



# Phylogenetic signal in the evolution of body colour and spicule skeleton in calcareous sponges

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Some of the morphological characters used in Porifera taxonomy have often been shown to be inconsistent. In the present study, we tested the phylogenetic coherence of currently used taxonomic characters of the calcarean genus *Clathrina*. For this, 20 species of *Clathrina* and three other calcarean genera (*Ascandra*, *Guancha*, and *Leucetta*) were sequenced for the ITS and D2 region of the 28S ribosomal DNA. Maximum-likelihood and maximum-parsimony algorithms were used to reconstruct phylogenetic trees. Deep divergences were observed in our tree and *Clathrina* was shown to be paraphyletic. The major split in our topology showed a clear-cut distinction between sponges with and without tetractine spicules. Moreover, a group of yellow-coloured *Clathrina* was clearly separated from the remaining white-coloured species. Our results show that the presence of diactines, water-collecting tubes, the degree of cormus anastomosis, and actine shapes do not correlate with the major clades of the calcarean phylogeny. On the other hand, the presence of tripods, the absence of tetractines, and the presence of spines in the apical actine of tetractines seem to be good synapomorphies for clades in our tree. Our results demonstrate that skeleton characters can be reliably used in higher level taxonomy in Clathrinida.

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## INTRODUCTION

A phylogenetic tree illustrates the flow that determines shared homologous characteristics within groups of organisms. More than a century and a half ago, the publication of *On the Origin of Species* set the stage for the idea of a phylogenetically sound classification (Darwin, 1859). Currently, there is an increasing trend toward using phylogenetics to understand the diversity of life, but the traditional

typological approach is still applied by research groups around the world. The reason is simple: consistent phylogenies are scarce for many groups of organisms (Manuel *et al.*, 2003; Dohrmann *et al.*, 2006; Pick *et al.*, 2010) and the typological approach is the method of choice to determine the taxonomic position of research specimens. Invertebrate animal groups with simple morphology and a meagre fossil record fit well into this category.

For those groups, molecular data seem to be the key to reliable phylogenetic reconstructions, as evolution at the molecular level may be accurately described by statistical models, and topology inference with

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molecular data becomes a statistical issue (Nei & Kumar, 2000). Once a well-supported molecular phylogeny is available, however, dubious morphological characters should be checked and the taxonomy re-evaluated (e.g. Waeschenbach *et al.*, 2009; Cárdenas *et al.*, 2010). In the case of morphologically simple organisms, such as sponges, the problem becomes critical (Borchiellini *et al.*, 2000). Porifera systematics is mainly based on the spicule skeleton, but morphological characters in many sponge groups have often been shown to be plastic (e.g. Klautau *et al.*, 1999; Bell, Barnes & Turner, 2002; Valderrama *et al.*, 2009) or simply absent (Lazoski *et al.*, 2001).

In the cosmopolitan genus *Clathrina*, for instance, species diagnoses are mainly based on absent or dubious characters (Klautau & Valentine, 2003). Progressively complex body organizations, however, are clearly recognized among species of the genus. Simpler forms have loosely anastomosed tubes with several oscula, whereas more complex forms present tightly anastomosed tubes with a sharply distinct cornus and fewer oscula with water-collecting tubes (Klautau & Valentine, 2003). In addition, as species in the genus *Clathrina* exhibit several combinations of spicule types, these sponges constitute an excellent model system to test the evolution of skeleton and body organization using a molecular evolutionary approach. Thus, this study aims to test the phylogenetic coherence of relevant characters in the taxonomy of the calcarean genus *Clathrina*.

## MATERIAL AND METHODS

### TAXA

The class Calcarea is currently divided into two subclasses, Calcinea and Calcaronea. Both are monophyletic, according to extensive morphological and molecular data (e.g. Manuel *et al.*, 2003; Dohrmann *et al.*, 2006). Due to a current dispute on the status of most Calcinean genera and families (Dohrmann *et al.*, 2006) and considering that *Clathrina* belongs to the Calcinea subclass, we have selected two calcaronean species, *Paraleucilla magna* Klautau, Monteiro & Borojevic, 2004 and *Sycettusa tenuis* Borojevic & Klautau, 2000, as outgroups.

Species names, collection sites, voucher numbers, and GenBank accession numbers for the newly generated sequences and for those downloaded from GenBank are listed in Table 1. Twenty species of *Clathrina* were sequenced for our analysis (the number of specimens sequenced for each species is shown in parentheses): *Clathrina* sp. 1 (3); *Clathrina* sp. 2 (1); *C. antofagastensis* Azevedo *et al.*, 2009 (1); *C. aspina* Klautau, Solé-Cava & Borojevic, 1994 (3); *C. aurea* Solé-Cava *et al.*, 1991 (3); *C. brasiliensis*

Solé-Cava *et al.*, 1991 (2); *C. cerebrum* (Haeckel, 1872) (3); *C. clathrus* (Schmidt, 1864) (3); *C. conifera* Klautau & Borojevic, 2001 (2); *C. contorta* Minchin, 1905 (1); *C. corallicola* Rapp, 2006 (1); *C. coriacea* (Montagu, 1818) (1); *C. cylindractina* Klautau, Solé-Cava & Borojevic, 1994 (4); *C. fjordica* Azevedo *et al.*, 2009 (2); *C. helveola* Wörheide & Hooper, 1999 (1); *C. luteoculcitella* Wörheide & Hooper, 1999 (1); *C. nansenii* (Breitfuss, 1896) (2); *C. reticulum* (Schmidt, 1862) (3); *C. tetractina* Klautau & Borojevic, 2001 (1); and *C. wistariensis* Wörheide & Hooper, 1999 (1).

Based on the current taxonomy, *Clathrina* is distinguishable from closely related genera by character states that might vary even within a single specimen (Klautau & Valentine, 2003). Hence, we have added another six calcinean species from three different genera to test the monophyletic condition of the genus (the number of specimens sequenced is shown in parentheses): *Ascandra falcata* Haeckel, 1872 (2) (type species); *Guancha* sp. (3); *G. lacunosa* Johnston, 1842 (2); *G. ramosa* Azevedo *et al.* 2009 (1); *Leucetta chagosensis* Dendy, 1913 (1); and *L. microraphis* Haeckel, 1872 (1).

### DNA SEQUENCING

The internal transcribed spacer (ITS) and the D2 region of the 28S ribosomal DNA were sequenced for our phylogenetic analyses. Multi-copy ribosomal genes and spacers may be unreliable in low-level molecular taxonomy studies (Nei & Kumar, 2000). Nonetheless, in calcareous sponges, gene conversion seems to be effective (Wörheide, Nichols & Goldberg, 2004), eliminating paralogy-related problems using multicopy markers in the group.

In spite of the extensive experience of our group in the study of sponge genetics, we were unsuccessful when trying to amplify fragments of mitochondrial and other nuclear protein-coding genes. Sequencing mitochondrial DNA of calcareous sponges is not a trivial task, and Calcarea remains one of the few metazoan classes for which no mitochondrial genome has been released, despite several attempts (D. Lavrov, pers. comm.).

Genomic DNA was extracted from ethanol or liquid nitrogen-preserved specimens with the guanidine/phenol chloroform protocol (Lôbo-Hajdu *et al.*, 2004) or using a Viogene kit and following the manufacturer's instructions. The entire region comprising the two spacers (ITS1 and ITS2) and the 5.8S ribosomal DNA was amplified by PCR with primers anchored on 18S (5'TCATTAGAGGAAGTAAAAGTCG3') and 28S (5'GTTAGTTTCTTTTCCTCCGCTT3') (Lôbo-Hajdu *et al.*, 2004).

The D2 region of the 28S rDNA was amplified by PCR with primers C2F (5'GAAAAGAACTTTG

**Table 1.** Specimens used in this study with collection sites, voucher numbers, and GenBank accession numbers of DNA sequences

Species	Collection site	Voucher number	GenBank accession number	
			ITS	28S
<i>Ascandra falcata</i>	Mediterranean Sea	UFRJPOR 5856	HQ588962	HQ589006
<i>Ascandra falcata</i>	Mediterranean Sea	UFRJPOR 6320	HQ588963	–
<i>Clathrina antofagastensis</i>	Chile	MNRJ 9289	HQ588985	HQ589003
<i>Clathrina</i> aff. <i>aspina</i>	Brazil	UFRJPor 5211	HQ588969	–
<i>Clathrina</i> aff. <i>aspina</i>	Brazil	UFRJPor 5245	HQ588998	–
<i>Clathrina</i> aff. <i>aspina</i>	Brazil	URFJPor 5495	–	HQ589017
<i>Clathrina aurea</i>	Brazil	MNRJ 8998	HQ588968	HQ589005
<i>Clathrina aurea</i>	Brazil	MNRJ 8990	HQ588958	–
<i>Clathrina aurea</i>	Brazil	MNRJ 5170	HQ588960	–
<i>Clathrina brasiliensis</i>	Brazil	UFRJPor 5214	HQ588978	HQ589015
<i>Clathrina brasiliensis</i>	Brazil	UFRJPor 5230	HQ588999	HQ589005
<i>Clathrina cerebrum</i>	Mediterranean Sea	UFRJPor 6322	HQ588964	HQ589008
<i>Clathrina cerebrum</i>	Mediterranean Sea	UFRJPor 6323	HQ588971	–
<i>Clathrina cerebrum</i>	Mediterranean Sea	UFRJPor 6324	HQ588975	–
<i>Clathrina clathrus</i>	Mediterranean Sea	UFRJPOR 6315	HQ588974	HQ589009
<i>Clathrina clathrus</i>	Mediterranean Sea	UFRJPor 6325	HQ588965	–
<i>Clathrina clathrus</i>	Mediterranean Sea	UFRJPor 6326	HQ588972	–
<i>Clathrina conifera</i>	Brazil	MNRJ 8991	HQ588959	HQ589010
<i>Clathrina conifera</i>	Brazil	MNRJ 8997	HQ588957	–
<i>Clathrina contorta</i>	Mediterranean Sea	UFRJPor 6327	HQ588970	HQ589011
<i>Clathrina corallicola</i>	Norway	UFRJPor 6329	HQ588994	HQ589012
<i>Clathrina coriacea</i>	Norway	UFRJPor 6330	HQ588986	HQ589001
<i>Clathrina cylindractina</i>	Brazil	UFRJPor 5206	HQ588979	HQ589007
<i>Clathrina cylindractina</i>	Brazil	UFRJPor 5213	HQ588980	–
<i>Clathrina cylindractina</i>	Brazil	UFRJPor 5221	HQ588981	–
<i>Clathrina cylindractina</i>	Brazil	UFRJPor 5413	HQ588993	–
<i>Clathrina fjordica</i>	Chile	MNRJ 8143	HQ588984	–
<i>Clathrina fjordica</i>	Chile	MNRJ 9964	–	HQ589016
<i>Clathrina helveola</i>	Australia	G313680	HQ588988	AM180987.1
<i>Clathrina luteoculcitella</i>	Australia	G313684	HQ588989	AM180988.1
<i>Clathrina nansenii</i>	Greenland	UFRJPor 6332	HQ588982	HQ589013
<i>Clathrina nansenii</i>	Norway	UFRJPor 6333	HQ588983	–
<i>Clathrina reticulum</i>	Mediterranean Sea	UFRJPOR 6258	HQ588973	HQ589014
<i>Clathrina reticulum</i>	Mediterranean Sea	UFRJPOR 6263	HQ588966	–
<i>Clathrina reticulum</i>	Mediterranean Sea	UFRJPOR 6260	HQ588977	–
<i>Clathrina</i> sp. 1	Brazil	UFRJPor 5172	HQ588961	HQ589004
<i>Clathrina</i> sp. 1	Brazil	UFRJPor 5173	HQ588976	–
<i>Clathrina</i> sp. 1	Brazil	UFRJPor 5174	HQ588967	–
<i>Clathrina</i> sp. 2	Brazil	UFRJPor 6107	–	HQ589018
<i>Clathrina tetractina</i>	Brazil	UFRJPor 5183	HQ589000	HQ589021
<i>Clathrina wistariensis</i>	Australia	G313663	HQ588987	AM180990.1
<i>Guancha lacunosa</i>	Norway	UFRJPor 6334	HQ588991	HQ589020
<i>Guancha lacunosa</i>	Norway	UFRJPor 6335	HQ588992	–
<i>Guancha ramosa</i>	Chile	MNRJ 10313	HQ588990	HQ589002
<i>Guancha</i> sp.	Norwegian Sea	UFRJPor 6336	HQ588996	HQ589019
<i>Guancha</i> sp.	Norwegian Sea	UFRJPor 6337	HQ588995	–
<i>Guancha</i> sp.	Norwegian Sea	UFRJPor 6338	HQ588997	–
<i>Leucetta microraphis</i>	Australia	QMG313659	AJ633873.1	AM180995.1
<i>Leucetta chagosensis</i>	Australia	–	AF458864.1	AY563543.1
<i>Paraleucilla magna</i>	Brazil	–	–	AM181005.1
<i>Sycettusa tenuis</i>	Brazil	QMG313685	–	AM181006.1

Voucher specimens were deposited at the Universidade Federal do Rio de Janeiro (Brazil), Instituto de Biologia, Departamento de Zoologia (UFRJPOR), and Museu Nacional do Rio de Janeiro (MNRJ). All specimens in the table were used in a preliminary analysis that recovered all nominal species as monophyletic with 100% bootstrap support. The final topology shown in Figure 1 was constructed using individuals with ITS1, 5.8S, ITS2 (ITS) and the D2 region of 28S (28S) GenBank accession numbers.

RARAGAGAGT3') and D2R (5'TCCGTGTTTCAA-GACGGG3') (Chombard *et al.*, 1997). The PCR mixes contained buffer (75 mM Tris-HCl, pH 8.8, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween 20), 1 µg/µL bovine serum albumin, 0.4 mM dNTPs, 0.5 pmol µL<sup>-1</sup> of each primer, 1 mM MgCl<sub>2</sub>, and one unit of Taq DNA-polymerase. The PCR steps included 5 min at 95 °C, 35 cycles of 1 min at 92 °C, 1 min at 50–55 °C, and 1 min at 72 °C, followed by 5 min at 72 °C. Forward and reverse strands were automatically sequenced by Macrogen. The obtained sequences were edited using the program DNASTAR – SeqMan, and BLAST searches (<http://www.ncbi.nlm.nih.gov/blast/>) were performed to confirm their source.

#### SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

ITS and 28S sequences were individually aligned using the ClustalW algorithm in MEGA 4.0 (Tamura *et al.*, 2007). As calcinean and calcaronean species are quite distantly related, we decided to eliminate the ITS sequence of the alignment for both outgroup species. Hence, for rooting purposes, we relied solely on the conservative 28S ribosomal sequences. ClustalW alignments were visually inspected, resulting in alignment lengths of 1078 bp (454 parsimony-informative) for ITS1, 5.8S and ITS2, and 495 bp (186 parsimony-informative) for the D2 region of 28S. Two different tree-building methods were used to generate phylogenetic trees with each marker independently: maximum-likelihood (ML) and maximum-parsimony (MP). Gap sites were removed from the phylogenetic analyses.

Four different tree topologies were individually generated with the tree-building methods using the two nuclear markers. As all the sequences from each single nominal species were grouped on preliminary trees, they were removed from the final analyses. The tree-building methods presented strikingly similar topologies with consistent confidence values on most branches for both markers (available upon request). Thus, we decided to assemble the alignments of both markers (1451 bp long with 525 parsimony-informative sites) and run a concatenated analysis using ML and MP methods.

The model for the ML analysis was selected using Modeltest 3.7 and the Akaike Information Criterion (Posada & Crandall, 1998), which indicated general time reversal (GTR) with six gamma categories (gamma parameter = 0.42). ML trees for both segments were constructed with the PHYML package (Guindon & Gascuel, 2003). The algorithm requires an input tree, and a BIONJ tree (Gascuel, 1997) was used for each marker independently. A heuristic tree bisection and reconnection algorithm was

performed on the respective BIONJ tree to find the ML tree for each marker. Nodal support for ML trees was also estimated using 1000 bootstrap pseudo-replicates.

All MP trees were estimated with PAUP 4.0 (Swofford, 2003). A MP heuristic search performed a tree-bisection-and-reconnection as the branch-swapping algorithm. The starting tree in this case was constructed via stepwise random addition of taxa. Ten trees were retained at each step and 100 replicates of addition were performed to select for the MP tree. One thousand bootstrap pseudo-replicates were performed in both ML and MP trees.

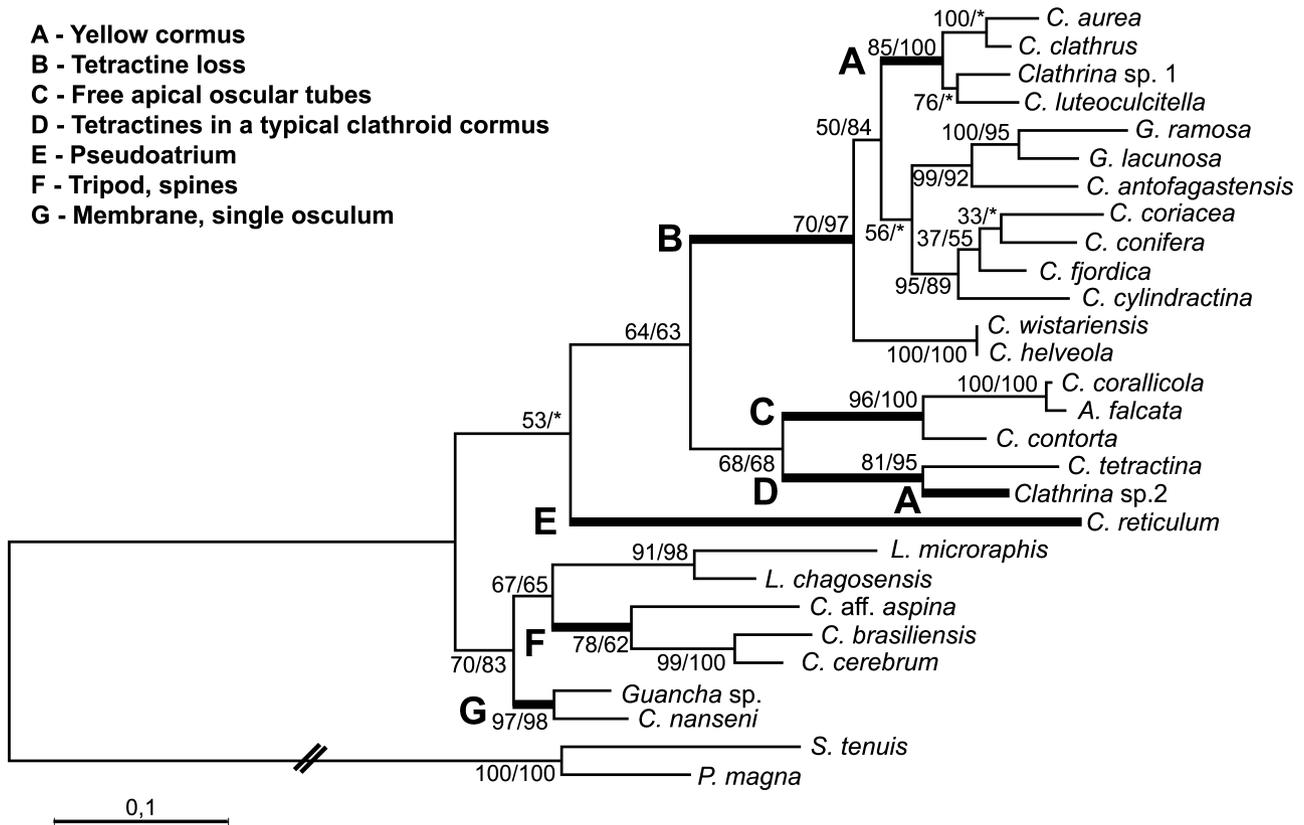
## RESULTS AND DISCUSSION

The results of the analyses are shown with bootstrap values for ML and MP trees given at interior branches of the ML topology (Fig. 1). Our phylogeny showed several high support values in the interior branches of the ML tree. The major split in our topology showed a clear-cut distinction between sponges with and without tetractine spicules. As tetractines are widely distributed within the Calcarea, the second group of sponges probably lost that spicule type earlier in their evolutionary history. Table 2 shows the selected taxonomic features of all the species analysed.

#### SPECIES WITHOUT TETRACTINES

Among the group without tetractines, the first split separated the Great Barrier Reef sponges *Clathrina wistariensis* and *C. helveola* from the remaining species. These species live in sympatry and they may only be distinguished by subtle differences in spicule sizes (Wörheide & Hooper, 1999; see Table 2). In our results, they showed no molecular divergence even for the highly variable ITS marker. Given the molecular and the morphological similarity for the species, one must consider the possibility that *C. helveola* is a junior synonym of *C. wistariensis*.

The second lineage in the group split the yellow-coloured *Clathrina* from the remaining white-coloured species. This result indicates that *C. aurea*, *C. clathrus*, *Clathrina* sp. 1, and *C. luteoculcitella* evolved from a yellow common ancestor. Therefore, body colour in calcarean sponges is not as plastic as it was previously thought (but see below, *Clathrina* sp. 2). Based on this conclusion, we hypothesize that *C. chrysea* Borojevic & Klautau, 2000, although not included in this study, is part of this group, as it is also a yellow-coloured clathroid sponge with no tetractines. Among the yellow clathrinans, *Clathrina* sp. 1 clustered with *C. luteoculcitella* (76/\*), and both were grouped (85/100) with the clade of *C. aurea* and



**Figure 1.** Maximum-likelihood (ML) tree based on a concatenated sequence of ITS1, 5.8S, ITS2 and the D2 region of 28S using the GTR model with six gamma categories. Bootstrap values for ML and maximum-parsimony (MP), respectively, are given on interior branches. An asterisk indicates ML interior branches that were not present on the MP topology. Thick branches specify those on which listed character changes were inferred.

*C. clathrus* (100/\*), the latter the type species of the genus.

The remaining species (56/\*) were separated into two clusters of white-coloured species with no tetractines. The first clade contained *Guancha ramosa* and its closely related *G. lacunosa* (100/95); both then joined *C. antofagastensis* (99/92). The second cluster of white clathrinas contained the group of *C. coriacea* and *C. conifera* (33/\*) that joined with *C. fjordica* (37/55) and then with *C. cylindractina* (95/89). No other distinct morphological character was found to match interior branches in this part of the tree.

The group of species with no tetractines should also include: *C. broendstedii* Rapp, Janussen & Tendal, 2011; *C. ceylonensis* (Dendy, 1905); *C. chrysea*; *C. cribrata* Rapp, Klautau & Valentine, 2001; *C. heronensis* Wörheide & Hooper, 1999; *C. parva* Wörheide & Hooper, 1999; *C. procumbens* (Von Lendenfeld, 1885); *C. sinusarabica* Klautau & Valentine, 2003; *C. hondurensis* Klautau & Valentine, 2003; *C. clara* Klautau & Valentine, 2003; *C. primordialis* Haeckel, 1872; *C. laminoclathrata* Carter, 1886; *C. rotunda* Klautau

& Valentine, 2003; *C. angraensis* Azevedo & Klautau, 2007; and *C. jorunnae* Rapp, 2006.

#### SPECIES WITH TETRACTINES

The other groups in our phylogenetic tree presented species that do exhibit tetractine spicules. In the sister group of the species without tetractines, the lineage with *Clathrina* sp. 2 and *C. tetractina* (81/95) joined a well-resolved cluster (96/100) that contained *C. contorta* and the group of *C. corallicola* and *Ascan-dra falcata* (100/100). However, support for the entire group was only moderate (68/68).

A group that includes the new species *Clathrina* sp. 2 and *C. tetractina* can be characterized by the clathroid cormus and the presence of tetractines in addition to the triactines. Interestingly, *Clathrina* sp. 2 was the only yellow species of the genus in our data set that also presented tetractines. Based on the characteristics of this species, there were two possibilities to resolve this phylogenetic consistency: either this species joins the yellow group (with no

**Table 2.** Morphological characters of the analysed clathroid species

Species	Spicules	Actine			und.	sp.	Cormus		Colour
		Shape	Tip				Anastomosis	wct	
<i>C. antofagastensis</i>	tri I	con	Blunt	no			irreg, tight	No	White
<i>C. aspina</i>	tri II	con	Blunt	no			reg, tight	Yes	White
	tri	con	Blunt	no					
<i>C. aurea</i>	tetra	con	Blunt	no			irreg, loose	No	Yellow
	apical	con	Sharp	no	vst				
<i>C. brasiliensis</i>	trip	con	Blunt	no			reg, tight	Yes	White
	tri	cyl	Rounded	yes					
<i>C. cerebrum</i>	tri	con	Blunt	no			reg, tight	Yes	White
	tetra	con	Blunt	no					
	apical	con	Sharp	no	Yes				
	trip	con	Blunt	no					
<i>C. clathrus</i>	tri	cyl	Rounded	yes			irreg, loose	Yes	Yellow
	tri	con	Blunt	no			irreg, loose	No	White
<i>C. contorta*</i>	tri	con	Sharp	yes			irreg, tight	Yes	White
	tetra	con	Sharp	yes					
<i>C. corallicola*</i>	apical	cyl	Sharp	no	No				
	di	fus	Sharp	curved					
	tric	cyl							
	tri I	cyl	Sharp	no			irreg, loose (no apical anastomosis)	No	White
	tri II	cyl	Sharp	no					
	tetra I	cyl	Sharp	no	No				
<i>C. coriacea</i>	apical	cyl	Sharp	no	No				
	tetra II	cyl	Sharp	no	No				
<i>C. cylindractina</i>	apical	cyl	Sharp	no	No				
	tri	con	Rounded	yes			irreg, loose	Yes	White
<i>C. fjordica</i>	tri	cyl	Blunt	no			irreg, loose	No	White
<i>C. helveola</i>	tri	con	Blunt	yes			irreg, loose	No	White
<i>C. luteoculcitella</i>	tri	con	Sharp	yes			irreg, tight	No	Yellow
	di	fus							
<i>C. nanseni</i>	tri	cyl/con	Blunt	no			reg, tight	Yes	White
	tetra I	cyl/con	Blunt	no					
	apical	cyl	Blunt	no	No				
	tetra II	cyl/con	Blunt	no					
<i>C. reticulum</i>	apical	rud.	Blunt	no	No				
	tri	cyl	Sharp	no			reg, tight	Yes/pseud	White
	tetra	cyl	Sharp	no					
	apical	cyl	Sharp	no	No				
<i>C. tetractina*</i>	di	arrow							
	tri	con	Sharp	yes			irreg, loose	Yes	White
<i>C. wistariensis</i>	tetra	con	Sharp	yes					
	apical	cyl	Sharp	no	No				
<i>Clathrina</i> sp. 1	tri	cyl	Blunt	no			irreg, loose	No	White
<i>Clathrina</i> sp. 2	tri	con	Rounded	yes			reg, tight	No	Yellow
	tetra	con	Rounded	yes			irreg, loose	Yes	Yellow
<i>Ascandra falcata*</i>	apical	con	Blunt	yes	No				
	tri	con	Sharp				irreg, loose (no apical anastomosis)		White
<i>Guancha lacunosa</i>	tetra	con	Sharp		No				
	apical				No				
<i>Guancha ramosa</i>	di						irreg, tight	Yes	White
	tri	cyl	Blunt						
<i>Guancha</i> sp.	tri paras.	cyl	Blunt				irreg, loose	Yes	White
	b	tri	Rounded	yes					
<i>Guancha</i> sp.	p	tri paras.	Rounded	yes			reg, tight	Yes	White
	b	tri							
	p	tetra			No				
		apical							
		tri			No				
		tetra							
		apical							

\*Species with tetractines as the most abundant spicules. Abbreviations: b, body; p, peduncle; tri, triactines; tetra, tetractines; trip, tripods; di, diactines; tric, trichoxeas; con, conical; cyl, cylindrical; und, undulation; sp., spines at the apical actine; vst, vestigial; reg, regularly; irreg, irregularly; tight, tightly; wct, water-collecting tubes; pseud, pseudoatrium.

tetractines), which would mean an independent gain of tetractines, or it clusters with the white clathrinas with tetractines, which would mean an independent gain of yellow body colour. The latter possibility was supported by our analysis (Fig. 1).

Hence, the high statistical support for this group (81/95) indicates that this lineage represents a true clade that is distinguished by a common clathroid cormus (without pseudoatrium or free apical oscular tubes) and tetractines. Other species that might belong to this clade are: *C. adusta* Wörheide & Hooper, 1999; *C. africana* Klautau & Valentine, 2003; *C. alcatraziensis* Lanna *et al.*, 2007; *C. canariensis* (Miklucho-Maclay, 1868); *C. dubia* (Dendy, 1891); *C. hirsuta* Klautau & Valentine, 2003; *C. quadri-radiata* Klautau & Borojevic, 2001; *C. sagamiana* (Hôzawa, 1929); *C. septentrionalis* Rapp *et al.*, 2001; *C. sueziana* Klautau & Valentine, 2003; and *C. tenuipilosa* (Dendy, 1905).

The second cluster in this lineage included *C. con-torta* and *C. corallicola*, as well as *Ascandra falcata* (96/100), the type species of the genus. In addition, these species showed morphological similarities, such as a clathroid body with free apical oscular tubes. Other species of *Clathrina* that also present these morphological features and might be part of the group are: *C. atlantica* Thacker, 1908; *C. ascandroides* Borojevic, 1971; and *C. biscayae* Borojevic & Boury-Esnault, 1987.

The next tetractine lineage comprised just *C. reticulum*, the only species with a pseudoatrium (a cavity below the osculum with no choanoderm). Other *Clathrina* species that also present a distinct pseudoatrium and might cluster in the group are *C. gardineri* (Dendy, 1913) and *C. panis* (Haeckel, 1870).

The last clade consisted of three different lineages. The first lineage joined the two *Leucetta* species (*L. microraphis* and *L. chagosensis*) (91/98), characterized by a leuconoid aquiferous system and a cortex. The second lineage is a group that includes *C. aff. aspina*, *C. cerebrum*, and *C. brasiliensis* (clustered with 78/62). This group is characterized by the presence of a clathroid body, tripods, and spines (or vestigial spines) in the apical actine of the tetractines. It might also include *C. paracerebrum* Austin, 1996 and *C. tetrapodifera* Klautau & Valentine, 2003, as they share morphological characters.

The last lineage includes *C. nanseni* and *Guancha* sp. (97/98), both with a well-delimited clathroid cormus surrounded by a membrane, at least in the young, and with a single osculum. Guanchas are very similar to clathrinas. In fact, only the presence of a peduncle and parasagittal spicules in *Guancha* distinguish the two genera. In our tree, *Guancha* spp. were spread across the topology, raising doubts on the

validity of these characters as diagnostic for the genus.

## FINAL REMARKS

Deep divergences were observed in our tree and *Clathrina* was shown to be paraphyletic. The paraphyly of *Clathrina* had already been observed in the most extensive *Clathrina* sampling to date (Dohrmann *et al.*, 2006). In their molecular analysis, the authors included seven *Clathrina* species that were sequenced for 18S and 28S markers. Considering that their GenBank *C. cerebrum* sequence was probably not actually derived from *C. cerebrum*, it is possible to recognize the same pattern we found, i.e. two clear clusters, one with and one without tetractines, corroborating our main result. If further evidence confirms these results, *Clathrina* should be divided into at least five different genera indicated by the clades discussed above. In that case, only the species of *Clathrina* with no tetractines should remain as true clathrinas, as the type species of the genus (*C. clathrus*) lies within this clade.

Given the highly debatable status of calcarean systematics (e.g. Borojevic *et al.*, 2002; Manuel *et al.*, 2003), the presence of phylogenetic signal in the skeleton of Clathrinida is a very promising result. In the past, characters such as cormus organization (tightly or loosely anastomosed), osculum type (with or without water-collecting tubes), presence of granules, spicule composition, shape of actines, and presence of spines were considered to be reliable for the systematics of *Clathrina* (Klautau & Valentine, 2003).

In the present study, we have observed that all of them remain reliable to distinguish between species, but not above this level. This is the case of all cited characters except those related to the skeleton. As extant calcarean sponges show low diversity of spicules (diactines, triactines, tetractines, and pentactines), these characters were never considered for phylogenetic purposes (Dendy & Row, 1913; Borojevic, Boury-Esnault & Vacelet, 1990). Among the various spicule types, diactines remain unreliable, but the presence of tripods, the absence of tetractines, and the presence of spines in the apical actine of tetractines seem to be good synapomorphies for clades in our tree.

In the 19th Century, Haeckel (1872) had already established the importance of spicule composition as a diagnostic character at the genus level in calcareous sponges. In his pioneering study, he proposed that asconoid sponges (Ascones), for example, should be divided into seven genera according to spicule composition. The major problem, however, with that proposal was that he did not distinguish between

calcarean and calcaronean species, for which the monophyletic status is very well supported by morphological and molecular analyses (e.g. Manuel *et al.*, 2003; Dohrmann *et al.*, 2006). In a molecular analysis of Demospongiae, spicule composition has already been shown to present a strong phylogenetic signal above species level (Chombard, Boury-Esnault & Tillier, 1998). Our results confirm Haeckel's hypothesis and extend the importance of spicule composition to the systematics of higher level calcarean taxa.

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