Phylogeny of Tetillidae (Porifera, Demospongiae, Spirophorida) based on three molecular markers

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ABSTRACT

Tetillidae are spherical to elliptical cosmopolitan demosponges. The family comprises eight genera: namely, *Acanthotetilla* Burton, 1959, *Amphitethya* Lendenfeld, 1907, *Cinachyra* Sollas, 1886, *Cinachyrella* Wilson, 1925, *Craniella* Schmidt, 1870, *Fangophilina* Schmidt, 1880, *Paratetilla* Dendy, 1905, and *Tetilla* Schmidt, 1868. These genera are characterized by few conflicting morphological characters, resulting in an ambiguity of phylogenetic relationships. The phylogeny of tetillid genera was investigated using the *cox1*, 18S rRNA and 28S rRNA (C1–D2 domains) genes in 88 specimens (8 genera, 28 species). Five clades were identified: (i) *Cinachyrella*, *Paratetilla* and *Amphitethya* species, (ii) *Cinachyrella levantinensis*, (iii) *Tetilla*, (iv) *Craniella*, *Cinachyra* and *Fangophilina* and (v) *Acanthotetilla*. Consequently, the phylogenetic analysis supports the monophyly of *Tetilla*, a genus lacking any known morphological synapomorphy. *Acanthotetilla* is also recovered. In contrast, within the first clade, species of the genera *Paratetilla* and *Amphitethya* were nested within *Cinachyrella*. Similarly, within the fourth clade, species of the genera *Cinachyra* and *Fangophilina* were nested within *Craniella*. As previously postulated by taxonomists, the loss of ectodermal specialization (i.e., a cortex) has occurred several times independently. Nevertheless, the presence or absence of a cortex and its features carry a phylogenetic signal. Surprisingly, the common view that assumes close relationships among sponges with porocalices (i.e., surface depressions) is refuted.

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1. Introduction

Tetillidae is a family of mostly spherical to elliptical spirophorid demosponges. Due to their spherical shape and the circular depressions that often appear on their surface (porocalices) they are frequently referred to as "golf ball sponges" and "moon sponges". Tetillidae are distributed worldwide and across a wide range of depths (van Soest et al., 2012; van Soest and Rützler, 2002). They have been found to be a source of secondary metabolites with potential medical applications (Aiello et al., 1991; Atta et al., 1989; Cardellina et al., 1983; McClintock and Gauthier, 1992).

Tetillid sponges are characterized by a spherical, spiraling growth form, the presence of specialized pits called porocalices that contain the inhalant ostia and occasionally exhalant orifices, and the possession of unique contorted and microspined sigma-spires. Sigmaspires are a synapomorphy of the order Spirophorida that contains Tetillidae. However, not all members of the family possess these diagnostic tetillid characteristics. For instance, the genus *Tetilla*, after which the family is named, does not possess porocalices, and *T. euplocamos* Schmidt, 1868, the type species of the genus, also lacks sigmaspires (Rützler, 1987).

Tetillid taxonomy fluctuated greatly both prior to and following the establishment of Tetillidae by Sollas (1886). Stability was achieved by Rützler (1987), who revealed the ambiguity of certain diagnostic characters and defined more reliable ones (Table 1). Today, Tetillidae comprise eight genera: *Acanthotetilla* Burton, 1959, *Amphitethya* Lendenfeld, 1907, *Cinachyra* Sollas, 1886, *Cinachyrella* Wilson, 1925, *Craniella* Schmidt, 1870, *Fangophilina* Schmidt, 1880, *Paratetilla* Dendy, 1905, and *Tetilla* Schmidt, 1868 (van Soest and Rützler, 2002). The family is perceived as having few diagnostic taxonomic characters that can be applied in order to clearly differentiate genera. In addition, some characters are difficult to recognize consistently. The first character used for identification is the

Table 1

Morphological characters with taxonomic importance in tetillids. Modified from Rützler (1987) and van Soest and Rützler (2002). + = presence, − = absence.

Genus	Cortex (reinforced by)	Porocalices (shape)	Accessory spicule (type)
Acanthotetilla	+ (Megacanthoxeas)	+ (Narrow)	+ (Megacanthoxeas)
Amphitethya	+ (Amphiclads)	−	+ (Amphiclads)
Cinachyra	+ (Minute smooth oxeas)	+ (Flask)	−
Cinachyrella	−	+ (Hemi-spherical or narrow)	−
Craniella	+ (Minute smooth oxeas)	−	−
Fangophilina	−	+ (Narrow)	−
Paratetilla	−	+ (Hemi-spherical or narrow)	+ (Calthrops-like triaenes)
Tetilla	−	−	−

presence or absence of porocalices, although several porocalyx morphologies exist. Another important character used is the presence or absence of a protein cortex reinforced by special cortical spicule types. Finally, the presence or absence of specific megasclere types (i.e., acanthoxea, amphiclads and calthrops-like triaenes) is also used (Table 1) (van Soest and Rützler, 2002).

Rützler (1987) argued that the presence of a protein cortex in tetillids is an inappropriate taxonomic character, since it can be easily overlooked. Instead, he considered the spicular reinforcement of the cortex as the true stable marker. Species identified or revisited, considering Rützler's guidelines, have remained stable regarding their generic affiliation. The morphological characteristics of each of the eight genera are described in Table 1 and in Supplementary file 1 as stated in van Soest and Rützler (2002).

It should be noted that the presence of porocalices and the presence of a spiculated cortex would conflict in a phylogenetic analysis. Indeed, the presence of a cortex spiculated with minute oxeas is a characteristic shared by *Craniella* and *Cinachyra* while porocalices are found in *Acanthotetilla*, *Cinachyra*, *Cinachyrella*, *Paratetilla* and *Fangophilina*, but not in *Craniella*. In terms of phylogeny, the presence of porocalices is viewed as a more informative character than the presence of a spiculated cortex (van Soest and Rützler, 2002). Consequently, *Acanthotetilla*, *Cinachyrella*, *Paratetilla*, *Cinachyra* and *Fangophilina* are considered to be closely related genera, although shape differences have been noted between porocalices of different genera (e.g., flask-shape versus hemispherical). Based on the shared presence of porocalices, it has been suggested that *Fangophilina* might be a junior synonym of *Cinachyrella* (Supplementary file 1; van Soest and Rützler, 2002). The only molecular study that includes more than ten different tetillid species suggests that some tetillid genera need to be revised (Szitenberg et al., 2010). Specifically, a *Paratetilla* species was found to be nested among *Cinachyrella* representatives.

The goals of this study were thus twofold: (1) to determine the phylogenetic relationships among tetillid genera; and (2) to evaluate the importance of the porocalices and spiculated cortex as phylogenetic markers. We employed three molecular markers (the *cox1*, 18S rRNA and the 28S rRNA genes) and representatives of 28 species in order to reconstruct a reliable phylogeny of Tetillidae (Tables 2 and S1).

2. Material and methods

2.1. Taxon sampling, DNA extraction, amplification and sequencing

A total of 88 tetillid specimens were included in this study, comprising two *Acanthotetilla* species, one *Amphitethya*, two *Cinachyra*, nine *Cinachyrella*, eight *Craniella*, one *Fangophilina*, one *Paratetilla* and four *Tetilla* species (Table 2). Genomic DNA was extracted from ethanol-preserved tissue samples following the procedure of Fulton et al. (1995). In many cases, the DNA obtained was found to be degraded and therefore PCR products were difficult to obtain. The *cox1* gene was the first marker we attempted to amplify since it is used as a DNA barcoding and phylogenetic marker in sponges (e.g., Cárdenas et al., 2009; Erpenbeck et al., 2007; Erpenbeck et al., 2006; Morrow et al., 2012; Poeppe et al., 2010; Redmond et al., 2010; Solé-Cava and Wörheide, 2007) and previous work on Tetillidae had been done using this marker (Szitenberg et al., 2010). The *cox1* gene was sequenced for 57 specimens. For 28 degraded samples only a small fragment of the *cox1* gene, smaller than 600 bp, could be obtained (Table 2). Twenty-three primer pairs were used to amplify between one to five overlapping fragments of varying lengths of the *cox1* gene for each species (Table S2). The fragments were obtained after several reamplifications, conducting nested or semi-nested PCRs. Up to three reamplifications were performed to obtain each fragment. The sequencing of the *cox1* gene yielded contigs of 462–1215 bp in length (Table 2).

Preliminary results showed that the 18S rRNA gene was less informative than the *cox1* gene, at both the species and genus level (see Section 3). Given the great difficulty in amplifying most of the museum samples we received, the 18S rRNA gene was sequenced for only 27 samples representative of six tetillid genera. The 18S rRNA gene was first amplified using the 18S1 forward primer (Borchiellini et al., 2004) and the 18S2-mod reverse (modified from Borchiellini et al., 2004; Table S2). Each PCR product then was reamplified once or twice using diverse nested primers (Table S2). The length of the amplicons ranged from 800 to 1722 bp (Table 2).

In order to complement the phylogenetic signal obtained with the *cox1* and the 18S rRNA genes, the variable C1–D2 region of the 28S rRNA gene was amplified for 41 samples which had reasonable DNA quality (Table 2). Semi-nested PCRs were also conducted to amplify the C1–D2 fragment of the 28S rRNA gene, using the C'1-mod forward primer (modified from Chombard et al., 1998; Table S2) and the D2 reverse primer (Chombard et al., 1998) in the first reaction, and the ITS4F forward primer (modified from Chombard et al., 1998; Table S2) and the D2 reverse primer in the first reamplification. A second reamplification of the PCR product was often required, in which the SN47F-mod forward primer (modified from Kober and Nichols, 2007; Table S2) and the D2 reverse primer were used. This procedure yielded 760–820 bp long fragments. In cases in which this approach failed, the amplification of a shorter fragment was attempted. In this case the first amplification was conducted using the C'1-mod forward primer and the 28S-tetR1 reverse primer (Table S2). The first nested reamplification was conducted with ITS4F forward primer and 28S-tetR2 reverse primer (Table S2). Finally the second nested reamplification was conducted with SN47F-mod forward primer and 28S-tetR2 reverse primer. This procedure yielded 450–670 bp long fragments (Table 2).

Most PCR products were directly sequenced using Big Dye Terminator v1.1 (Applied Biosystems) on an ABI 310 sequencer. In a few cases, sequencing revealed more than one sequence. In such cases PCR products were ligated into the pSC-A vector. Ligation products were then transformed into StrataClone™ SoloPack® competent cells (Stratagene). At least three clones per species were sequenced. Both strands of all genes were sequenced. The 125

Table 2

Sequences used in the phylogenetic analyses. Accession – GenBank accession numbers. Length: bp length of the analyzed (total) fragment, *cox1* sequence lengths are given excluding the length of mitochondrial introns when present. Specimens included in the combined analysis are shaded. Acronyms: RMNH – Rijksmuseum van Natuurlijke Historie, Leiden, Nederland; UFBA – Universidade Federal da Bahia, Brazil; MNRJ – Museu Nacional do Rio de Janeiro, Brazil; Bioice – The inter-Nordic BIOICE project; VM – Museum of Natural History and Archaeology, a part of the University of Science and Technology, Trondheim, Norway; ZMBN – Zoologisk Museum Bergen Norge; NIWA – National Institute of Water and Atmospheric Research, New Zealand; TAU – Steinhardt National Collection of Natural History, Zoological Museum at Tel Aviv University, Israel; SAM – South Australia Museum; QM – Queensland Museum, Australia; MHNM – Muséum d'histoire naturelle Palais Longchamp, Marseille, France; DH, LB, MI – lab collections of the authors. Sequences taken from GenBank are marked with an asterisk.

Taxonomy	Tissue number	*Cox1*		28S rRNA		18S rRNA	
		Accession	Length	Accession	Length	Accession	Length
Acanthotetilla							
A. celebensis	RMNH POR 2877	JX177909	622 (746)	JX177921	654 (655)		
A. walteri	UFBA 1897 POR	JX177908	462 (462)				
A. walteri	UFBA 2021 POR	JX177907	622 (771)	JX177922	765 (789)		
A. walteri	UFBA 2028 POR	JX177911	400 (436)				
Tetilla							
T. radiata	MNRJ 576	*HM032742	622 (1215)	JX177958	774 (820)		
T. japonica	TAU 25619	JX177901	622 (766)	JX177957	673 (709)	JX177913	615 (654)
T. japonica						*D15067	1598 (1716)
T. pentatriaena	UFBA 3646 POR	JX177899	499 (607)				
T. muricyi	UFBA 2568 POR	JX177898	592 (628)	JX177959	553 (563)		
Craniella							
Craniella sp.	Bioice 3659	*HM032750	622 (1134)				
C. zetlandica	VM 14754	*HM032751	622 (1110)	JX177961	663 (583)	JX177986	1515 (1516)
C. cranium	ZMBN 85239	*HM592669	622 (658)	JX177960	693 (725)	JX177987	1042 (1108)
Craniella sp 3318	QM G 318785	*HM032752	622 (822)			JX177985	818 (820)
Craniella sp 3878	QM G 316342	*HM032747	622 (984)	JX177956	777 (816)	JX177983	1611 (1718)
Craniella sp 3878	QM G 316372	*HM032748	622 (984)			*HE591469	1602 (1701)
Craniella cf. *leptoderma*	NIWA 27816			JX177948	518 (520)		
Craniella cf. *leptoderma*	NIWA 52077	JX177917	622 (1178)	JX177949	658 (669)		
Craniella cf. *leptoderma*	NIWA 28524	JX177895	616 (729)	JX177946	758 (763)	JX177976	1540 (1541)
Craniella cf. *leptoderma*	NIWA 28496	JX177897	622 (701)	JX177947	769 (780)		
Craniella cf. *leptoderma*	NIWA 28507	JX177896	622 (1120)	JX177944	759 (764)	JX177975	1577 (1541)
Craniella cf. *leptoderma*	QM G 315031	*HM032749	622 (1134)	JX177943	778 (816)	JX177974	1314 (1359)
Craniella cf. *leptoderma*	NIWA 28910	JX177865	551 (551)			JX177982	1526 (1527)
Craniella cf. *leptoderma*	NIWA 36097	JX177866	585 (661)	JX177945	770 (780)		
C. sagitta	NIWA 25206	JX177918	622 (727)			JX177981	1520 (1520)
C. sagitta	NIWA 28491	JX177916	618 (776)				
C. sagitta	NIWA 28929	JX177863	586 (612)	JX177952	770 (780)		
C. neocaledoniae	NIWA 28591					JX177984	1540 (1542)
Cinachyra							
C. antarctica	QM G 311149	JX177915	622 (765)				
C. antarctica	NIWA 28957	JX177867	622 (651)	JX177950	770 (780)		
C. antarctica	NIWA 28951	JX177868	622 (636)				
C. barbata	NIWA 28877	JX177864	584 (611)	JX177951	774 (785)	JX177977	1540 (1542)
Fangophilina							
Fangophilina sp.	NIWA 28586	JX177920	622 (1190)	JX177954	769 (780)	JX177978	1540 (1541)
Fangophilina sp.	NIWA 28601	JX177919	621 (1190)	JX177955	644 (647)	JX177979	1540 (1541)
Fangophilina sp.	NIWA 28614			JX177953	749 (760)		
Fangophilina sp.	NIWA 28617	JX177912	622 (1192)			JX177980	1540 (1541)
Cinachyrella							
C. apion	Flatts Inlet	*AJ843895	622 (654)				
C. apion	B25	*EF519601	582 (584)				
C. apion	ZMBN 81785	*FJ711645	622 (673)				
C. apion	ZMBN 1789	*HM592667	622 (658)				
C. kuekenthali	B79	*EF519602	582 (584)				
C. kuekenthali	K75	*EF519603	582 (584)				
C. kuekenthali	ZMBN 81787	*FJ711646	622 (673)				
C. kuekenthali		*NC_010198	622 (1221)				
C. levantinensis	TAU 25456	*AM076987	622 (1206)			*HM629802	1575 (1762)
C. levantinensis	MHNM 16194	JX177905	622 (1180)	JX177942	777 (799)	*HM629803	1574 (1716)
C. levantinensis	TAU 25618	JX177903	622 (1180)	JX177939	777 (797)		
C. levantinensis	TAU 25568	JX177904	622 (1183)	JX177941	513 (525)	JX177969	1574 (1716)
C. levantinensis	TAU 25529	JX177906	622 (1182)	JX177940	777 (797)	JX177970	1573 (1713)

Table 2 (continued)

Species	Voucher	cox1		28S		18S	
C. australiensis	QM G 320216	JX177902	622 (752)			JX177966	1554 (1630)
C. australiensis	QM G 321405			JX177931	451 (480)	JX177968	1563 (1657)
C. australiensis	QM G 320656					JX177967	1575 (1716)
C. australiensis	LB 637	JX177869	533 (533)				
C. australiensis	LB 436	JX177883	500 (500)				
C. australiensis	LB 645	JX177878	483 (483)				
C. australiensis	LB 664	JX177873	483 (483)	JX177929	621 (624)		
C. australiensis	LB 662	JX177872	533 (533)				
C. australiensis	LB 424	JX177876	500 (500)				
C. australiensis	LB 91	JX177875	500 (500)				
C. australiensis	LB 425	JX177877	500 (500)				
C. australiensis	LB 80	JX177874	500 (500)				
C. australiensis	LB 647	JX177879	483 (483)	JX177938	509 (515)		
C. australiensis	LB 1223	JX177882	483 (483)				
C. australiensis	LB 817	JX177881	483 (483)	JX177933	776 (808)		
C. australiensis	LB 815	JX177880	461 (461)				
C. australiensis	LB 1232	JX177871	533 (533)				
Cinachyrella sp.	TAU 25621	*HM032740	622 (1213)			JX177964	1499 (1499)
Cinachyrella sp.	TAU 25622	*HM032739	622 (1213)			JX177962	1497 (1497)
Cinachyrella sp. 3473	QM G 320270	*HM032741	600 (1076)	JX177932	777 (808)	JX177963	1563 (1590)
C. alloclada	TAU 25623	*HM032738	622 (1215)				
C. alloclada	TAU 25617	JX177914	622 (1105)	JX177936	776 (801)	JX177965	1488 (1488)
C. anomala	LB 913	JX177888	483 (483)				
C. anomala	LB 1756	JX177886	500 (500)	JX177934	765 (776)		
C. anomala	LB 1750	JX177887	500 (500)				
C. anomala	LB 45	JX177884	484 (484)	JX177935	513 (514)		
C. anomala	LB 1757	JX177885	500 (500)				
C. schulzei	QM G 320636	*HM032745	(1194)			JX177971	1575 (1719)
C. schulzei	QM G 320143	*HM032746	622 (1185)	JX177923	772 (810)	JX177973	804 (805)
C. paterifera	LB 112	JX177889	440 (440)				
C. paterifera	LB 113	JX177890	622 (440)	JX177937	662 (667)		
Amphitethya							
Amphitethya cf. microsigma	SAM S1189	JX177910	622 (767)	JX177930	778 (804)		
Paratetilla							
P. bacca	RMNH POR 3206	JX177892	564 (896)	JX177926	776 (813)		
P. bacca	RMNH POR 3100	JX177891	534 (872)				
P. bacca	TAU 25620	JX177900	622 (964)	JX177927	419 (419)		
P. bacca	QM G 314224	*HM032744	622 (1116)	JX177925	428 (485)		
P. bacca	QM G 306342			JX177928	664 (669)		
P. bacca	LB 622	JX177894	600 (844)				
P. bacca	LB 671	JX177893	597 (840)	JX177924	653 (672)	JX177972	1553 (1601)
P. bacca	LB 1231	JX177870	536 (536)				
Geodia neptuni		*AY320032	622 (1221)			*AY737635	1617 (1763)
Pachymatisma johnstonia		*EF564339	622 (673)	*HM592832	760 (778)		
Geodia barretti		*EU442194	622 (658)				
Calthropella geodioides		*HM592705	622 (658)	*HM592826	770 (813)		
Thenea levis		*HM592717	622 (658)	*HM592765	772 (813)		
Theonella swinhoei		*HM592745	622 (658)	*HM592820	769 (806)		
Geodia cydonium						*AY348878	1606 (1722)

newly obtained sequences were submitted to GenBank under accession numbers JX177863–JX177987 (Table 2).

2.2. Phylogenetic analyses

2.2.1. Datasets

Phylogenetic trees were reconstructed from four datasets, one for each marker separately and a combined dataset. In each analysis, Astrophorida species were used as outgroup since this order has been established to be the sister clade of Spirophorida (e.g., Borchiellini et al., 2004; Nichols, 2005). The cox1 gene dataset comprises 81 tetillid sequences and six astrophorid sequences. The 28S rRNA gene dataset comprises 41 tetillid sequences and four astrophorid sequences. The 18S rRNA gene dataset comprises 33 tetillid sequences and two astrophorid sequences. The combined dataset comprises 53 specimens, for which the cox1 gene and at

least one of the two rRNA genes were sequenced (Table 2). The datasets are available in Supplementary file 2 and in the Dryad repository doi:10.5061/dryad.10gh5.

2.2.2. Multiple alignments

The *cox1* gene dataset was aligned twice, once with the L-ins-i algorithm implemented in Mafft 6 (Katoh et al., 2005; Katoh and Toh, 2008) and once with the online version of MUSCLE (Edgar, 2004a,b) with default options (we verified manually that no frame shift was introduced in the alignment). The two alignments were compared with SOAP 1.2a4 (Löytynoja and Milinkovitch, 2001). All positions were found to be identically aligned in the two alignments. Following Szitenberg et al. (2010), in order to exclude potential co-conversion tracts which originated from the insertion of self-splicing introns (some tetillid species possess group I introns in their *cox1* sequence), the 18 nucleotides located downstream to each known intron insertion site were removed from the alignment. In addition, positions with a proportion of missing data higher than 0.4 were also removed.

The two rRNA genes datasets were aligned according to the consensus secondary structures derived from Voigt et al. (2008) and from Morrow et al. (2012) for the 18S rRNA gene and the 28S rRNA gene, respectively. Specifically, preliminary 18S rRNA and 28S rRNA alignments were performed including the tetillid sequences and sequences from the datasets of Voigt et al. (2008) and of Morrow et al. (2012). The sequence of *Tetilla japonica* (GenBank accession number D15067) was used to align the 18S rRNA dataset with the consensus structure scheme. Since there are no tetractinellid sequences in the Morrow et al. (2012) dataset, the sequence of *Axinella damicornis* (GenBank accession number HQ379198, one of the most complete G4 sequences) was used to align the 28S rRNA dataset with the consensus structure scheme. For both genes, the tetillid alignments fitted the structure scheme without any need for manual correction (i.e., the tetillid sequences did not include additional stems or loops). Positions with a proportion of missing data higher than 0.4 were also excluded from the rRNA genes datasets, unless participating in a stem structure.

2.2.3. Phylogenetic reconstruction

For all datasets, phylogenetic reconstructions were performed using the maximum likelihood (ML) criterion and the Bayesian framework. ML analyses were all performed with RAxML 7.2.6 (Stamatakis, 2006) while Bayesian analyses were either conducted with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) for the *cox1* gene dataset, or with PHASE 2 (www.bioinf.manchester.ac.uk/resources/phase/index.html) for the combined dataset and the rRNA datasets.

To facilitate comparison between analyses, the same substitution models were chosen in the Bayesian and in the ML analyses. The *cox1* gene was partitioned according to codon positions. The rRNA genes were partitioned into stem and loop partitions. For the *cox1* partitions and the loop partitions of the rRNA genes, tree searches were conducted under the GTR + Γ model as recommended in the RAxML manual. For the stem partitions of the 18S rRNA and 28S rRNA genes, the best fitting secondary structure constrained substitution models were selected with the program Optimizer of the PHASE 2 package (www.bioinf.manchester.ac.uk/resources/phase/index.html) following Tsagkogeorga et al. (2009). For the stem partitions of both rRNA genes, the RNA6D doublet-substitution matrix was identified as best-fitting. Since RAxML allows only a single stem partition per analysis, in the combined ML analysis the stem partitions of the two rRNA datasets were considered as a single partition evolving under the RNA6D + Γ model. In the Bayesian analysis of the combined dataset the two stem partitions were analyzed separately (each one under the RNA6D + Γ model).

RAxML tree reconstructions were carried out using 100 random starting trees. Branch support was computed based on 1000 boot-

strap replications. In the MrBayes analysis (*cox1* bayesian analysis), two runs with eight chains each were conducted, with default temperatures and prior distributions. The chains were sampled every 100 generations. Model parameters were allowed to be optimized independently for each codon position partition. Convergence was achieved at 600,000 generations when standard deviation of split frequencies reached 0.009. After convergence, the sampling continued until the analysis reached 3,000,000 generations. The first 600,000 generations, amounting to 20% of the total number of generations, were discarded as burnin. In PHASE (combined and rRNA analysis), 15,000,000 iterations were conducted, out of which every 100th tree was sampled. Convergence of the parameters was assessed with the program Tracer v1.4 (www.http://tree.bio.ed.ac.uk/software/tracer/). The first 20% of the trees were discarded as burnin. To further confirm convergence, computations were run twice, specifying a different random seed in each MCMC run. In all cases, similar results were obtained for the two independent runs (only the results of the first run are presented).

2.2.4. Testing alternative tree hypotheses

An AU (Approximate Unbiased) test was performed to compare the *cox1* and combined RAxML trees to seven or five alternative topologies respectively, each presenting the monophyly of one lineage that was not recovered in the unconstrained phylogenetic reconstructions (i.e., 1 – the genus *Cinachyrella* monophyly, 2 – the genus *Craniella* monophyly, 3 – *Paratetilla bacca* monophyly, 4 – *Cinachyrella australiensis* monophyly, 5 – *Cinachyrella kuekenthali* monophyly, 6 – *Cinachyrella alloclada* monophyly, 7 – *Cinachyra antarctica* monophyly). The alternative topologies were reconstructed using constrained ML searches. Specifically, for each lineage tested an ML tree, which constrained the monophyly of the corresponding lineage, was built using RAxML 7.2.6 (Stamatakis, 2006), under the models and parameters indicated above. A separate set of constrained trees was built from each of the *cox1* and combined datasets. The resulting constrained trees were compared with the unconstrained ML tree using CONSEL V0.1i (Shimodaira and Hasegawa, 2001) with 100,000 bootstrap replicates and RELL optimization. Other parameters were set to default values. The null hypothesis in the AU test is that the enforced relationships are as likely as the observed ones.

2.3. Evolution of morphological characters

The ancestral states of four morphological characters were reconstructed by calculating their proportional likelihoods (P_L). The characters were the presence or absence of: 1 – porocalices; 2 – proteinous subectosomal layer; 3 – subectodermal smooth minute oxeas; 4 – acanthose monoaxonic megascleres; 5 – calthrops-like short shafted triaenes (Table S3). The presence of amphiclad spicules, which is a character found exclusively in *Amphitethya*, could not be included in our analysis since this genus was represented by a single specimen. Following Cárdenas et al. (2011), the P_L of each ancestral character state was computed with Mesquite (Maddison and Maddison, 2006, 2011) using the phylogenetic relationships of the *cox1* tree (in this analysis each monophyletic species was represented by a single terminal taxon, polyphyletic and paraphyletic species were represented by one terminal node per lineage) and the default Mk1 evolutionary model for categorical characters (Lewis, 2001).

3. Results

3.1. Molecular phylogeny

3.1.1. Comparison of molecular markers

In this study, we sequenced three different markers, the *cox1*, 18S rRNA and 28S rRNA genes, in order to resolve tetillid
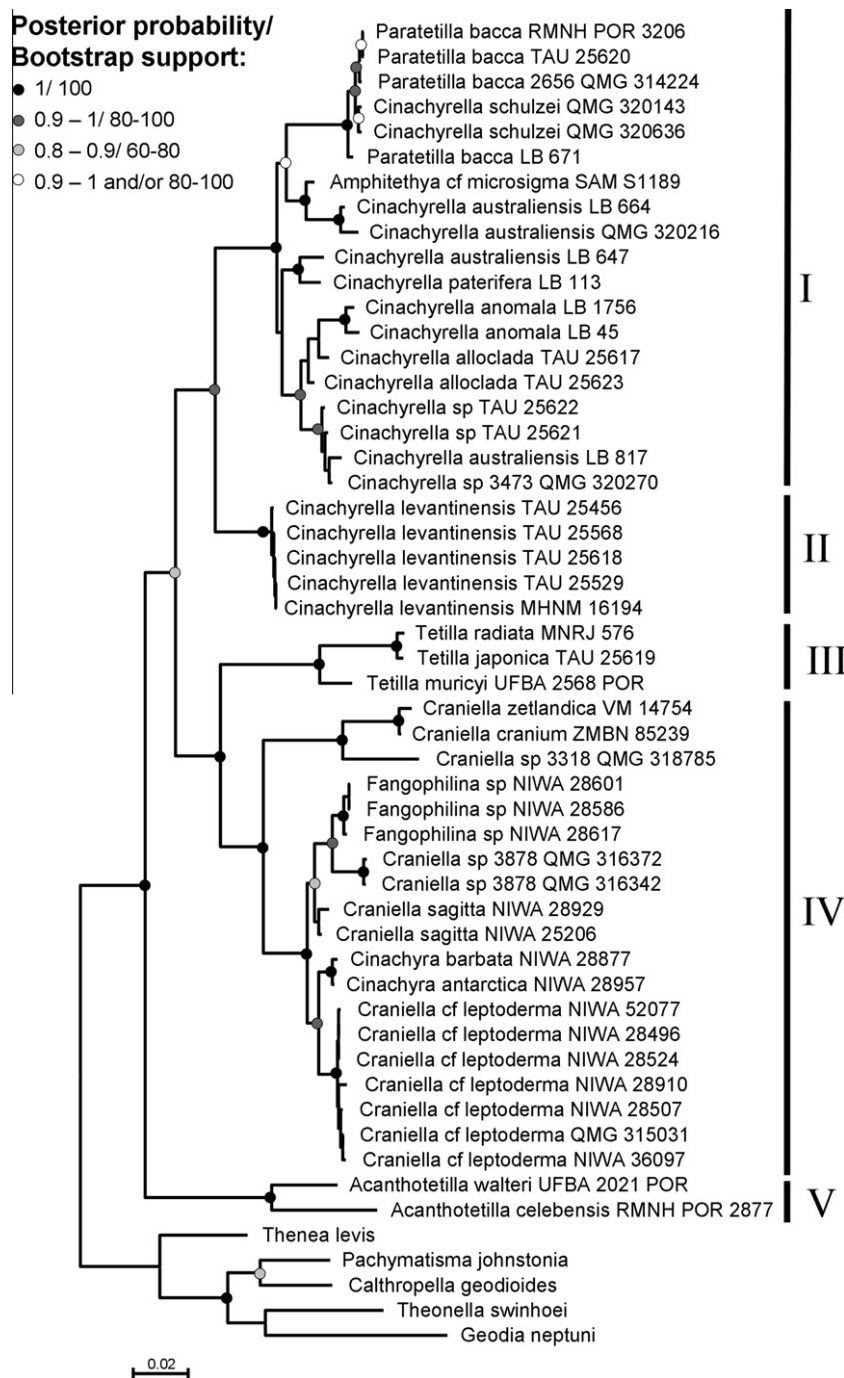
Fig. 1. Bayesian phylogenetic tree reconstructed from a combined dataset of the *cox1* gene and the 18S and 28S rRNA sequences. Circles represent node supports. Solid circles: PP = 1, BP = 100. Dark gray circles: 0.9 < PP < 1, 80 < BP < 100. Light gray circles: 0.8 < PP < 0.9, 60 < BP < 80. White circles: a support of either PP > 0.9 or of BP > 80.

phylogeny. The performance of the three markers varied considerably. The 18S rRNA gene marker provided little phylogenetic signal (Fig. S1), which resulted in lower resolution, shorter branch lengths and lower node supports than those obtained with the *cox1* (Fig. 2) and 28S rRNA (Fig. S2) genes. A quick visual comparison of Figs. S1, 2, and S2 is sufficient to make these differences evident. However, it is worth noting that the addition of the 18S rRNA gene in the combined dataset considerably improved the support of several nodes (Fig. 1). In agreement with Cárdenas et al. (2010), we noticed that the C1–D2 region of the 28S rRNA gene evolves faster than the *cox1* gene (Fig. S3). However, the *cox1* gene provides a better phylogenetic signal for several nodes. For example, the relationships

between *P. bacca* and *C. schulzei* are better resolved with the *cox1* gene. The two most informative analyses are thus those of the combined dataset (Fig. 1) and of the *cox1* gene (Fig. 2). The combined analysis resolves the relationships among the major tetillid clades with high support, while the large taxonomic sampling of the *cox1* analysis allows evaluation of the monophyly of several species. We here only discuss the results of these two analyses.

3.1.2. Major tetillid clades

In the combined tree (Fig. 1), five well supported clades emerge. Clade I comprises *Cinachyrella* species, *Paratetilla* and *Amphitethya* (Posterior Probability, PP = 1; Bootstrap Percentage, BP = 100).
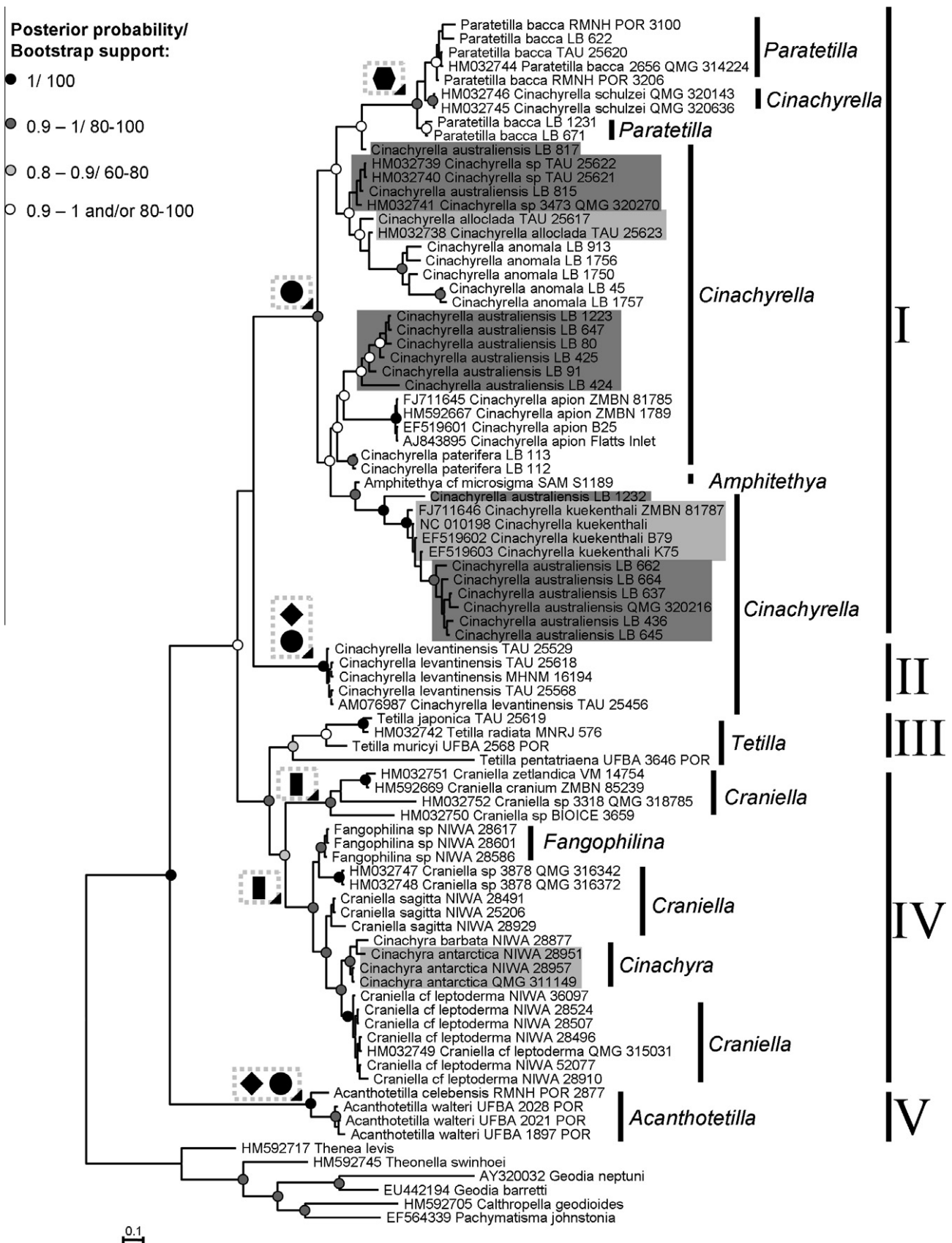
Fig. 2. Bayesian phylogenetic tree reconstructed from the *cox1* gene. Circles represent node supports. Black circles: PP = 1, BP = 100. Dark gray circles: 0.9 < PP < 1, 80 < BP < 100. Light gray circles: 0.8 < PP < 0.9, 60 < BP < 80. White circles: a support of either PP > 0.9 or of BP > 80. Species shaded in light gray are paraphyletic. Species shaded in dark gray are polyphyletic. The presence of diagnostic features found to be likely (proportional likelihood P_L > 0.85) in the ancestors of major clades is indicated within gray dashed frames at the base of nodes as follows: Circles: porocalices, diamonds: spined monoaxons, rectangles: minute ectosomal oxeas, hexagons: calthrops-like triaenes.

Paratetilla bacca was paraphyletic as it contained *C. schulzei* (PP = 1, BP = 100). Clade II comprises *Cinachyrella levantinensis* (PP = 1, BP = 100) and it is sister to clade I (PP = 1, BP = 92). Clade III comprises the three *Tetilla* species (PP = 1; BP = 100). Clade IV encompasses all species from the genera *Craniella*, *Cinachyra* and *Fangophilina* (PP = 1; BP = 100). This clade comprises two distant sister subclades. The first subclade includes *Craniella zetlandica*, *Craniella cranium* and *Craniella* sp. 3318 (PP = 1; BP = 100). The second subclade includes all other *Craniella*, *Cinachyra* and *Fangophilina* species considered (PP = 1; BP = 100). Within this subclade, *Cinachyra* species cluster together (PP = 1; BP = 100). Clade IV is sister to Clade III (*Tetilla*) with high support (PP = 1; BP = 98). Clade V is the first to diverge among the tetillid clades (PP = 0.97; BP = 72) and includes the *Acanthotetilla* species (PP = 1; BP = 100). The five clades that emerge in the combined analysis are also recovered by the analysis of the *cox1* dataset, but with lower support (Fig. 2).

3.1.3. Phylogenetic hypotheses testing

The large species sampling of the *cox1* gene allowed us to test the monophyly of lineages that are recovered as paraphyletic (Table 3). At the genus level, the AU test rejected the monophyly of *Cinachyrella* and *Craniella* (*p*-value <0.001 for both genera in both the *cox1* and combined analyses). At the species level, the AU test could not reject the monophyly of *Cinachyrella kuekenthali*, *Cinachyrella alloclada*, *Cinachyra antarctica* and *Paratetilla bacca* which appear paraphyletic in the ML *cox1* and combined trees (*p*-value >0.05 for all four species in both the *cox1* and combined analyses). However, the monophyly of *Cinachyrella australiensis* was clearly rejected (*p*-value <0.001 in both analyses). *C. australiensis* is polyphyletic and is represented by five distinct lineages. Interestingly, the different clades do not correspond to different geographical areas. All other species were found to be monophyletic.

3.2. The evolution of morphological characters

3.2.1. Porocalices

Both the presence and absence of porocalices were equally likely in the ancestor of Tetillidae (P_L = 0.6 for presence, and thus P_L = 0.4 for absence). Therefore, the analysis does not allow us to determine whether porocalices are symplesiomorphic in tetillids. The presence of porocalices was found to be slightly more likely than their absence in the ancestor of all *Cinachyrella* species (clades I + II, P_L = 0.8 for presence), and an almost certain presence in the common ancestors of clades V (*Acanthotetilla*; P_L = 0.99), I and II (P_L = 0.98 and P_L = 1 respectively). In the ancestors of clades III and IV, the absence of porocalices was found to be the most likely (P_L = 0.97, for absence in both clades), suggesting that the porocalices found in clade IV (in *Cinachyra* and *Fangophilina*) evolved independently from those found in clade I (in *Cinachyrella* and *Paratetilla*), clade II (*Cinachyrella levantinensis*) and clade V (*Acanthotetilla*).

3.2.2. Subectodermal smooth minute oxeas

Out of the five emerging clades, the presence of a distinct size-class of subdermal smooth oxeas was only found to be likely in the common ancestor of clade IV (P_L = 0.72). In the two subclades of clade IV the P_L was even higher (P_L = 0.99 and P_L = 0.87). Accordingly, in the tetillid ancestor the presence of such spicules was found to be unlikely (P_L = 0.03). Therefore, the subectodermal smooth minute oxeas are probably a synapomorphy of clade IV.

3.2.3. Proteinous subectosomal layer

The presence of a collagenous subdermal layer was inferred to be unlikely in the ancestor of clades V (P_L = 0.05) and II (comprising a single species). For all other clades, the presence or absence of this character was equivocal (0.39 < P_L for presence <0.51). This result reflects the fact that several unrelated lineages possess this character (i.e., *Cinachyrella paterifera* + *C. apion*, *C. alloclada*, *C. kuekenthali*, *P. bacca* + *C. schulzei*, all *Cinachyra* species and all *Craniella* species).

3.2.4. Acanthose monoaxons

The presence or absence of acanthose monoaxons in the ancestral tetillid has remained undetermined (P_L = 0.49 for presence and P_L = 0.51 for absence). Consequently, we cannot determine whether acanthose monoaxons are homoplastic or plesiomorphic in Tetillidae. Within Tetillidae, only the ancestors of *Acanthotetilla* (Clade V; megacanthoxea; P_L = 0.97) and *Cinachyrella levantinensis* (clade II; P_L = 1) were likely to have possessed such spicules. In clade I, the presence of acanthose monoaxons was somewhat unlikely (P_L = 0.3) since this clade contains six unrelated lineages possessing acanthose monoaxons. The ancestors of clade III and IV are likely to have lacked this character (P_L = 0.03) as do all their known descendants.

3.2.5. Calthrops-like triaenes

Calthrops-like triaenes are only found in *Paratetilla* which is represented in our tree by *P. bacca*. The curent *cox1* topology suggests that the common ancestor of *P. bacca* and *C. schulzei* was highly likely to possess calthrops-like triaenes (P_L = 0.98), although the *C. schulzei* specimens do not seem to possess them.

4. Discussion

4.1. The evolution of morphological characters

4.1.1. Porocalices

The presence of porocalices is considered to be a phylogenetically informative trait by taxonomists (van Soest and Rützler, 2002). However, in the past, Wilson (1925) assumed a polyphyletic relationship between porocalices bearing Tetillidae, when he allocated *Cinachyrella* to be a subgenus of *Tetilla*, considering the lack of a cortex in both groups as more informative than the presence

Table 3
AU test results. The constraint imposed on each topology is specified in the column "Monophyly imposed". The log likelihood of each alternative topology and the corresponding *p*-value of the AU test are given for the analysis based on the *cox1* dataset and the combined dataset. NA: The node was represented by one or no specimen in the combined analysis and could not be tested. Clades rejected by the AU test are shaded in gray.

Monophyly imposed	ln*L*		Δln*L*		*p*-Value	
	cox1	Combined	*cox1*	Combined	*cox1*	Combined
C. antarctica	4112.340603	NA	0	NA	0.822	NA
P. bacca	4114.045420	11462.548849	1.7	4	0.462	0.23
C. alloclada	4114.626566	11462.619122	2.3	4.1	0.203	0.222
C. kuekenthali	4118.711379	NA	6.4	NA	0.136	NA
Craniella	4147.701820	11621.097304	35.4	162.5	0.0001	2×10^{-68}
Cinachyrella	4237.370700	11719.729177	125	261.2	8×10^{-61}	10^{-11}
C. australiensis	4237.721009	11630.388854	125.4	171.8	10^{-8}	6×10^{-6}

of porocalices in only one of them. Our analyses show that genera possessing porocalices (i.e., *Acanthotetilla*, *Cinachyrella*, *Cinachyra*, *Fangophilina* and *Paratetilla*) are indeed polyphyletic (Figs. 1 and 2), refuting the current view of this character. Although the proportional likelihood values are inconclusive concerning the presence of porocalices in the tetillid ancestor, it is more likely that the porocalices of *Cinachyra* and *Fangophilina* evolved independently from those of *Cinachyrella* and *Acanthotetilla* than that they were lost independently in several lineages. The fact that different porocalyx shapes have been described (e.g., flask-shaped, hemispherical) supports this view.

It is possible to conceive a scenario in which some underlying developmental mechanism controlling the formation of porocalices is shared among all tetillids. However, the porocalices themselves can neither be considered a synapomorphy of the family nor an indicator of close relationships among genera. Our results suggest that further studies should investigate, with a larger sampling, whether or not porocalyx shapes are taxonomically informative. For instance, flask-shaped porocalices only occur in *Cinachyra* (clade IV) and hemispherical porocalices occur only in some *Cinachyrella* and *Paratetilla* (clade I) (Table 1). In addition, the deep and narrow porocalices of *Fangophilina* (clade IV) are more similar to the flask-shaped porocalices of *Cinachyra* (also clade IV) than to the hemispherical porocalices of *Cinachyrella* and *Paratetilla* (clade I) (AS personal observation).

4.1.2. Subectodermal smooth minute oxeas

Another morphological character used as a taxonomic marker is the presence of a cortex, composed of a distinct size-class of smooth oxeas (Rützler, 1987). The ancestor of clade IV may have possessed these subdermal small oxeas, as do most of its descendants, since clade IV groups the genera *Craniella* and *Cinachyra* together for the first time. The only clade IV species lacking this character belongs to *Fangophilina*. Since most species included in clade IV (potentially 49 out of 56 according to the WPD) have such spicules, small cortical oxeas can be considered a synapomorphy of clade IV that *Fangophilina* secondarily lost.

4.1.3. Proteinous subectosomal layer

The presence of a proteinous subectosomal layer does not appear to be a phylogenetically informative character. Our analyses confirm the common view that species of *Cinachyrella* possessing a prominent subectodermal collagen layer are not closely related. As stated by Rützler (1987), in addition to being homoplastic, this character can also be inadvertently overlooked, which complicates its use as a taxonomic marker.

Rützler (1987) reassigned some former *Cinachyra* species to *Cinachyrella* (i.e., *C. alloclada*, *C. apion*, and *C. kuekenthali*). His decision was based on the absence of cortical spicules, disregarding the presence of a proteinous subdermal layer. In agreement with Rützler (1987), these species cluster together with other *Cinachyrella* rather than with *Cinachyra* representatives.

4.1.4. Acanthose monoaxons

The earliest diverging tetillid group is *Acanthotetilla*, based on our phylogenetic analysis (Figs. 1 and 2). Sponges of this genus possess megacanthoxeas, which are spined monoaxons. Seven lineages of *Cinachyrella* (i.e., *C. levantinensis*, *C. kuekenthali* and five polyphyletic lineages of *C. australiensis*) also possess spined monoaxons, but of a different size than the megacanthoxeas. *C. levantinensis* is the earliest species to diverge in clade I + II and it is therefore possible that *C. levantinensis* shares this feature with *Acanthotetilla* as a result of the presence of spined monoaxons in the tetillid common ancestor. However, the remaining mentioned lineages are distantly related to *C. levantinensis* and the origin of spined monoaxons in the latter species is less clear. Unfortunately,

the ancestral character states analysis is inconclusive and does not allow us to differentiate homoplasy from plesiomorphy in this case.

4.1.5. Calthrops-like triaenes

Paratetilla is distinguished from *Cinachyrella* by the presence of unique spicules among tetillids, called calthrops-like triaenes. Since *P. bacca* was found to be nested within *Cinachyrella*, the presence of calthrops-like triaenes does not appear to justify a distinct genus for the species possessing them. In addition, *P. bacca* form a paraphyletic clade containing *C. schulzei* (Figs. 1 and 2). This suggests that calthrops-like triaenes were lost in the lineage of *C. schulzei*, or that they are rare in our *C. schulzei* specimens and are easily overlooked, thus examplifing a claim made by Rützler and Smith (1992), saying that perhaps calthrops-like triaenes should not be valued above the species level. However, since the AU test did not reject the monophyly of *P. bacca*, more data are necessary in order to confirm this result. Still, the close relationship among our *C. schulzei* specimens and *P. bacca* is supported by the presence of a different type of spicule, the smooth microxeas, in both of them. This spicule type is not reported to occur in other species included in clade I (LEB and NS pers. obs., Cárdenas et al., 2009).

4.2. Phylogeny and taxonomy of tetillid genera

The phylogenetic analysis presented here confirms that several tetillid genera are not valid in their current concept or rank, as suggested by Szitenberg et al. (2010). Phylogenetic definitions of the emerging clades and their implications for tetillid taxonomy are summarized in Supplementary file 3.

4.2.1. Clade I, Amphithethya, Cinachyrella and Paratetilla

Clade I contains all *Amphithethya*, *Cinachyrella* and *Paratetilla* species except *C. levantinensis*. *Amphithethya*, and *Paratetilla* specimens appear nested among *Cinachyrella* species. Based on the Principle of Priority, species of this clade should be synonymized under *Paratetilla*. As a consequence, *Paratetilla* loses its prevalent concept associating it with the presence of calthrops-like triaenes. However, no morphological character could be identified which characterized all members of this proposed *Paratetilla* clade. Alternatively, a subgeneric structure should be formed within clade I, with the clade of *P. bacca* and *C. schulzei* as a candidate for one subgenus that is characterized by smooth microxeas. Additional sampling from diverse geographic ranges is needed to answer the question of whether or not other subclades of clade I can be justified as constituting subgenera based on geographic distribution or morphology.

Within clade I, the phylogenetic results also conflict with the current taxonomic classification in the case of *C. australiensis*, which is represented by five unrelated lineages. Our results indicate that several cryptic *C. australiensis* exist in sympatry.

4.2.2. Clade II, Cinachyrella levantinensis

Cinachyrella levantinensis was found to deeply diverge from all the other *Cinachyrella* species examined. In addition, *C. levantinensis* is the only tetillid species known to exist in the Levantine basin of the Mediterranean Sea. Clade II is therefore a candidate to be a new genus.

4.2.3. Clade III, Tetilla

Unlike clade I, clade III supports the validity of an existing tetillid genus, *Tetilla*. So far, the justification for this genus has relied on the intuition of taxonomists since no synapomorphy has been identified, except the absence of common tetillid characteristics (van Soest and Rützler, 2002). Indeed, tetillids lacking porocalices, cortex, cortical spicules or auxiliary megascleres, were affiliated to *Tetilla*. The molecular analysis thus supports this intuition, and

provides the first positive evidence for the validity of *Tetilla*. However, unrepresented *Tetilla* species may still cluster within other genera.

4.2.4. Clade IV, Craniella, Cinachyra and Fangophilina

Clade IV contains all *Fangophilina*, *Cinachyra* and *Craniella* species. The genus *Fangophilina* Schmidt, 1880, which possesses porocalices, has been considered to be of "dubious nature" (Supplementary file 1) and has been suggested to be a junior synonym of *Cinachyrella* (Rützler, 1987; van Soest and Rützler, 2002). Since *Cinachyrella* species cluster in clades I and II, the position of the *Fangophilina* species within clade IV indicates that this is not the case.

Within clade IV, two well supported subclades emerge. The first subclade includes *Craniella zetlandica*, *C. cranium*, *Craniella* sp. 3318 and *Craniella* sp. Bioice 3659. The second clade includes the remaining *Craniella* species as well as *Fangophilina* and *Cinachyra* which appear to be nested within *Craniella*. *Fangophilina* and *Cinachyra* should therefore be synonymized under *Craniella* either as junior synonyms or as subgenera of *Craniella*. Consequently, the current concept of *Craniella*, which is characterized by the absence of porocalices, is rejected since both *Fangophilina* and *Cinachyra* possess porocalices. Since most of the species in this clade possess a distinct class of small oxeas reinforcing their subdermal region, this character can be considered to be a synapomorphy of the newly conceptualized *Craniella*.

As for clade I, it would seem that a subgeneric classification will properly reflect the diversity within clade IV and will express the current taxonomic knowledge. A larger species sampling is needed to establish subgenera within the two subclades. Within the second subclade, *Cinachyra* nonetheless appears to be monophyletic and is a candidate to form a subgenus, since it possesses both a characteristic Antarctic geographic range as well as an established synapomorphy, the flask-shaped porocalices.

Most clade III and clade IV species (e.g., *Tetilla* spp., Fernandez et al., 2011; and *C. barbata* as described in van Soest and Rützler, 2002) possess a root system unlike those observed in other clades. What differentiates it from root systems of other groups is the existence of a unique spicule class within the root. Future work should evaluate whether or not this character is a synapomorphy of the clade III + IV. Unfortunately, the literature is not always clear regarding the presence or absence of a root system (e.g., the presence or absence of a root system is not mentioned for *C. barbata* as described in Campos et al., 2007; and *C. zetlandica*, Carter, 1872), and we could not examine this character in most of our specimens, having only a small tissue fragment from each. An assessment of this character should include a thorough morphological revision of several species.

4.2.5. Clade V, Acanthotetilla

The earliest tetillid lineage to diverge comprises solely *Acanthotetilla* species, thus supporting the genus validity, and that of its synapomorphy, the megacanthoxea megascleres. This is the only genus containing encrusting (e.g., *A. walteri*; Peixinho et al., 2007), semiglobular (e.g., *A. celebensis*; de Voogd and Van Soest, 2007) as well as irregular species (*A. gorgonosclera*; van Soest, 1977).

5. Conclusions

In this study we present the first comprehensive molecular phylogeny of Tetillidae. Tetillidae was considered to be a relatively simple case in sponge taxonomy since it contains a small number of species (only 158 valid species; van Soest et al., 2012) and clear synapomorphies (e.g., porocalices). However, as in many other molecular phylogeny studies of sponges (reviewed in Wörheide et al., 2012), our findings confirm that morphological characters are affected by rampant convergence and are not always good predictors of phylogenetic relationships in sponges. This has been well accepted for the spicule characters within Tetractinellida (Cárdenas et al., 2011). However, our study shows that this is also the case for less common features, such as the porocalices. We divide Tetillidae into five well-supported clades, out of which three lack clear morphological synapomorphies. We believe that the phylogenetic model presented here will provide important information for consideration in taxonomic revision of the family.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.02.018.

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