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Research Article



Molecular and morphological data from Thoosidae in favour of the creation of a new suborder of Tetractinellida

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The Thoosidae (Porifera, Demospongiae, Tetractinellida) currently includes the genera *Thoosa*, *Alectona*, and *Delectona*. To this date, molecular data are only available for *Alectona*. In this study, the phylogenetic affinities of the genera *Thoosa* and *Alectona* have been investigated with the species *T. mismalolli*, *T. calpulli*, and *T. purpurea* from the Mexican Pacific using morphology and three molecular loci: the mitochondrial cytochrome oxidase subunit 1 (CO1 mtDNA), 28S rRNA (fragment D2), and 18S rRNA. Morphology and embryology showed that these genera are quite different from the rest of the tetractinellids because larvae of *Alectona* and *Thoosa* have unique features in sponges, such as the presence of monaxonic discs in *Thoosa* and tetraxonic discs in *Alectona* which disappear in the adult stages. A phylogenetic analysis using selected species from the order Tetractinellida revealed that *Thoosa* groups with *Alectona* thus confirming morphological studies. The peculiarities in spiculation and embryology of the *Thoosa* and *Alectona* larvae, which are markedly different from species belonging to the suborders Astrophorina and Spirophorina and their distant phylogenetic position (based on three molecular loci), suggest that Thoosidae could be placed in the new suborder Thoosina.

Key words: Astrophorina, molecular markers, Porifera, systematics, synapomorphy, Thoosidae

Introduction

The combination of molecular phylogeny with the traditional morphological characters in sponge systematics has showed incongruence in some orders and families (Morrow & Cárdenas, 2015; Morrow et al., 2013; Redmond et al., 2011), which has led to major changes in the higher classification of the Demospongiae; the class with the greatest number of species (Van Soest et al., 2012). The current classification divides Demospongiae into three subclasses: Verongimorpha, Keratosa, and Heteroscleromorpha with 22 orders, some of them recently created, or resurrected, such as the order Tetractinellida, divided into two suborders: Astrophorina and Spirophorina. The species of this order possess monactine

megascleres and triaenes in various shapes; microscleres include sigmaspires, asters, microrhabds, microxeas, or raphides (Morrow & Cárdenas, 2015).

The monophyly of Tetractinellida is clearly supported by morphological and molecular data (Borchiellini, Alivon, & Vacelet, 2004; Chombard, Boury-Esnault, & Tillier, 1998; Erpenbeck et al., 2007; Kelly & Cárdenas, 2016; Lavrov, Wang, & Kelly, 2008; Nichols, 2005). However, the first comprehensive molecular phylogenetic study of the suborder Astrophorina showed that some families and genera are polyphyletic (Cárdenas, Xavier, Reveillaud, Schander, & Rapp, 2011). One of those polyphyletic families is Aletonidae Rosell, 1996 with a particularly unstable taxonomic history due to its boring habitus, spicule morphology, and larval features. *Alectona* was first classified in the family Clionaidae d'Orbigny, 1851 (order Hadromerida) (Rützler & Stone, 1986), and

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later it was moved to the new family Alectonidae with the genera *Thoosa* Hancock, 1849 and *Delectona* de Laubenfels, 1936, as a part of the order Hadromerida (Rosell, 1996).

Vacelet (1999) suggested moving *Alectona* Carter, 1879 to Astrophorida because of the morphology of the spicules in the larvae (tetraxonic discs and amphiasters) which are similar to some astrophorid species. Nevertheless, Rützler (2002a) included this genus with other boring sponges such as *Dotona* Carter, 1880, *Spiroxya* Topsent, 1896, *Neamphius* de Laubenfels, 1953, *Scolopes* Sollas, 1888 and *Delectona* de Laubenfels, 1936, in the family Alectonidae while *Thoosa* remained in the family Clionaidae (Rützler, 2002b).

Borchiellini et al. (2004) by using a fragment of the 28S rRNA confirmed that *Alectona millari* Carter, 1879 (type species of the genus) was more closely related to astrophorids than to hadromerids. Cárdenas et al. (2011) reached the same conclusion by using the COI mtDNA; although the sequence of *A. millari* did not cluster within any of the astrophorid clades. Interestingly, the sequence diverged between the major clades of Astrophorina and Spirophorina which seems to suggest a pivotal evolutionary step between the two suborders. However, the sequence of *Neamphius huxleyi* (Sollas, 1888), (with amphiasters but no triaenes), grouped more closely to desma-bearing astrophorids (Cárdenas et al., 2011). Thus, the family Thoosidae Cockerell, 1925 was resurrected (non Alectonidae because *Thoosa* was described before *Alectona*) to include *Thoosa*, *Alectona*, *Delectona* but not *Neamphius* de Laubenfels, 1953, considered as *incertae sedis* (Cárdenas & Rapp, 2012).

In summary, different authors suggested the reallocation of *Thoosa* and *Delectona* along with *Alectona* in Thoosidae, because they share important morphological and biological features. They are excavating, and at least *Thoosa* and *Alectona* produce an unusual hoplitomella larva, which has particular spicules that are different from those observed in adult stages (Bautista-Guerrero, Carballo, Aguilar-Camacho, & Sifuentes-Romero, 2016; Topsent, 1920; Vacelet, 1999). Another

interesting character shared by *Thoosa* and *Alectona* is the pit pattern made during the process of bioerosion, different to those produced by other boring sponges (Borchiellini et al., 2004; Calcinai, Bavestrello, & Cerrano, 2004).

However, despite the morphological, cytological, and especially embryological evidence that suggest the grouping of *Alectona* with *Thoosa*, and their uniqueness amongst the Tetractinellida, we are still missing molecular data from *Thoosa* species to support this recommendation. In the Mexican Pacific, three *Thoosa* species have been described (Carballo, Cruz-Barraza, & Gómez, 2004; Cruz-Barraza, Carballo, Bautista-Guerrero, & Nava, 2011), and the reproductive cycle is known for one of them (Bautista-Guerrero, Carballo, & Maldonado, 2010). The aim of this work was to assess the systematic position of species belonging to the genus *Thoosa* using three independent loci: COI mtDNA, 28S rRNA (D2), and 18S rRNA. Our results confirm that *Thoosa* and *Alectona* are closely related in the family Thoosidae. On the basis of morphological and molecular differences with suborders Astrophorina and Spirophorina, we propose the creation of the new suborder Thoosina.

Materials and methods

Specimens of *Thoosa mismalolli* Carballo, Cruz-Barraza & Gómez, 2004 and *T. calpulli* Carballo, Cruz-Barraza & Gómez, 2004 were collected by scuba diving in a shallow (5–9 m depth) coral community surrounding Isabel Island, Mexico (21°52'30"N, 105°54'54"W). Specimens of *T. purpurea* Cruz-Barraza, Carballo, Bautista-Guerrero & Nava, 2011 were collected from Islas Revillagigedo (19°34'57"N, 111°03'57"W) also by scuba diving at 5–7 m depth. Specimens were deposited in 'Colección de Esponjas del Pacífico' (LEB-ICML-UNAM) (Table 1).

Alectona sp. 1 was collected in the Flemish Cap, off Newfoundland (48°00.0031'N, 44°45.0644'W) at a depth of 1554 m (NEREIDA 0509 campaign, field# DR10-056) (GeneBank accession COI MH256567; 18S MH256569).

Table 1. Localities of sponge specimens, museum voucher numbers, GB and ENA accession numbers used in this study.

<i>Thoosa</i> Species	Locality	Collection accession number	GenBank accession number (COI/ 18S/28S)
<i>T. calpulli</i> Carballo et al. 2004	Isabel Island Mexico, 21°52'30"N, 105°54'54"W, 2m depth, 21 Jul/2005	LEB-ICML-UNAM-1332	KU559625 -6/ MH236103
<i>T. mismalolli</i> Carballo et al. 2004	Isabel Island Mexico, 21°52'30"N, 105°54'54"W, 2m depth, 21 Jul/2005	LEB-ICML-UNAM-1050	KU559627 -8/ MH236102
<i>T. purpurea</i> Cruz-Barraza et al. 2011	Revillagigedo Island, 18°44'10"N, 110°57'37"W, 5 m depth, 6 May 2008	LEB-ICML-UNAM-1674	MH233578/ MH238470/ MH233579

Alectona sp. 2 was collected in deep-sea in the Mozambique Channel, Banc du Geyser (station DW4790: 12°22'S, 46°25'E), at a depth of 360–375 m (MNH-BIOMAGLO 2017 campaign) (GeneBank accession COI MH256568). *Alectona* sp. 2 has large acanthoxeas similar to the ones in *Alectona* species but it also has centrotylote thin oxeads identical to the ones in *Delectona* so we are unsure of the genus identification at this point.

All samples were fixed in 96% ethanol and stored at room temperature before DNA extraction.

Total genomic DNA of *Thoosa* specimens was extracted using standard proteinase K digestion following the protocol from Aljanabi and Martínez (1997), but also the methodology described by Soler-Jiménez, García-Gasca, and Fajer-Ávila (2012). Specimens for which these protocols failed were subjected to an extraction using a SV Promega kit (Promega) following the manufacturer's instructions. The DNA of *Alectona* spp. were extracted using a DNeasy Blood and Tissue kit (Qiagen).

Partial sequences of the mitochondrial cytochrome oxidase subunit 1 gene (COI), and ribosomal 18S rRNA and 28S rRNA were amplified. For amplification of the *Thoosa* COI fragment (659 bp), we used the degenerated primers of Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994) proposed by Meyer, Geller, and Paulay (2005): dgLCO1490 (5'-GGTCAACAAATCATAAAGAY-ATYGG-3') and dgHCO12198 (5'-TAAACTTCAGG-GTGACCAAARAAYCA-3'). The *Alectona* spp. COI fragment (659 bp) was obtained using the standard primers LCO1490 and HCO2198 (Folmer et al., 1994). The *Thoosa* 18S rRNA gene was amplified using primers SP18aF (5'-CCT GCC AGT AGT CAT ATG CTT-3') and SP18gR (5'-CCT GCC AGT AGT CAT ATG CTT-3') (Redmond et al., 2013). The *Alectona* sp. 1, the 18S rRNA gene was amplified in two parts with two sets of primers (4FB/1806R and S30/5FR), as described in Cárdenas et al. (2013). The D2 domain of the 28S rRNA gene was amplified with universal primers C2 (5'-GAAAAGAACTTTGRARAGAGAGT-3') and D2 (5'-TCCGTGTTT CAAGACGGG-3') (Chombard et al., 1998). In most cases each amplification was carried out in 11.5 µL volume reaction containing: 7.23 µL of distilled H₂O (sterile MilliQ), 0.75 µL MgCl₂, 2.5 PCR buffer 5×, 0.66 µL dNTPs, 0.2 µL primer F, 0.2 µL of Primer R, 0.1 µL of *Taq* DNA polymerase (Promega), and 1 µL of a 1/100 dilution of the DNA extracts. Other reactions were made in 12.5 µL, as described in Cruz-Barraza, Vega, and Carballo (2014). Thermal cycling conditions were: an initial denaturation for 5 min at 94°C, followed by 35–40 cycles of 1 min at 94°C, 1 min at 48.4–55°C, 1 min at 72°C and final extension of 5 min at 72°C. The PCR reaction was visualized on a 2% agarose gel, using TAE 1× as electrophoresis buffer for 25 min at 90 V. The final

products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and sequencing was performed at Macrogen Inc. (Seoul, Korea).

Sequences were assembled using Geneious 5.6.4 software (Kearse et al., 2012) and CodonCode Aligner 2.0.1 (CodonCode Corporation). BLAST (NCBI/Blast) searches were used to verify the identity of sequences. For COI and 18S rRNA phylogenetic analyses we used the previously assembled datasets by Kelly and Cárdenas (2016), which include all Tetractinellida species sequenced to this date with outgroups from several other demosponge orders. For the 28S rRNA analyses we gathered published Tetractinellida sequence available in GenBank. Sequences of species of *Cliona* and *Cliothesa* (Clionaida order), were used as outgroup for this new 28S alignment.

All sequences were aligned using MEGA 7 (Kumar, Stecher, & Tamura, 2016) for each of the three loci, using the CLUSTALW alignment under the default gap opening–gap extension parameters (15.0–6.66). Maximum likelihood (ML) analyses were generated with RAxML 8.1.11 (Stamatakis, 2014) on the CIPRES science gateway v.3.3 portal (www.phylo.org) (Miller, Pfeiffer, & Schwartz, 2010) using the GTRGAMMA model. In addition, a Bayesian inference (BI) analysis was performed with MrBayes 3.2.2 (Ronquist et al., 2012) using the GTR+I+G model of sequence evolution as obtained with jModelTest 2.1.7 (Darriba, Taboada, Doallo, & Posada, 2012). The program was run with four Markov chains each 5,000,000-generations long, which were sampled every 200th trees and a burn-in of 25%. Posterior probabilities were computed from the remaining trees. Convergence was evaluated by viewing the log files in Tracer v1.5 (Rambaut & Drummond, 2007), all parameters had effective sample sizes (ESS) >300.

Results

Two *Alectona* COI (sp.1 and sp.2) and one *Thoosa purpurea* COI sequence was obtained. The COI alignment (1216 bp) included 146 sequences, 698 sites were conservative and 518 were variable, of which 408 were parsimony informative. *Alectona* sp. 1 and three *Thoosa* species of the 18S rRNA sequences were obtained. The 18S alignment (1741 bp) included 96 sequences, 1378 sites were constant and 328 were variable, of which 224 were parsimony informative. As for the 28S rRNA (D2) marker, only one sequence of *Thoosa purpurea* was obtained. The 28S alignment (509 bp) included 36 sequences, 137 sites were constant and 347 were variable, of which 278 were parsimony informative.

Phylogenetic reconstructions of the three molecular markers (COI, 18S, and 28S) were mostly congruent in both ML and BI analysis. The topologies obtained by ML analyses are presented, indicating for each node the support found by the different methods, bootstrap

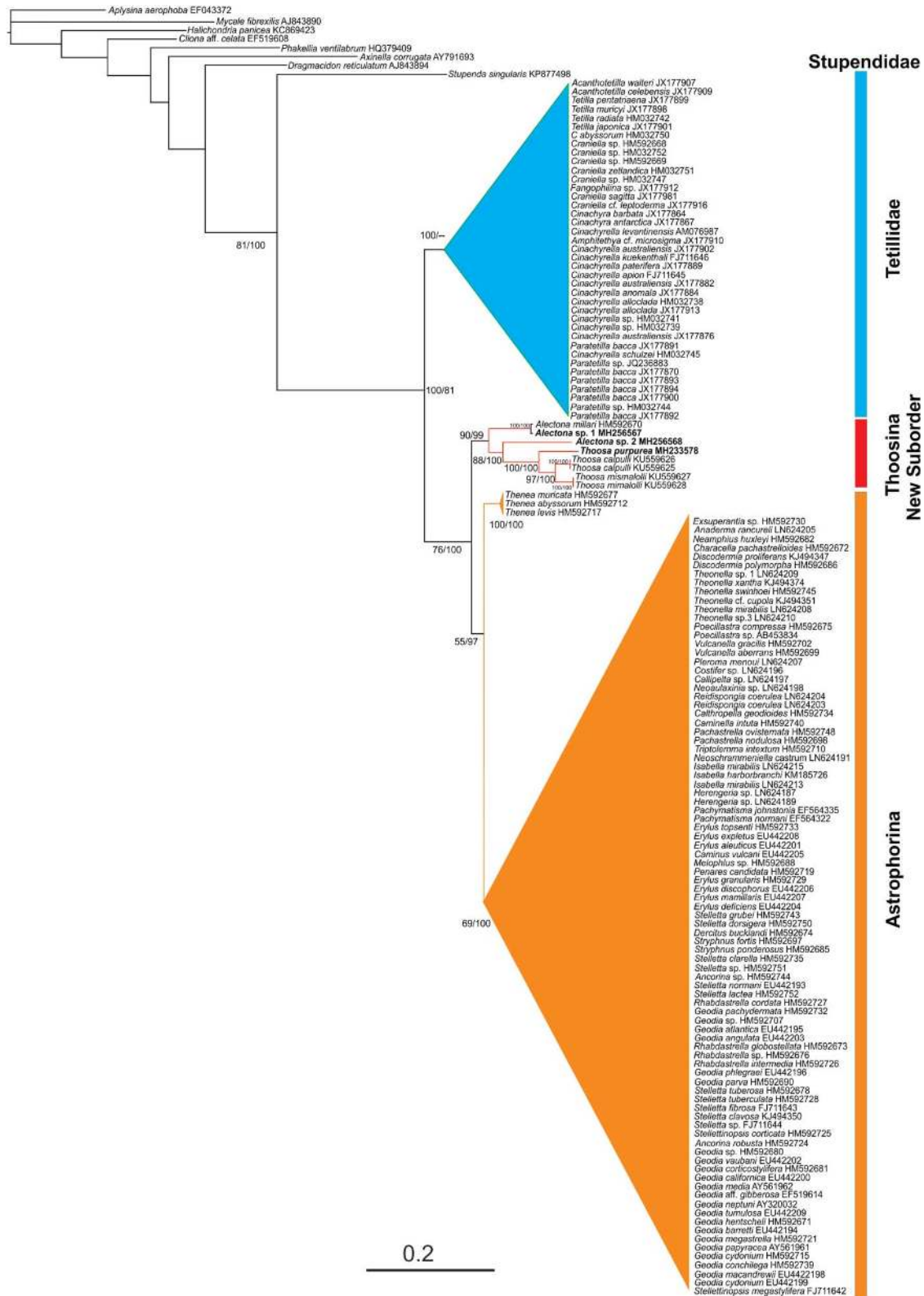


Fig. 1. Phylogenetic reconstruction of cytochrome c oxidase subunit (CO1) mitochondrial marker. The maximum likelihood (ML) topology (presented) was obtained by RAxML. The number at each node represents the ML bootstrap supports (100 bootstrap replicates) and Bayesian posterior probabilities (%), only bootstrap supports above 50 are shown. GenBank accession numbers are given after each taxa name. New Thoosidae sequences are in bold.

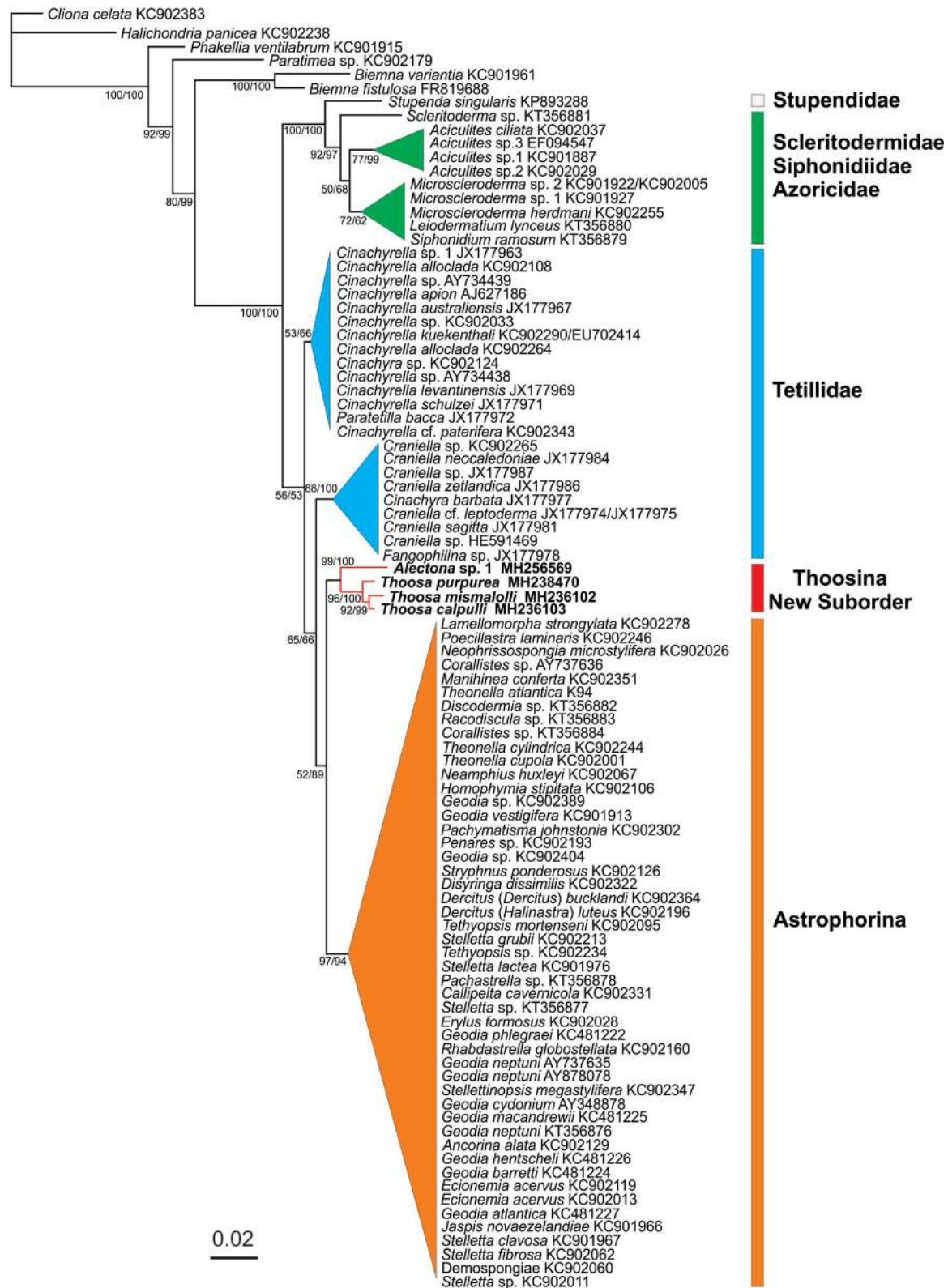


Fig. 2. Phylogenetic reconstruction of 18S rRNA nuclear-ribosomal marker. The maximum likelihood (ML) topology (presented) was obtained by RAxML. The number at each node represents the ML bootstrap supports (100 bootstrap replicates) and Bayesian posterior probabilities (%), only bootstrap supports above 50 are shown. GenBank accession numbers are given after each taxa name. New Thoosidae sequences are in bold.

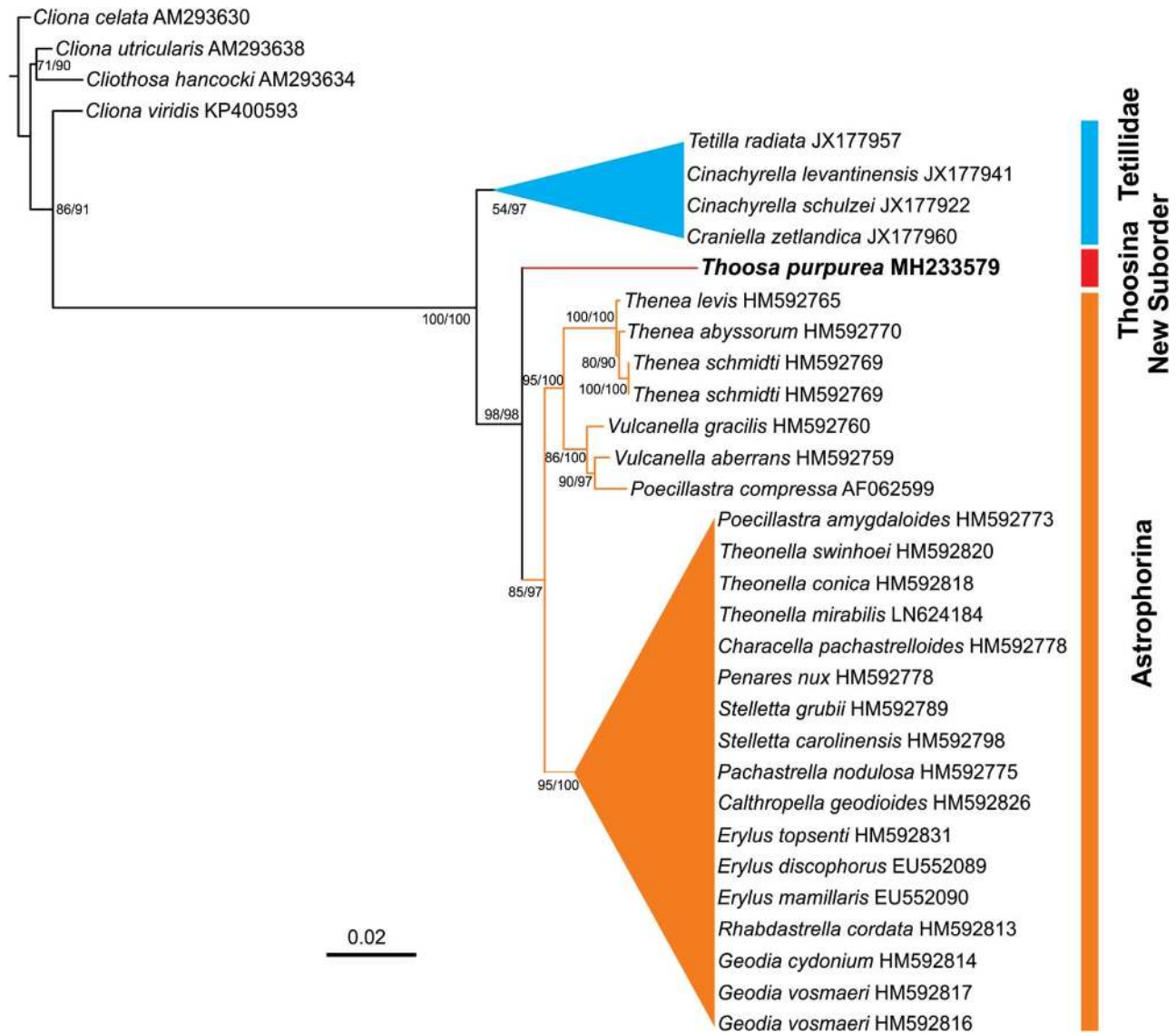


Fig. 3. Phylogenetic reconstruction 28S rRNA gene fragment ‘D2’ marker. The maximum likelihood (ML) topology (presented), was obtained by RAXML. The number at each node represents the ML bootstrap supports (100 bootstrap replicates) and Bayesian posterior probabilities (%), only bootstrap supports above 50 are shown. GenBank accession numbers are given after each taxa name.

proportion followed by posterior probabilities (BP/PP) (Figs 1, 2, 3). Both CO1 and 18S confirmed a well-supported grouping of *Thoosa* and *Alectona*. Specifically, CO1 topology showed that *Alectona* sp.2 was sister to all *Thoosa* spp. but in a separate clade than the other *Alectona* spp., which makes *Alectona* paraphyletic. All three markers agreed concerning the phylogenetic relationships of the *Thoosa* + *Alectona* clade. The three topologies suggested that the Thoosidae arose earlier than the Astrophorina (BP/PP: CO1 = 76/100, 18S = 52/89, 28S = 98/98). With both markers CO1 and 18S, *Thoosa* was monophyletic.

In general, the phylogenetic relationships obtained between Tetractinellida groups were congruent with previous molecular hypotheses (Cárdenas et al., 2011; Kelly &

Cárdenas, 2016; Redmond et al., 2013; Schuster et al., 2015). The family Stupendidae was separated from the Tetillidae and Astrophorina clades (CO1), but closely related with the Scleritodermidae, Siphonidiidae, and Azoricidae (represented in 18S rRNA tree). The family Tetillidae was recovered as a monophyletic clade in CO1 and 28S rRNA trees, but split in two clades in the 18S tree: the *Craniella*/*Antarctotetilla*/*Cinachyra*/*Fangophilina* clade formed a sister group to the Thoosidae + Astrophorina clade.

We now feel that we have enough evidence, based on the morphological and molecular data presented in this study, to say that the Thoosidae represents a unique demersal sponge clade for which we propose to create a new suborder of Tetractinellida.

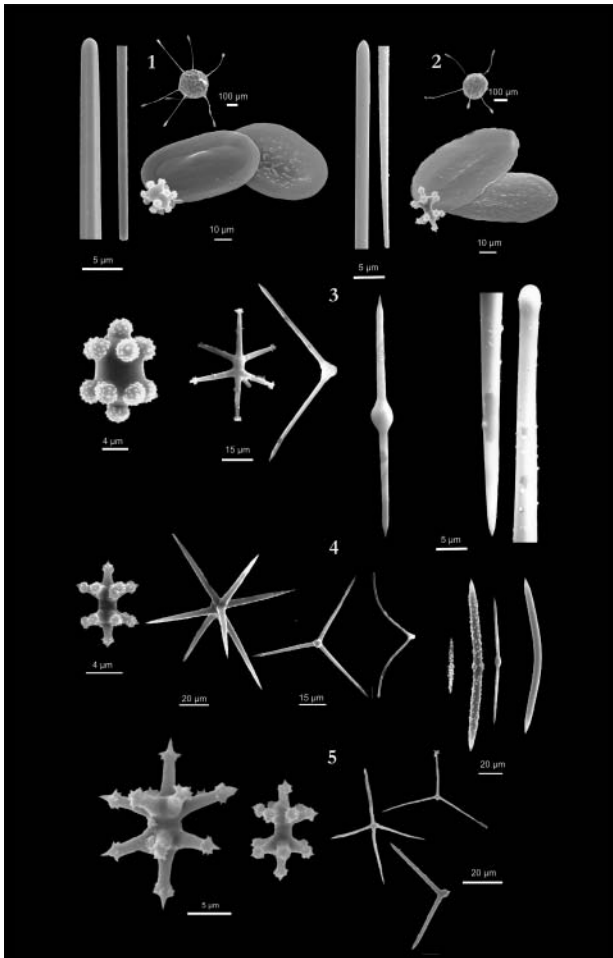


Fig. 4. SEM spicular elements of hoplitomella larva and adults of the three Mexican species of *Thoosa*. (1) Hoplitomella larva of *T. mismalolli*. Style, amphiaser, and view of the inner (right) and outer (left) surface of monaxonic disc. (2) Hoplitomella larva of *T. calpulli*. Style, amphiaser, and view of the inner (right) and outer (left) surface of monaxonic disc of *T. calpulli*. (3) Spicular elements of *T. mismalolli*; (left to right) Nodulose amphiaser, different forms of oxyasters, centrotylote oxeas, and head and tip of subtylostyles. (4) Spicular elements of *T. calpulli*; (left to right) Amphiaser, different forms of oxyasters and smooth and spined centrotylote oxea. (5) Spicules of *T. purpurea*; (left to right) Amphiasers and oxyasters bi- tri- tetra-radiate.

Thoosina new suborder

Definition. Excavating astrophorid sponges with planktonic armoured larvae (named 'hoplitomella'). Megascleres includes monactinal (styles and subtylostyles) or diactinal (acanthoxeas and acanthostrongyles) spicules. These spicules change or disappear in adult stages. Microscleres in adult stages are amphiasers, oxyasters, pseudosterrasters, spined triactines, and toxas. Monaxonic (*Thoosa*) and tetraxonic (*Alectona*) discs are present only in planktonic stages. Pits produced by species of this

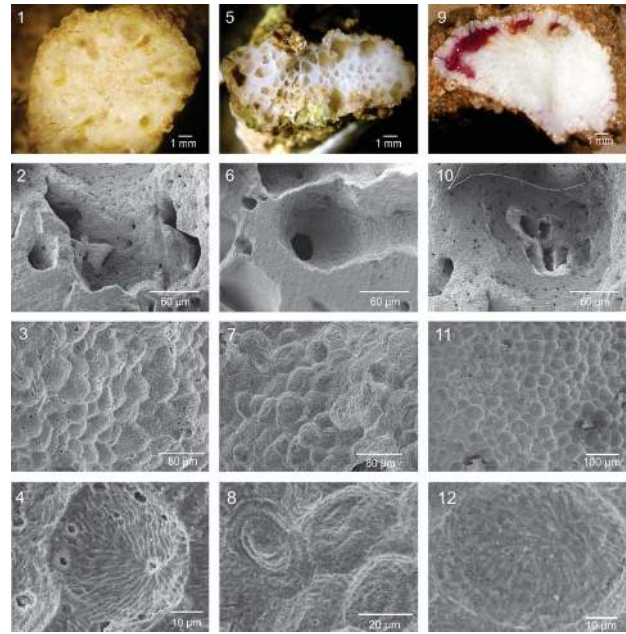


Fig. 5. Excavating pattern of *Thoosa* spp. in a cross-section fragment of the corals of the genus *Pocillopora*. (1–4) *T. mismalolli* (5–8) *T. calpulli*. (9–12) *T. purpurea*. In the first-row, cross-section view of a network of the excavating pattern. In the second row, details of a chamber to SEM where the pitting pattern is visible. In the third row, detail of the scars where chips were removed by the sponge (SEM). Last row, detail of a scar where there are clearly visible unusual micro-ornamentations with deep concentric etching marks with a characteristic radial pattern that overlaps the concentric (SEM).

group have unusual micro-ornamentations with deep concentric etching marks with a characteristic radial pattern that overlaps the concentric bands (Fig. 5).

Remarks. The new suborder contains the family Thoosidae Cockerell, 1925 with three genera: *Thoosa* Hancock, 1849, *Alectona* Carter, 1879, and *Delectona* de Laubenfels, 1936 (to be confirmed when molecular data are available). This family represents 29 valid species (Van Soest *et al.*, 2017).

Thoosa is the most diverse genus with 16 species. The type species is *Thoosa cactoides* Hancock, 1849, found in a shell of *Meleagrina margaritifera* (Linnaeus, 1758) in the northern Indian Ocean, syntype is in the Hancock Museum, Newcastle, UK (4.17.05-06). *Alectona* Carter, 1879, with 10 species is the second most diverse genus. The type species is *Alectona millari* Carter, 1879. *Delectona* Laubenfels, 1936 has four species. This genus was erected by Laubenfels (1936) for the type species *Delectona higgini* (Carter, 1879) from the Indian Ocean. All the species known in the suborder Thoosina are excavators of corals, mollusc shells, and other carbonate substrates.

Discussion

The taxonomic status of the genera *Alectona* and *Thoosa* has been controversial (Alander, 1942; Topsent, 1891, 1928). These genera have no sign of radial architecture and have acquired an ovoviviparous mode of reproduction, which occurs through a larval type unique in the Porifera, which present spicules absent in adult stages (Fig. 4). Precisely, the presence of tetraxonics discs (discotriaenes) in the larvae of *Alectona* suggested its reallocation into the order Astrophorida (Vacelet, 1999). Subsequently, molecular studies corroborated the taxonomic affinities of *Alectona millari* with the astrophorins (Borchiellini et al., 2004; Cárdenas et al., 2011).

Due to similar pit structures, reproductive, and larval traits to *Alectona*, *Thoosa* was suggested to be included in the astrophorins as well (Bautista-Guerrero et al., 2010; Borchiellini et al., 2004; Vacelet, 1999), but this had never been supported by molecular data.

The molecular results obtained in this work showed congruence with the morphological peculiarities. Thus, the molecular phylogenetic trees using three independent loci confirm that *Thoosa* and *Alectona* are closely related, because the sequences are clustered in a monophyletic clade confirming the validity of the family Thoosidae, suggested by previous authors (Alander, 1942; Vacelet, 1999) and officially resurrected by Cárdenas et al. (2011) (Figs 1, 2, 3). Most importantly, the bootstrap and branches length shown in the molecular trees clearly separated Thoosidae (*Thoosa* and *Alectona*) from the rest of the families showing that it diverged after the Tetillidae and before the rest of the astrophorines.

Indeed, several morphological features shared by *Alectona* and *Thoosa* show they are quite different from the rest of the tetractinellids. The larvae of *Alectona* and *Thoosa* have spicules that are absent in adult stages (Bautista-Guerrero et al., 2010, 2016; Topsent, 1904; Vacelet, 1999) (Fig. 4). This is considered an ancient adult character because it is only observed during the embryogenesis and larval stages. Most importantly, the planktonic discs are homologous structures within Thoosidae: monaxonics in *Thoosa* lacking the short rhabdome (erroneously called discotriaenes by Bautista-Guerrero et al., 2010), and tetraxonics in *Alectona* (Bautista-Guerrero et al., 2016; Topsent, 1891; Vacelet, 1999). The discotriaenes are also found in all Theonellidae genera but one (*Manihinea*), but the monaxonic discs are so far exclusive of *Thoosa* larvae. The tetraxon megasclere and aster-type microsclere are considered morphological synapomorphies for Tetractinellida (Cárdenas et al., 2011). Aster-type microscleres are observed in *Thoosa* and *Alectona*, but the tetraxon feature is lacking in *Thoosa* (adults and larvae) while it is present in *Alectona* (larvae).

Interestingly the pits produced by *Alectona* during the excavating processes have unusual micro-ornamentations with deep concentric etching marks (Calcinai et al., 2004) which also occurred in Mexican *Thoosa* species (Fig. 5), all of them very different of those of other boring species (Calcinai, Arillo, Cerrano, & Bavestrello, 2003).

Our study suggests that embryological and larval features must be considered to infer phylogenetic relationships in the Demospongiae.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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