supported.

*Crella cyathophora* sequences differ from those of the Atlanto-Mediterranean Crella spp. in 2% (18S rRNA), 2.19 % (28S rRNA), and 10.24 % (COI). These genetic distances suggest that, despite some spicule similitude (presence of acanthoxeas and smooth diactines with Atlanto-Mediterranean Crella spp.), the former species belongs in a different genus, closer to *Hemimycale arabica* (0.71% with 18S rRNA, 1.37% with 28S rRNA and none with COI) than to the Atlanto-Mediterranean Crella spp.

*H. arabica* differs from the Atlanto-Mediterranean Hemimycale spp. in 1.43-1.86% with 18S rRNA, 1.78-2.19 with 28S rRNA, and in 8.38-8.64% with COI. These strong COI differences and the contrasting morphological traits (blue external color, irregular, rim-free, aerolate areas and abundance of true styles in *H. arabica* vs. orange-pinkish color, circular,
rimmed aerolate areas, and slightly asymmetrical anystrongyles almost exclusively in *Hemimycale* spp.) also indicate that *H. arabica* would belong in a different genus, which might also include *C. cyathophora*, as there are not COI differences between these two species.

Moreover, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S rRNA phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*, which suggests higher affinities of this genus with Crellidae than with Hymedesmiidae (its current family). However, more complete analyses including additional Crellidae and Hymedesmiidae OTUs are needed to move *Hemimycale* from Hymedesmiidae to Crellidae.

The phylogenetic trees with any of the three gene partitions either separately or concatenated confirm the presence of two cryptic *Hemimycale* species in the Mediterranean within what was considered until now *Hemimycale columella*. The new species that we name *Hemimycale mediterranean* sp. nov. (see description below) has a shallower distribution across the whole Mediterranean than *H. columella*, which has Atlantic affinities. *H. columella* differs from *H. mediterranea* in 0.85% (18S rRNA), 1.23% (28S rRNA), and in 1-1.2% (COI).

The lack of genetic diversity among the distant populations of *H. mediterranea* analyzed points to its recent presence in the Mediterranean, which is compatible with a recent introduction. However, the new species has not been recorded out of the Mediterranean and thus, its origin cannot be established at the present time.

Many cryptic species that were revealed by molecular markers have never been formally described owing to the difficulty of finding diagnostic phenotypic characters. Although after exhaustive observation, only slight, morphological differences have been found to differentiate *H. mediterranea* sp. nov. from *H. columella* (see species description below), these phenotypic differences are consistent across individuals and thus, add to molecular differences and biological traits (Garate et al., unpublished) to consistently differentiate these two species.

Species description

Genus *Hemimycale* Burton, 1934

Sequence accession Numbers GenBank (Table 1)

Type species *Hemimycale columella* (Bowerbank 1874)

*Hemimycale* is the only genus of Hymedesmiidae that has smooth diactines and monactines exclusively (Van Soest, 2002). The genus was described by Burton (1934) as “reduced Mycaleae
with skeleton of loose fibers of styli, sometimes modified into anisostrongyles, running vertically to the surface; fibers tending to branch and anastomose; no special dermal skeleton, no microscleres”.

The spicule complement described by Burton, however, seems different from that reported in the several modern redescriptions of *H. columella* (Vacelet, Donadey & Froget, 1987), which report predominant straight anisostrongyles with rare or absent styles. Indeed, Burton stated that the Bowerbank representation of *H. columella* spicules was wrong because it figured anisostrongyles instead of styles, and was precisely the dominance of styles what induced Burton to place the species among the *Mycaleae*. The termination of the diaactines either round or pointed ends may be the result of different silica concentration in the water masses, as reported for other siliceous sponge skeletons (Uriz, 2006), but it cannot be totally discarded that the Burton *H. columella* belonged in another *Hemimycale* species.

Species: *H. columella* (Bowerbank, 1874)

Sequence accession Numbers GenBank (Table 1)

Description (Fig. 3A-D): Encrusting to massive sponges. Surface smooth, covered with circular inhaling, areas up to 6 mm in diameter with an up to 3 mm high rim. Morbid and fleshy consistence. Translucent to whitish ectosome, difficult to separate from the choanosome. Thousands of calcareous spherules, 1μm in diameter formed by intracellular calcifying bacteria (Uriz et al., 2012) are spread through the sponge mesohyl and specially accumulated at the sponge periphery of whitish individuals (Garate et al., unpublished data).

Color from pinkish-orange to whitish outside, dark orange inside.

Spicules (Table 3, Fig. 4F): asymmetric strongyles (anysotrongyles), straight, 302-435 μm x 3-4 μm in size. Styles rare or completely absent from the Mediterranean specimens (this study) and Canary Islands (Cruz 2002).

Skeletal arrangement: plumose branching bundles of anysostrongyles together with spread spicules. A palisade of vertical anysotrongyles forms the rim around the inhaling areas.

Distribution: Northeastern Atlantic (United Kingdom and Ireland coasts) Canarias Islands (Cruz 2002), western Mediterranean: Tossa de Mar, Arenys de Mar, from 28 to 60 m depth (this study).

It is not possible to confirm whether previous Mediterranean records of the species (see Vacelet & Donadey, 1977) belonged to *H. columella* or to *H. mediterranea*. 
Biology: multiannual life span, ca. 60% survival after two monitoring years; maximum growth in autumn-winter (Garate et al., unpublished data). Larval release occurs at the beginning of November in Mediterranean populations (authors unpublished obs.).

Species: *H. mediterranea* sp. nov. (Fig. 3E-H)

Sequence accession Numbers GenBank (Table 1)

Description: thick encrusting sponges with aerolate inhaling areas up to 3mm in diameter, surrounded by an up to 1.5-2mm high rim, which in some cases barely surpasses the sponge surface. Thousands of calcareous spherules, 1μm in diameter formed by intracellular calcifying bacteria are spread through the sponge mesohyl and specially accumulated at the sponge periphery (Garate et al., in press).

Ectosome: firmly attached to the choanosome.

Color: flesh to clear brownish externally, more or less whitish depending on calcibacteria accumulation at the surface, sometimes partially covered by an epibiotic (reddish or pinkish) cyanobacteria.

Spicules (Table 3, Fig. 4A-E): smooth, uniform in size, straight, anysostrongyles, 200-296 x 3-4 μm in size. Styles completely absent.

Skeletal arrangement: plumose undulating bundles of anysostrongyles together with spread spicules. A palisade of vertical anysotrongyles forms the rim around the inhaling areas.

Known distribution: Northwestern Mediterranean, central Mediterranean, Adriatic, eastern Mediterranean (Spain: Cap De Creus, Tossa, Blanes, Arenys, South Italy: Croatia, Tremiti, Turkey, Greece) between 3 and 17 m deep.

Biology: annual life span, maximum growth rates in summer (authors unpublished data). Larval release at the end of September beginning of October (authors unpublished obs.).

In most cases, it is difficult to ascertain whether individuals of *H. columella* recorded by other authors belong in *H. columella* or *H. mediterranea*. The redescription of *H. columella* by Van Soest (2002) based on the holotype (from the Atlantic) reported large aerolate porefields with elevated rims, which are shared with the deep Mediterranean specimens of *H. columella* (Fig. 3A-D) in contrast to the small, short-rimmed porefields showed by *H. mediterranea* sp. nov. Both species have mainly straight slightly asymmetric strongyles but the spicule sizes are
systematically larger in *H. columella* (Table 3). However, while styles were rarely present in *H. columella* individuals, they have not been found in specimens of *H. mediterranea* sp. nov. The external color also differs between the two species, being orange to pinkish in *H. columella* and flesh color to brownish *H. mediterranea* sp. nov. (Fig. 3E-H). Vacelet & Donadey (1977) reported two different color forms occurring side by side on the littoral of Provence (France), one pink cream and the other one brownish. Likely the second color morph, which besides had smaller strongyles, corresponded to the *H. mediterranea* sp. nov.

Color has not received much attention as a diagnostic character in sponges because it has been generally considered to be a response to higher or lower light irradiance at the sponge habitat, or to the presence of epibiotic or symbiotic cyanobacteria. However, color has proven to be taxonomically relevant to distinguish other invertebrates such as shrimp species (Knowlton & Mills, 1992) and also sponge species of the genus *Scopalina* (Blanquer & Uriz, 2008), and thus it seems worthy to be taken into account in sponge taxonomy.

The slight phenotypic differences found between the two species appear, however, consistent across individuals and localities within the Atlanto-Mediterranean basin. Moreover, their ecological distribution and bacterial symbionts, strongly differentiate these two cryptic species. For instance, although calcareous spherules produced by intracellular bacteria are present in the two species, the producer bacteria belong in different species (Garate et al., in press), and the respective microbial communities totally differ (Garate et al., in press). Symbionts, as predators do (e.g. Wulff, 2006), often distinguish their target sponge preys or hosts while the species remain morphologically cryptic to taxonomists. Moreover, *H. mediterranea* sp. nov. shows an annual life span, with individuals disappearing after larval release, while *H. columella* has a multiannual life span (authors unpublished data) and growth dynamics also differs between the two species, as *H. mediterranea* sp. nov. grows more in summer, while *H. columella* grows preferentially in autumn-winter (authors unpublished data).

The contrasting ecological distribution of these two cryptic species in the Mediterranean helps to predict their identity in the field. *H. mediterranea* sp. nov. inhabits shallower zones than *H. columella*. However, it is likely that both species may share occasionally habitat, as the record of the two color morphs side by side (Vacelet & Donadey, 1977) indicate. *H. mediterranea* sp. nov. seems to be more abundant and widespread in the Mediterranean than *H.
columella. Molecular differences between groups of individuals of *H. columella* suggest the possible presence of additional cryptic species among the deep Mediterranean *Hemimycale*.

The presence of two morphologically cryptic *Hemimycale* species in the Mediterranean, which show contrasting biological traits, reinforces the idea that cryptic species represent something more than wrong taxonomic identifications or biodiversity underestimates. They may feature contrasting biological cycles and life spans, and puzzle biological studies, which may invalidate conservation policies based on those studies.

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Figure 1

Phylogenetic tree using concatenated (18S rRNA+COI) partitions

BI, NJ and ML gave the same topologies. Posterior probability, neighbor joining, and maximum likelihood supporting values are at the base of clades.
18S + 28S

Monanchora arbuscula (KC902225.1 + KC869447.1)

Crella elegans (KC902282 + HQ393898.1)
Crella rosea (KC902058 + HQ379299.1)

Crella cyathophora (Daedalus, EG) ind1
Crella cyathophora (Elphistone, EG) ind2
Crella cyathophora (Bempton, PAC) ind3
Crella cyathophora (Bempton, PAC) ind4

Hemimycale arabica (Daedalus, EG) ind 1
Hemimycale arabica (Elphistone, EG) ind 2

Hemimycale columella (KC902127 + HQ379300)

Hemimycale columella (Tossa, SP) ind 1
Hemimycale columella (Tossa, SP) ind 2

Hemimycale sp. (Tossa, SP) ind 1
Hemimycale sp. (Tossa, SP) ind 2
Hemimycale sp. (Othonoi, GR) ind 3
Hemimycale sp. (Othonoi, GR) ind 4
Hemimycale sp. (Karaburun, ALB) ind 5
Hemimycale sp. (Karaburun, ALB) ind 6
Hemimycale sp. (Kornati, CRO) ind 7
Hemimycale sp. (Porto Cesareo, IT) ind 8
Hemimycale sp. (Porto Cesareo, IT) ind 9

0.02

BI/NJ/ML
Figure 2

Phylogenetic tree using concatenated (18S+28S rRNA) partitions

BI, NJ and ML gave the same topologies. Posterior probability, neighbor joining, and maximum likelihood supporting values are at the base of clades.

18S + COI
Figure 3

*In situ* pictures of Atlanto-Mediterranean *Hemimycale* spp

A, B, C, D) *Hemimycale columella* from 35-40 m of depth. E, F, G, H) *Hemimycale mediterranea* sp. nov. from 12-17 m of depth. Whitish tinge is due to calcibacteria accumulation. Red tinges are due to several species of epibiotic cyanobacteria. Arrows point to aerolate inhaling areas; arrowheads indicate the epibiont cyanophycea on *H. mediterranea* specimens.
Figure 4

Spicules of *Hemimycale* spp. and *Crella cyathophora* though SEM

A, B, C, D, E) Anisostongyles (*Hemimycale mediterranea*). F) Anisostongyles (*Hemimycale columella*). G) Anisostongyles and one style (*Hemimycale arabica*). H) Anisostongyles and acantoxeas (*Crella cyatophora*). Inserts on each picture correspond to magnifications of the spicule ends.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
Manuscript to be reviewed
**Table 1** (on next page)

Geographical origin and ecological distribution of the individuals used in the phylogenetic study, with accession numbers

Individuals sequenced *de novo* are in bold
| Species                      | Sea/Ocean          | Locality                  | Voucher numbers | Accession numbers |
|------------------------------|--------------------|---------------------------|-----------------|-------------------|
| *H. arabica* ind. 1          | Red Sea            | Dedalos-Brother Islands   | CEAB.POR.G.EN.001 | COI: KY002124     |
|                              |                    |                           |                 | 18S: KY002171     |
|                              |                    |                           |                 | 28S: KY002181     |
| *H. arabica* ind. 2          | Red Sea            | Elphistone-Brother Islands| CEAB.POR.G.EN.002 | COI: KY002125     |
|                              |                    |                           |                 | 18S: KY002172     |
|                              |                    |                           |                 | 28S: KY002182     |
| *H. columella*               | Northeastern Atlantic | Plymouth, Wales-UK       |                 | 28S: HQ379300.1   |
|                              |                    |                           |                 | 18S: KC902127.1   |
| *H. columella* ind. 1        | Northwestern Mediterranean | Arenys de Mar- | CEAB.POR.G.EN.003 | 28S: KY002183     |
|                              |                    | Spain                     |                 |                   |
| *H. columella* ind. 2        | Northwestern Mediterranean | Arenys de Mar- | CEAB.POR.G.EN.004 | 28S: KY002184     |
|                              |                    | Spain                     |                 |                   |
| *H. columella* ind. 3        | Northwestern Mediterranean | Arenys de Mar- | CEAB.POR.G.EN.005 | COI: KY002126     |
|                              |                    | Spain                     |                 |                   |
| *H. columella* ind. 1        | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.006 | COI: KY002127     |
|                              |                    | Spain                     |                 | 18S: KY002160     |
|                              |                    |                           |                 | 28S: KY002185     |
| *H. columella* ind. 2        | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.007 | COI: KY002128     |
|                              |                    | Spain                     |                 | 18S: KY002161     |
|                              |                    |                           |                 | 28S: KY002186     |
| *H. columella* ind. 3        | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.008 | COI: KY002129     |
|                              |                    | Spain                     |                 | 28S: KY002187     |
| *H. columella* ind. 4        | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.009 | 28S: KY002188     |
| *H. mediterranea* sp. nov. ind. 1 | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.010 | COI: KY002130     |
|                              |                    | Spain                     |                 | 18S: KY002162     |
|                              |                    |                           |                 | 28S: KY002189     |
| *H. mediterranea* sp. nov. ind. 2 | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.011 | 18S: KY002163     |
|                              |                    | Spain                     |                 | 28S: KY002190     |
| *H. mediterranea* sp. nov. ind. 4 | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G.EN.012 | COI: KY002131     |
| *H. mediterranea* sp.        | Northwestern Mediterranean | Tossa de Mar- | CEAB.POR.G        |                   |
|                              |                    |                           |                 |                   |
| Nov. Ind. | Location | Country | Catalogue Code | GenBank Accession Numbers |
|----------|----------|---------|----------------|--------------------------|
| 5        | Adriatic Sea | Koznati-Croatia | CEAB.POR.G.EN.013 | COI: KY002132 |
| H. mediterránea sp. nov. ind. 3 | Adriatic Sea | Koznati-Croatia | CEAB.POR.G.EN.014 | 18S: KY002170 28S: KY002193 |
| H. mediterránea sp. nov. ind. 7 | Adriatic Sea | Koznati-Croatia | CEAB.POR.G.EN.015 | 18S: KY002194 |
| H. mediterránea sp. nov. ind. 8 | Adriatic Sea | Koznati-Croatia | CEAB.POR.G.EN.016 | 18S: KY002133 |
| H. mediterránea sp. nov. ind. 2 | Adriatic Sea | Tremiti-Italy | CEAB.POR.G.EN.017 | 18S: KY002199 |
| H. mediterránea sp. nov. ind. 11 | Adriatic Sea | Tremiti-Italy | CEAB.POR.G.EN.018 | 28S: KY002164 |
| H. mediterránea sp. nov. ind. 8 | Central Mediterranean | Porto Cesareo-Italy | CEAB.POR.G.EN.019 | 18S: KY002165 28S: KY002197 |
| H. mediterránea sp. nov. ind. 9 | Central Mediterranean | Porto Cesareo-Italy | CEAB.POR.G.EN.020 | 28S: KY002198 |
| H. mediterránea sp. nov. ind. 10 | Central Mediterranean | Porto Cesareo-Italy | CEAB.POR.G.EN.021 | 18S: KY002166 28S: KY002191 |
| H. mediterránea sp. nov. ind. 5 | Adriatic Sea | Karaburum-Albania | CEAB.POR.G.EN.022 | 18S: KY002167 28S: KY002192 |
| H. mediterránea sp. nov. ind. 6 | Adriatic Sea | Karaburum-Albania | CEAB.POR.G.EN.023 | 18S: KY002168 28S: KY002195 |
| H. mediterránea sp. nov. ind. 3 | Eastern Mediterranean | Othonoi-Greece | CEAB.POR.G.EN.024 | 18S: KY002169 28S: KY002196 |
| H. mediterránea sp. nov. ind. 4 | Eastern Mediterranean | Othonoi-Greece | CEAB.POR.G.EN.025 | COI: KY002120 18S: KY002173 28S: KY002177 |
| Crella cyatophora ind. 1 | Red Sea | Dedalos-Brother Islands | CEAB.POR.G.EN.026 | COI: KY002121 18S: KY002174 28S: KY002178 |
| Crella cyatophora ind. 2 | Red Sea | Elphistone-Brother Islands | CEAB.POR.G.EN.027 | COI: KY002122 18S: KY002175 28S: KY002179 |
| Crella cyatophora ind. 3 | Pacific | Bempton Patch Reef (between New Caledonian and Australia) | CEAB.POR.G.EN.028 | COI: KY002122 18S: KY002175 28S: KY002179 |
| **Crella cyatophora** ind. 4 | Pacific | Bempton Patch Reef (between New Caledonian and Australia) | CEAB.POR.GEN.029 | COI: KY002123 18S: KY002176 28S: KY002180 |
|---------------|-------|-------------------------------------------------|----------------|-----------------------------------|
| Crella elegans | Mediterranean | France | | 18S: KC902282 |
| Crella elegans | Mediterranean | France | | 18S: AY348882 |
| Crella elegans | Mediterranean | France | | 28S: HQ393898 |
| Crella plana | Northeastern Atlantic | Northern Ireland | | 18S: KC902309 |
| Crella rosea | Northeastern Atlantic | Northern Ireland | | 28S: HQ37929 |
| Crella rosea | Northeastern Atlantic | Northern Ireland | | 18S: KC902282 |
| **Phorbas bihamiger** | Northeastern Atlantic | English Channel | | 18S: KC901921.1 28S: KC869431 |
| **Phorbas punctatus** | Northeastern Atlantic | Wales | | 18S: KC869439.1 28S: KC869439.1 |
| **Phorbas dives** | Northeastern Atlantic | English Channel | | 28S: HQ37930 |
| **Phorbas fictitiioides** | North Pacific | - | | COI: HE61161 7.1 |
| **Phorbas tenacior** | Northeastern Atlantic | - | | 18S: AY348881 |
| **Phorbas glaberrimus** | Antarctic | Ross Sea | | COI: LN85021 6.1 |
| **Hymedesmia paupertas** | Northeastern Atlantic | | | 18S: KC902073.1 28S: KF018118.1 |
| **Hymedesmia pansa** | Northeastern Atlantic | | | 18S: KC90207.1 |
| **Hymedesmia paupertas** | Northeastern Atlantic | | | 28S: KF018118.1 |
| **Kirkpatrickia variolosa** | Antarctic | Ross Sea | | COI: LN850202.1 |

Table 1. Geographical origin and ecological distribution of the individuals used in the phylogenetic study, with accession numbers. Individuals sequenced *de novo* are in bold.
Table 2 (on next page)

PCR conditions for the three partitions used (COI, 28S and 18S).
| PCR Stage     | COI (M1-M6) | 28S (D3-D5) | 18S |
|---------------|-------------|-------------|-----|
| Denaturalization | 94°C 2min   | 94°C 5min   | 94°C 5min |
| Denaturalization | [94°C 1min] | [94°C 1min] | [94°C 30s] |
| Annealing     | 43°C 1min   | 50-55°C 1min | 53°C 30s |
| Elongation    | 72°C 1min   | 72°C 1min   | 72°C 30s |
| Final elongation | 72°C 5min   | 72°C 5min   | 72°C 5min |

Table 2. PCR conditions for the three partitions used (COI, 28S and 18S).
Table 3 (on next page)

Locality and spicule sizes of the studied individuals, and comparison with descriptions by other authors.
| Species            | Author               | Locality             | Depth (m)/assemblage | styles                  | strongyles (range/mean) | acanthoxeas |
|--------------------|----------------------|----------------------|----------------------|-------------------------|-------------------------|-------------|
| *H. arabica* ind. 1 | This study           | Red Sea (Egypt)      | 14/coral reef        | 160-189 (179.6) x 7-8 (7.5) | 210-290 (273) x 2.8-4.1 (3.6) | -           |
| *H. arabica*       | Illan et al. 2004    | Red Sea (Egypt)      | 190-250 (218) x 3.5-5 (4.7) | 200-290(266) x 2.5-4 (3.5) | -           |
| *H. mediterránea* ind. 7 | This study       | Adriatic (Croatia)   | 10-15/rocky sub-horizontal | -                       | 233-330 (274.8) x 3-4.6 (4.0) | -           |
| *H. mediterránea* ind. 11 | This study    | Adriatic (Italy)     | 10-15/rocky sub-horizontal | -                       | 251-300 (276.6) x 2.1-4 (3.0) | -           |
| *H. mediterránea* ind. 5 | This study    | Adriatic (Albania)   | 10-15/rocky sub-horizontal | -                       | 274-317 (296.4) x 2.9-4.5 (4.0) | -           |
| *H. mediterránea* ind. 10 | This study   | Central Med. (Italy) | 10-15/rocky sub-horizontal | -                       | 229-328 (291.3) x 2.4-5.2 (3.5) | -           |
| *H. mediterránea* ind. 3 | This study    | Eastern Med. (Greece)| 10-15/rocky sub-horizontal | -                       | 242-340 (272.7) x 2.6-4 (3.2) | -           |
| *H. mediterránea* ind. 1 | This study    | NW Med. (Spain)      | 12-16/rocky wall      | -                       | 261-320(296.3) x 3.1-3.8 (3.5) | -           |
| *H. columella* ind. 1 | This study      | NW Med. (Spain)      | 27-29/coralligenous   | -                       | 302-435 (370) x 3-4 (3.7) | -           |
| “*H. columella*”   | Vacelet 1987       | NW Med. (France)     | -                    | -                       | 225-310 (285) x 2.4 (3)   | -           |
| *H. columella*     | Vacelet 1987       | NW Med. (France)     | -                    | -                       | 320-410(369) x 2.5-3.8(3.1)| -           |
| “*H. columella*”   | Vacelet 1987       | NW Med. (France)     | -                    | -                       | 220-320(273) x 2-4(2.7)  | -           |
| *H. columella*     | Vacelet 1987       | North Atlantic (France)| -                | -                       | 290-465 (394) x 4-7(5.1) | -           |
| *H. columella*     | Topsent 1925       | North Atlantic (France)| -                | -                       | 400 x 6                | -           |
| *H. columella*     | Foster 1995        | North Atlantic (UK)  | -                    | -                       | 330-420(373) x 5-6(5.85)| -           |
| *H. columella*     | Bowerbank 1874     | North Atlantic (UK)  | -                    | -                       | 376 x 7                | -           |
| *Crella* Cyatophora ind 3 | This study     | Indo-Pacific (Bemptom) | 18m/coral reef      | -                       | 205-308 (263.9)x 2.2-4.3 (3.4) | 92-115 (105.4)x 2-2.3(2) |
| C. cyatophora ind 1 | This study | Red Sea (Egypt) | 12/coral reef | – | 227-293 (267.8)x 2.5-3.9 (3.4) | 89-120 (109.4)x 1.8-2.5(2.47) |

Table 3. Locality and spicule sizes of the studied individuals, and comparison with descriptions by other authors.