The LINNEAN SOCIETY of London ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2011. With 1 figure

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

ANDRÉ LINHARES ROSSI,[1] CLAUDIA AUGUSTA DE MORAES RUSSO,[2] ANTONIO MATEO SOLÉ-CAVA,[2] HANS TORE RAPP[3,4] and MICHELLE KLAUTAU[1]*

[1]*Universidade Federal do Rio de Janeiro, Instituto de Biologia, Departamento de Zoologia, 21.941-902, Rio de Janeiro, Brazil*
[2]*Universidade Federal do Rio de Janeiro, Instituto de Biologia, Departamento de Genética, 21.941-902, Rio de Janeiro, Brazil*
[3]*University of Bergen, Department of Biology, N-5020, Bergen, Norway*
[4]*University of Bergen, Centre for Geobiology, N-5020, Bergen, Norway*

Received 16 November 2010; revised 4 February 2011; accepted for publication 11 February 2011

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 calcinean 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 calcinean 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.

doi: 10.1111/j.1096-3642.2011.00739.x

ADDITIONAL KEYWORDS: Calcarea – Calcinea – *Clathrina* – molecular phylogenetics – morphological evolution – Porifera.

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

*Corresponding author. E-mail: mklautau@biologia.ufrj.br

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 cormus 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. nanseni* (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′TCATTTAGAGGAAGTAAAAGTCG3′) 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
Ascandra falcata	Mediterranean Sea	UFRJPOR 5856	HQ588962	HQ589006
Ascandra falcata	Mediterranean Sea	UFRJPOR 6320	HQ588963	–
Clathrina antofagastensis	Chile	MNRJ 9289	HQ588985	HQ589003
Clathrina aff. *aspina*	Brazil	UFRJPor 5211	HQ588969	–
Clathrina aff. *aspina*	Brazil	UFRJPor 5245	HQ588998	–
Clathrina aff. *aspina*	Brazil	URFJPor 5495	–	HQ589017
Clathrina aurea	Brazil	MNRJ 8998	HQ588968	HQ589005
Clathrina aurea	Brazil	MNRJ 8990	HQ588958	–
Clathrina aurea	Brazil	MNRJ 5170	HQ588960	–
Clathrina brasiliensis	Brazil	UFRJPor 5214	HQ588978	HQ589015
Clathrina brasiliensis	Brazil	UFRJPor 5230	HQ588999	HQ589005
Clathrina cerebrum	Mediterranean Sea	UFRJPor 6322	HQ588964	HQ589008
Clathrina cerebrum	Mediterranean Sea	UFRJPor 6323	HQ588971	–
Clathrina cerebrum	Mediterranean Sea	UFRJPor 6324	HQ588975	–
Clathrina clathrus	Mediterranean Sea	UFRJPOR 6315	HQ588974	HQ589009
Clathrina clathrus	Mediterranean Sea	UFRJPor 6325	HQ588965	–
Clathrina clathrus	Mediterranean Sea	UFRJPor 6326	HQ588972	–
Clathrina conifera	Brazil	MNRJ 8991	HQ588959	HQ589010
Clathrina conifera	Brazil	MNRJ 8997	HQ588957	–
Clathrina contorta	Mediterranean Sea	UFRJPor 6327	HQ588970	HQ589011
Clathrina corallicola	Norway	UFRJPor 6329	HQ588994	HQ589012
Clathrina coriacea	Norway	UFRJPor 6330	HQ588986	HQ589001
Clathrina cylindractina	Brazil	UFRJPor 5206	HQ588979	HQ589007
Clathrina cylindractina	Brazil	UFRJPor 5213	HQ588980	–
Clathrina cylindractina	Brazil	UFRJPor 5221	HQ588981	–
Clathrina cylindractina	Brazil	UFRJPor 5413	HQ588993	–
Clathrina fjordica	Chile	MNRJ 8143	HQ588984	–
Clathrina fjordica	Chile	MNRJ 9964	–	HQ589016
Clathrina helveola	Australia	G313680	HQ588988	AM180987.1
Clathrina luteoculcitella	Australia	G313684	HQ588989	AM180988.1
Clathrina nanseni	Greenland	UFRJPor 6332	HQ588982	HQ589013
Clathrina nanseni	Norway	UFRJPor 6333	HQ588983	–
Clathrina reticulum	Mediterranean Sea	UFRJPOR 6258	HQ588973	HQ589014
Clathrina reticulum	Mediterranean Sea	UFRJPOR 6263	HQ588966	–
Clathrina reticulum	Mediterranean Sea	UFRJPOR 6260	HQ588977	–
Clathrina sp. 1	Brazil	UFRJPor 5172	HQ588961	HQ589004
Clathrina sp. 1	Brazil	UFRJPor 5173	HQ588976	–
Clathrina sp. 1	Brazil	UFRJPor 5174	HQ588967	–
Clathrina sp. 2	Brazil	UFRJPor 6107	–	HQ589018
Clathrina tetractina	Brazil	UFRJPor 5183	HQ589000	HQ589021
Clathrina wistariensis	Australia	G313663	HQ588987	AM180990.1
Guancha lacunosa	Norway	UFRJPor 6334	HQ588991	HQ589020
Guancha lacunosa	Norway	UFRJPor 6335	HQ588992	–
Guancha ramosa	Chile	MNRJ 10313	HQ588990	HQ589002
Guancha sp.	Norwegian Sea	UFRJPor 6336	HQ588996	HQ589019
Guancha sp.	Norwegian Sea	UFRJPor 6337	HQ588995	–
Guancha sp.	Norwegian Sea	UFRJPor 6338	HQ588997	–
Leucetta microraphis	Australia	QMG313659	AJ633873.1	AM180995.1
Leucetta chagosensis	Australia		AF458864.1	AY563543.1
Paraleucilla magna	Brazil	–	–	AM181005.1
Sycettusa tenuis	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′TCCGTGTTTCAAGACGGG3′) (Chombard *et al*., 1997). The PCR mixes contained buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 1 μg/μL bovine serum albumin, 0.4 mM dNTPs, 0.5 pmol μL^{-1} of each primer, 1 mM $MgCl_2$, 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 clathrinas, *Clathrina* sp. 1 clustered with *C. luteoculcitella* (76/*), and both were grouped (85/100) with the clade of *C. aurea* and

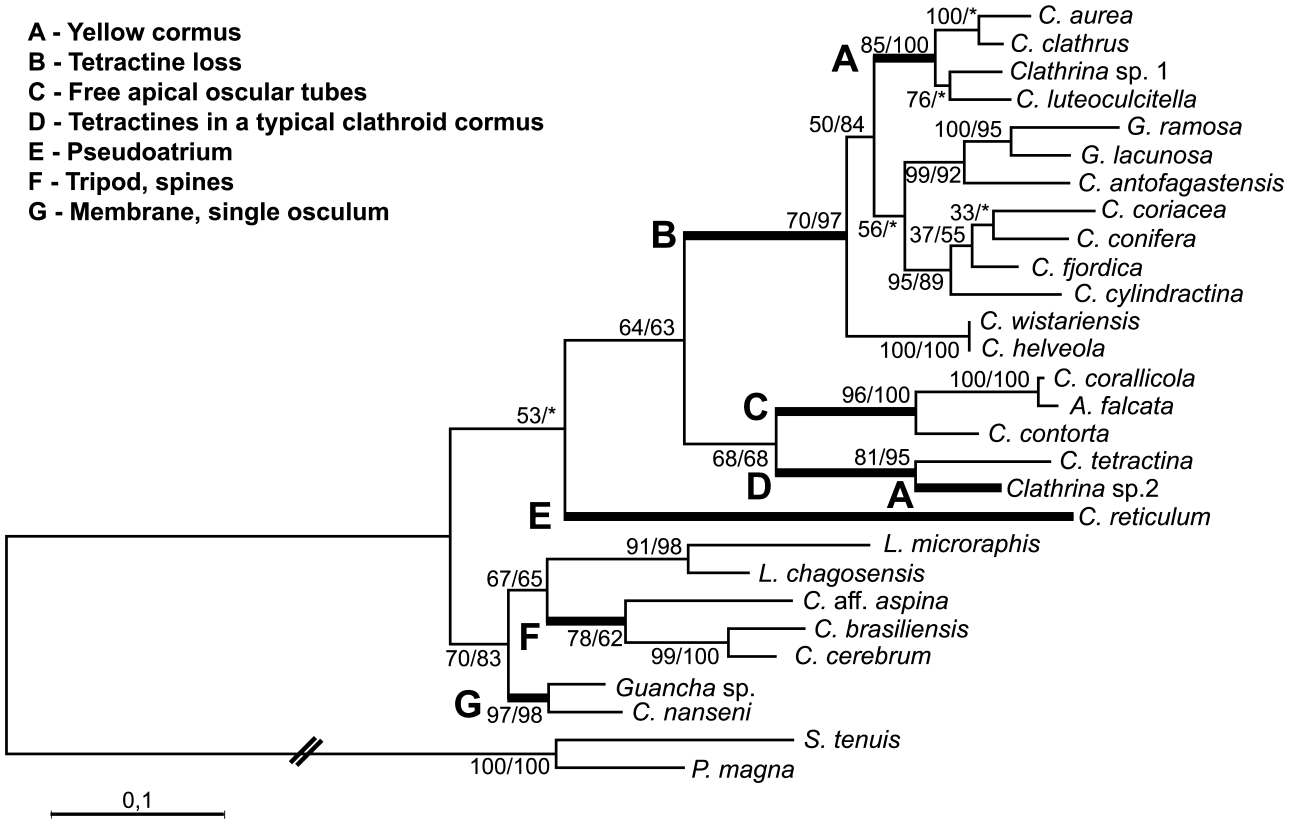

Figure 1. Maximum-likelihood (ML) tree based on a concatenated sequence of ITS1, 5.8S, ITS2 and the D2 region of 28S using the GTR model with six gamma categories. Bootstrap values for ML and maximum-parsimony (MP), respectively, are given on interior branches. An asterisk indicates ML interior branches 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. broendstedi* 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 *Ascandra 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				Cormus		Colour
			Shape	Tip	und.	sp.	Anastomosis	wct	
C. antofagastensis		tri I	con	Blunt	no		irreg, tight	No	White
		tri II	con	Blunt	no				
C. aspina		tri	con	Blunt	no		reg, tight	Yes	White
		tetra	con	Blunt	no				
		apical	con	Sharp	no	vst			
		trip	con	Blunt	no				
C. aurea		tri	cyl	Rounded	yes		irreg, loose	No	Yellow
C. brasiliensis		tri	con	Blunt	no		reg, tight	Yes	White
		tetra	con	Blunt	no				
		apical	con	Sharp	no	Yes			
		trip	con	Blunt	no				
C. cerebrum		tri	con	Blunt	no		reg, tight	Yes	White
		tetra	con	Blunt	no				
		apical	con	Sharp	no	Yes			
		trip	con	Blunt	no				
C. clathrus		tri	cyl	Rounded	yes		irreg, loose	Yes	Yellow
C. conifera		tri	con	Blunt	no		irreg, loose	No	White
*C. contorta**		tri	con	Sharp	yes		irreg, tight	Yes	White
		tetra	con	Sharp	yes				
		apical	cyl	Sharp	no	No			
		di	fus	Sharp	curved				
		tric	cyl						
*C. corallicola**		tri I	cyl	Sharp	no		irreg, loose (no apical anastomosis)	No	White
		tri II	cyl	Sharp	no				
		tetra I	cyl	Sharp	no				
		apical	cyl	Sharp	no	No			
		tetra II	cyl	Sharp	no				
		apical	cyl	Sharp	no	No			
C. coriacea		tri	con	Rounded	yes		irreg, loose	Yes	White
C. cylindractina		tri	cyl	Blunt	no		irreg, loose	No	White
C. fjordica		tri	cyl/con	Blunt	yes		irreg, loose	Yes	White
C. helveola		tri	con	Blunt	yes		irreg, loose	No	White
C. luteoculcitella		tri	con	Sharp	yes		irreg, tight	No	Yellow
		di	fus						
C. nanseni		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				
		apical	rud.	Blunt	no	No			
C. reticulum		tri	cyl	Sharp	no		reg, tight	Yes/pseud	White
		tetra	cyl	Sharp	no				
		apical	cyl	Sharp	no	No			
		di	arrow						
		tric							
*C. tetractina**		tri	con	Sharp	yes		irreg, loose	Yes	White
		tetra	con	Sharp	yes				
		apical	cyl	Sharp	no	No			
C. wistariensis		tri	cyl	Blunt	no		irreg, loose	No	White
Clathrina sp. 1		tri	cyl	Rounded	yes		reg, tight	No	Yellow
Clathrina sp. 2		tri	con	Rounded	yes		irreg, loose	Yes	Yellow
		tetra	con	Rounded	yes				
		apical	con	Blunt	yes	No			
*Ascandra falcata**		tri	con	Sharp			irreg, loose (no apical anastomosis)		White
		tetra	con	Sharp		No			
		apical				No			
		di							
Guancha lacunosa	b	tri	cyl	Blunt			irreg, tight	Yes	White
		di	cyl	Blunt					
	p	tri paras.	cyl	Blunt					
Guancha ramosa	b	tri	cyl	Rounded	yes		irreg, loose	Yes	White
	p	tri paras.	cyl	Rounded	yes				
Guancha sp.	b	tri					reg, tight	Yes	White
		tetra							
		apical				No			
	p	tri							
		tetra							
		apical				No			

*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. quadriradiata* 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. contorta* 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

calcinean 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.

ACKNOWLEDGEMENTS

We thank G. Wörheide for providing specimens of the Australian species. Grants and fellowships were provided by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (grants to A.M.S.C., C.A.M.R., M.K.), Conselho Nacional de Desenvolvimento Científico e Tecnológico (graduate student fellowship to A.L.R.), Universidade Federal do Rio de Janeiro/PIBIC (undergraduate student fellowship to A.L.R.), the Norwegian Research Council through project number 28630 (grant to H.T.R.), and The University of Bergen through the Mohn Foundation (grants to A.L.R., M.K., H.T.R.).

REFERENCES

Austin WC. 1996. Sponges from Rocas Alijos. In: Schmieder RW, ed. *Monographiae biologicae. Rocas Alijos. Scientific results from the cordell expeditions*. Dordrecht: Kluwer Academic Publishers, 238.

Azevedo F, Hajdu E, Willenz P, Klautau M. 2009. New records of Calcareous sponges (Porifera, Calcarea) from the Chilean coast. *Zootaxa* **2072:** 1–30.

Azevedo F, Klautau M. 2007. Calcareous sponges (Porifera, Calcarea) from Ilha Grande Bay, Brazil, with descriptions of three new species. *Zootaxa* **1402:** 1–22.

Bell JJ, Barnes DKA, Turner JR. 2002. The importance of micro and macro morphological variation in the adaptation of a sublittoral demosponge to current extremes. *Marine Biology* **140:** 75–81.

Borchiellini C, Chombard C, Lafay B, Boury-Esnault N. 2000. Molecular systematics of sponges (Porifera). *Hydrobiologia* **420:** 15–27.

Borojevic R. 1971. Éponges calcaires des côtes du sud-est Bresil, épibiontes sur *Laminaria brasilensis* et *Sargassum cymorum*. *Revista Brasileira de Biologia* **31:** 525–530.

Borojevic R, Boury-Esnault N. 1987. Calcareous sponges collected by N.O. Thalassa on the continental margin of the Bay of Biscay: I. Calcinea. In: Vacelet J, Boury-Esnault N, eds. *Taxonomy of Porifera from the NE Atlantic and Mediterranean Sea*. NATO Asi Series, G13. Berlin: Springer-Verlag, 1–27.

Borojevic R, Boury-Esnault N, Manuel M, Vacelet J. 2002. Order Clathrinida Hartman, 1958. In: Hooper JNA, van Soest RWM, eds. *Systema Porifera: a guide to the classification of sponges*. New York: Kluwer Academic/Plenum Publishers, 1141–1152.

Borojevic R, Boury-Esnault N, Vacelet J. 1990. A revision of the supraspecific classification of the subclass Calcinea (Porifera, class Calcarea). *Bulletin of the Muséum National d'Histoire Naturelle* Paris **12:** 243–276.

Breitfuss LL. 1896. Kalkschwämme der Bremer-Expedition nach Ost-Spitsbergen. *Zoologischer Anzeiger* **514:** 426–432.

Cárdenas P, Rapp HT, Schander C, Tendal OS. 2010. Molecular taxonomy and phylogeny of the Geodiidae (Porifera, Demospongiae, Astrophorida)–combining phylogenetic and Linnaean classification. *Zoologica Scripta* **39:** 89–106.

Carter HJ. 1886. Descriptions of sponges from the neighborhood of Port Philip Heads, S. Australia. *Annals and Magazine of Natural History* **18:** 34–55, 126–149.

Chombard C, Boury-Esnault N, Tiller A, Vacelet J. 1997. Polyphyly of 'sclerosponges' (Porifera, Demospongiae) supported by 28S ribosomal sequences. *Biological Bulletin* **193:** 359–367.

Chombard C, Boury-Esnault N, Tillier S. 1998. Reassessment of homology of morphological characters in tetractinellid sponges based on molecular data. *Systematic Biology* **47:** 351–366.

Darwin C. 1859. *The origin of species by means of natural selection*. London: John Murray.

Dendy A. 1891. A monograph of the Victorian sponges I. The organization and classification of the Calcarea Homocoela, with descriptions of the Victorian species. *Transactions of the Royal Society of Victoria* **3:** 1–82.

Dendy A. 1905. Report on the sponges collected by Professor Herdman, at Ceylon, in 1902. In: Herdman WA, ed. *Report to the Government of Ceylon on the Pearl Oyster Fisheries of the Gulf of Manaar*. 3 (Suppl. 18). London: Royal Society, 57–246, pls I–XVI.

Dendy A. 1913. The Percy Sladen trust expedition to the Indian Ocean in 1905 (V). I. Report on the Calcareous Sponges collected by HMS 'Sealark' in the Indian Ocean. *Transactions of the Linnean Society of London* **16:** 1–29.

Dendy A, Row RW. 1913. The classification and phylogeny of the calcareous sponges, with the reference list of all the described species, systematically arranged. *Proceedings of the Zoological Society of London* **47:** 704–813.

Dohrmann M, Voigt O, Erpenbeck D, Wörheide G. 2006. Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA. *Molecular Phylogenetics and Evolution* **40:** 830–843.

Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution* **14:** 685–695.

Guindon S, Gascuel O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52:** 696–704.

Haeckel E. 1870. Prodromus eines Systems der Kalkschwamme. *Jenaische Zeitschrift* **5:** 236–254.

Haeckel E. 1872. *Die kalkschwämme. Eine monographie*, Vols 1–3. Berlin: Reimer.

Hôzawa S. 1929. Report on the calcareous sponges obtained by the survey of the continental shelf bordering on Japan. *Sci. Rep. Tohuku Univ.* **8** (1)**:** 1–20.

Johnston G. 1842. *A history of British sponges and lithophytes*. Edinburgh.

Klautau M, Borojevic R. 2001. Sponges of the genus *Clathrina* Gray, 1867 from Arraial do Cabo, Brazil. *Zoosystema* **23:** 395–410.

Klautau M, Russo CAM, Lazoski C, Boury-Esnault N, Thorpe JP, Solé-Cava AM. 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. *Evolution* **53:** 1414–1422.

Klautau M, Solé-Cava AM, Borojevic R. 1994. Biochemical systematics of sibling sympatric species of *Clathrina* (Porifera: Calcarea). *Biochemical Systematics and Ecology* **22:** 367–375.

Klautau M, Valentine C. 2003. Revision of the genus *Clathrina* (Porifera, Calcarea). *Zoological Journal of the Linnean Society* **139:** 1–62.

Lanna E, Rossi AL, Cavalcanti FF, Hajdu E, Klautau M. 2007. Calcareous sponges from Sao Paulo State, Brazil (Porifera: Calcarea: Calcinea) with the description of two new species. *Journal of the Marine Biological Association of the United Kingdom* **87:** 1553–1561.

Lazoski C, Solé-Cava AM, Boury-Esnault N, Klautau M, Russo CAM. 2001. Cryptic speciation in a high gene flow scenario in the oviparous marine sponge *Chondrosia reniformis*. *Marine Biology* **139:** 421–429.

Lôbo-Hajdu G, Guimarães ACR, Salgado A, Lamarão FRM, Vieiralves T, Mansure JJ, Albano RM. 2004. Intragenomic, intra- and interspecific variation in the rDNA ITS of Porifera revealed by PCR-Single-Strand conformation polymorphism (PCR-SSCP). *Bollettino dei Musei e degli Istituti Biologici dell'Universitá di Genova* **68:** 413–423.

Manuel M, Borchiellini C, Alivon E, Le Parco Y, Vacelet J, Boury-Esnault N. 2003. Phylogeny and evolution of calcareous sponges: monophyly of Calcinea and Calcaronea, high level of morphological homoplasy, and the primitive nature of axial symmetry. *Systematic Biology* **52:** 311–333.

Miklucho-Maclay N. 1868. Beiträge zur Kenntnis der Spongien. I. Über *Guancha blanca* einen neuen Kalkschwamm. *Jenaische Zeitung* **4:** 221–240.

Montagu G. 1818. An essay on Sponges, with descriptions of all the species that have been discovered on the coasts of Great Britain (1812). *Memoires of the Wernerian Society of Edinburgh* **2:** 67–122.

Nei M, Kumar S. 2000. *Molecular evolution and phylogenetics*. New York: Oxford University Press.

Pick KS, Philippe H, Schreiber F, Erpenbeck D, Jackson DJ, Wrede P, Wiens M, Alié A, Morgenstern B, Manuel M, Wörheide G. 2010. Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. *Molecular Biology and Evolution* **27:** 1983–1987.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Rapp HT. 2006. Calcareous sponges of the genera *Clathrina* and *Guancha* (Calcinea, Calcarea, Porifera) of Norway (NE Atlantic) with the description of five new species. *Zoological Journal of the Linnean Society* **147:** 331–365.

Rapp HT, Janussen D, Tendal OS. 2011. Calcareous sponges from abyssal and bathyal depths in the Weddell Sea, Antarctica. *Deep Sea Research Part II*. Available at http://dx.doi.org/10.1016/j.dsr2.2010.05.022

Rapp HT, Klautau M, Valentine C. 2001. Two new species of *Clathrina* (Porifera, Calcarea) from the Norwegian coast. *Sarsia* **86:** 69–74.

Schmidt O. 1862. *Die Spongien des adriatischen Meeres*. Leipzig: Wilhelm Engelmann, i–viii, 1–88, pls 1–7.

Schmidt O. 1864. *Supplement der Spongien des adriatischen Meeres. Enthaltend die Histologie und systematische Ergänzungen*. Leipzig: Wilhelm Engelmann, i–vi, 1–48, pls 1–4.

Solé-Cava AM, Klautau M, Boury-Esnault N, Borojevic R, Thorpe JP. 1991. Genetic evidence for cryptic speciation in allopatric populations of 2 cosmopolitan species of the calcareous sponge genus *Clathrina*. *Marine Biology* **111:** 381–386.

Swofford DL. 2003. *PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4*. Sunderland, MA: Sinauer Associates.

Tamura K, Dudley J, Nei M, Kumar S. 2007. Mega 4: molecular evolutionary genetics analysis (MEGA), software version 4.0. *Molecular Biology and Evolution* **24:** 1596–1599.

Thacker AG. 1908. On collections of the Cape Verde Islands fauna made by Cyril Crossland, M.A. The Calcareous sponges. *Proceedings of the Zoological Society of London* **49:** 757–782.

Valderrama D, Rossi AL, Solé-Cava AM, Rapp HT, Klautau M. 2009. Revalidation of *Leucetta floridana* (Haeckel, 1872) (Porifera, Calcarea): a widespread species in the tropical western Atlantic. *Zoological Journal of the Linnean Society* **157:** 1–16.

Von Lendenfeld R. 1885. A monograph on the Australian sponges. III. The Calcispongiae. *Proceedings of the Linnean Society of New South Wales* **4:** 1083–1150.

Waeschenbach A, Cox CC, Littlewood DTJ, Porter JS, Taylor PD. 2009. The first molecular estimate of cyclostome bryozoan phylogeny confirms extensive homoplasy among skeletal characters used in traditional taxonomy. *Molecular Phylogenetics and Evolution* **52:** 241–251.

Wörheide G, Hooper JNA. 1999. Calcarea from the Great Barrier Reef. 1: cryptic Calcinea from Heron Island and Wistari Reef (Capricorn-Bunker Group). *Memoirs of the Queensland Museum* **43:** 859–891.

Wörheide G, Nichols SA, Goldberg J. 2004. Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. *Molecular Phylogenetics and Evolution* **33:** 816–830.