THE SYSTEMATIC POSITION OF *ALECTONA* (PORIFERA, DEMOSPONGIAE): A TETRACTINELLID SPONGE

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ABSTRACT

The affinities of the genus *Alectona* Carter and of *Spiroxya levispira* (Topsent) have been investigated using 28S rDNA sequences and SEM study of their boring pattern. The close relationships of *Alectona millari* with the tetractinellids are strongly supported by molecular data, although its affinities with the orders Astrophorida or Spirophorida remain unresolved. *Spiroxya levispira* has no relationship with the tetractinellids. These results imply modifications of the conventional classification of *Alectona* within Hadromerida. In the Systema Porifera, *Alectona* and *Spiroxya* were classified in the family Alectonidae, whereas *Thoosa* was placed in the Clionaidae. We propose maintaining *Spiroxya* in the Clionaidae, and *Alectona* and *Thoosa*, which share a unique hoplitomella larva, in the family Thoosidae of the tetractinellids. Morphological evidence suggests the placing, possibly temporary, of Thoosidae in the order Astrophorida. However, the molecular data, and the uniqueness of the reproduction pattern and of spiculation suggest that the family could represent a special order of Tetractinomorpha.

KEY WORDS

Alectona, *Spiroxya*, Astrophorida, DNA sequences, classification.

INTRODUCTION

Among excavating sponges, which are now classified in various orders and families, the genus *Alectona* Carter, 1879 is very puzzling. Its spicules, consisting of irregular spinose diactines and microscleres resembling the amphiaster type, are unique and do not provide an adequate basis for a precise classification. Perhaps more importantly, *Alectona* species reproduce by incubated larvae devoid of flagella and provided with a spicule armor and flotation devices, the hoplitomella larva (VACELET, 1999). This larva, which has long been regarded as an asexual propagule (TOPSENT, 1903, 1948; TRÉGOUBOFF, 1939, 1942) is highly unusual in sponges, and is also characterized by an unusually long planktonic life. This peculiarity in reproduction is shared with another excavating sponge genus, *Thoosa* Hancock, 1849 and possibly with the poorly known genera of excavating sponges, *Dotona* Carter, 1880 and *Delectona* Laubenfels, 1936 (TOPSENT, 1903).

The classification of these aberrant sponges has been very inconstant and remains controversial. On the basis of their excavating properties, they were traditionally classified in the family Clionidae (now Clionaidae), order Hadromerida. This choice was reinforced by the presence of amphiasters resembling those of

Alectona and *Thoosa* in the indisputable Clionaidae genus *Cliothosa* Topsent, 1905. The basis for this classification is debatable, as it is now well established that an excavating habit is not restricted to the Clionaidae, being found in other orders and families of demosponges, and that amphiasters occur in diverse Tetractinomorpha. The presence of discotriaenes derived from a tetraxonid spicule in the hoplitomella larva of *Alectona* is a clear indication of the affinity of the genus with the tetractinellids, especially with the order Astrophorida. (In this paper, we will use the terms 'tetractinellids' or 'Tetractinellida' for the clade including the orders Astrophorida, Spirophorida and a part of 'Lithistida', as discussed by CHOMBARD *et al.* 1998. This clade, although having strong molecular and morphological support, is not formally retained in the Systema Porifera, as discussed by HOOPER & VAN SOEST, 2002a, p. 106.) Tetractinellids, however, and hadromerids as well, are oviparous, without any known species incubating larvae. The genus was considered as "intermediary" between clionids and tetractinellids (TOPSENT, 1891, 1928). It was even firmly classified in Astrophorida (ALANDER, 1942), considering that tetraxonid spicules, never found in Hadromerida, are compelling evidence for membership of the tetractinellids. Alander's position, however, was not followed by most taxonomists, due to the fact that *Thoosa* which shares with *Alectona* the hoplitomella larva, does not have tetraxonid spicules, the larva armor being made of monaxonic plates instead of discotriaenes. In recent classifications, *Alectona* and *Thoosa* have often been isolated in a family Thoosidae classified either in the order Hadromerida or as *incertae sedis* (ROSELL, 1996; ROSELL & URIZ, 1997; VACELET, 1999). The most recent treatment of these sponges in the Systema Porifera proposed to classify all of them in the Hadromerida, placing *Alectona* in a separate family Alectonidae, together with other genera previously included in the Clionaidae, but excluding *Thoosa* which is still classified in the Clionaidae although it shares with *Alectona* its reproductive uniqueness (RÜTZLER, 2002).

This is a case where conflicting evidence from morphology and reproductive data, obtained from sponges that are small and rarely collected, makes it difficult to clearly establish affinities. The aim of this study is to examine the clues provided by DNA sequences, both on *Alectona millari* and on a representative of *Spiroxya* Topsent, 1896, another excavating genus which has been recently included in the family Alectonidae (RÜTZLER, 2002).

MATERIALS AND METHODS

The sponges were collected in the skeleton of deep-sea corals from NW Atlantic sampled off Ireland by means of ROV (Remotely Operated Vehicle) ("Victor" and an USNEL box-corer during the CARACOLE cruise of the N/O ATALANTE operated by IFREMER. The samples were obtained on Perseverance Mound (52°.18 N - 13°.01 W, 590 m depth, 06/VIII/2001, dive 127.5) and Theresa Mound (51°25 N - 11°.46 W, 880 m depth, 02/VIII/2001, box-corer sample KGS05). The specimens were preserved in 95 % ethanol.

Scanning electron microscopy (SEM) observations of the excavating pattern of *Alectona millari* Carter, 1879 and *Spiroxya levispira* (Topsent, 1898) (= *Cliona levispira* Topsent, 1898), were made on coral skeletons after cleaning in sodium hypochlorite. Complementary observations were made on skeletons of the sphinctozoan sponge *Vaceletia crypta* (Vacelet, 1977) from the bathyal zone of New Caledonia bored by a *Thoosa* sp. The skeletons were sputter-coated with gold-palladium and observed under a Hitachi S570 scanning electron microscope.

Before genomic DNA extraction, tissue samples were dehydrated and frozen in liquid nitrogen. Small pieces of frozen samples were ground to a powder in a precooled mortar with liquid nitrogen. Powder was poured in 500 µl lysis buffer (10 mM Tris-HCl pH8, 0.1 M EDTA pH 8, 20 µg/ml RNAse DNAse free, 0.5 % SDS) and incubated 1 h at 37° C. The mixture was digested after addition of Proteinase K (100 µg/ml) during 3 h at 50° C. After digestion, the aqueous lysate was extracted with water-saturated ultrapure phenol, followed by a single chloroform extraction of the aqueous phase. Genomic DNA was recovered by standard precipitation procedures with 0.1 volume of 3M pH 7 ammonium acetate and 2.5 volumes of absolute ethanol. Genomic DNA was finally resuspended in sterile water at 1 µg/µl after optic density measurement at 260/280 nm.

Amplification of 28S rRNA was performed as described in CHOMBARD *et al.* (1997), using a pair of primers to permit amplification of 413 bp: primer C'1 (5'ACCCGCTGAATTTAAG CAT-3') and primer Ep3 (5'ATKCGYTTCCCTCCYAACGG-3'). After amplification, each PCR fragment was cloned into pTZBlue T-Vector (Tebu) and sequenced using the dideoxy-nucleotide chain termination method (SANGER *et al.,* 1977). The sequences corresponding respectively to18S and 28S rRNA gene fragments resulting from this work have been deposited in GenBank database. Their respective accession numbers are listed in Tab. I. Others sequences used for this work came from GenBank (Tab. I).

Initial sequence alignment was performed using Clustal W and subsequently corrected by eye. Positions which showed variable alignment were excluded from phylogenetic analyses, only positions that could be unambiguously aligned were selected, leaving 1592 positions, 1081 of which are constant and 362 are parsimony informative. Phylogenetic analyses were performed using both parsimony and distance methods. We used PAUP 4.0 for all phylogenetic analyses. Distance analyses were performed using Neighbor-Joining (NJ) with distances corrected by Kimura's two-parameter model. For maximum parsimony (MP) analyses, characters were always treated as unordered and unweighted. MP trees were computed using heuristic searches with 50 replicates of random taxon addition sequence and TBR branch swapping. The confidence of the tree topology was assessed by 500 bootstrap resampling.

Tab. I. List of the species used in this work with their accession numbers. New sequences are indicated in bold characters.

	28S, numbers Genbank accession
Geodia cydonium	AY348893
Discodermia polydiscus	AF062603
Stelletta grubii	AY348892
Corallistes masoni	AF062602
Stryphnus mucronatus	AF062597
Spiroxya levispira	**AY552026**
Alectona millari	**AY552020**
Thenea muricata	**AY552019**
Erylus euastrum	AF062600
Poecillastra compressa	AF062599
Pachastrissa pathologica	AF062596
Pachymatisma johnstonia	AF062601
Cinachyrella sp.	AF062604
Aaptos aaptos	AY348889
Timea sp.	**AY552022**
Tethya aurantium	**AY552024**
Diplastrella bistellata	**AY552025**
Placospongia carinata	**AY552021**
Cliona orientalis	**AY552023**
Cliona viridis	AF062606
Suberites ficus	AY026381
Agelas oroides	AJ225830
Phorbas tenacior	AJ225832
Asbestopluma hypogea	AY348890
Axinella damicornis	AF062605
Spongilla lacustris	AY348894

RESULTS AND DISCUSSION

The strict consensus tree of 17 most parsimonious trees and Neighbor-Joining tree constructed with 28S rDNA sequences and rooted on *Spongilla lacustris* (Fig. 1) shows that *Alectona millari* belongs to a well supported clade Tetractinellida (bootstrap values of 100) as redefined according to morphological and molecular data (CHOMBARD *et al.,* 1998). This clade includes the two orders Astrophorida and Spirophorida, and *Alectona*. In this clade, however, the relationship of *Alectona* remains unresolved. There is no real molecular basis for affinities of *Alectona* either with the representative of Spirophorida or with those of the families Geodiidae, Corallistidae, Calthropellidae, Ancorinidae, Theonellidae and Pachastrellidae, which form a well supported Astrophorida clade with the exception of the pachastrellid

Thenea muricata. Unfortunately, no member of the genus *Thoosa* could be analysed and there is no molecular support to determine its phylogenetic affinities.

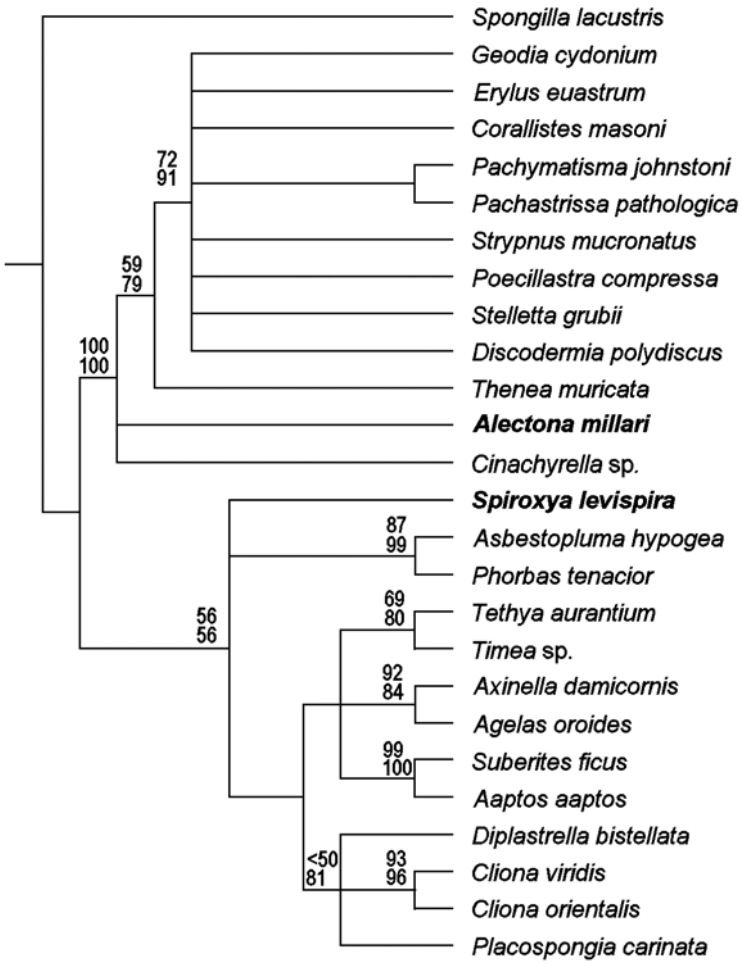

Fig. 1. Strict consensus tree rooted on *Spongilla* of 17 most parsimonious trees and NJ tree. The bootstrap values in Maximum Parsimony (upper) and in Neighbor-Joining (lower) are indicated at each node when > 50 %. The species analysed in this work are in bold.

The same tree also clearly indicates that *Spiroxya levispira* does not belong to the clade Tetractinellida and has thus no relationship with *Alectona*. The relationships of the genus *Spiroxya* with the family Clionaidae and order Hadromerida, however, are not supported by the molecular data. Considering the morphological evidences including a spicule complement composed of oxeas, spirasters and spiral microstrongyles and a boring pattern similar to that of clionids, we propose to maintain the genus *Spiroxya* in the family Clionaidae.

The observations of the boring patterns in corals confirm that *Alectona* has a special pit structure, with concentric lines complemented by clear radiating lines (Figs 2A, B), as already shown for boring patterns in other substrata (OMNES, 1991; VACELET, 1999). The pit structure of *Thoosa* sp. in the skeleton of the sphinctozoan *Vaceletia crypta*, which has a peculiar microstructure different from the coral skeletons (VACELET *et al.,* 1992), displays faint radiating lines in addition to concentric lines. The radiating lines, however, are less visible than in several species of *Alectona* (Fig. 2C). In contrast, *Spiroxya levispira* displays a pit structure similar to those of members of the family Clionaidae, with concentric lines without any traces of radiating lines (Fig. 2D). The peculiarities of the *Alectona* pits are not related to the microstructure of the excavated substrata, as they were observed in the same coral skeletons as those of *Spiroxya*.

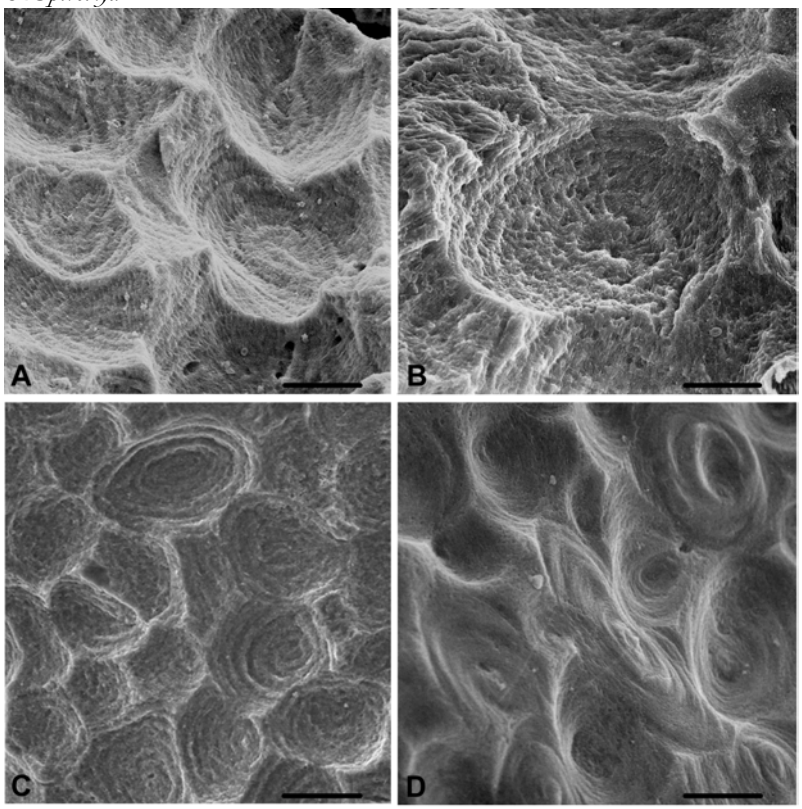

Fig. 2. SEM pictures of pit pattern. **A,** *Alectona wallichii* in coral skeleton; scale bar 30 μm. **B,** *Alectona millari* in deep-sea coral skeleton; scale bar 20 μm. **C,** *Thoosa* sp. in sphinctozoan *Vaceletia* sp. skeleton; scale bar 15 μm. **D,** *Spiroxya levispira* in deep-sea coral skeleton; scale bar 15 μm.

The evidence from molecular data that *Alectona* is a tetractinellid is congruent with the presence of tetraxonid spicules in the larva. There is now compelling

evidence that *Alectona* is not a hadromerid that would have secondarily acquired triaenes in its aberrant larva, but rather is a tetractinellid which has lost triaenes in the adult stage. It should consequently be classified in the clade Tetractinellida, which includes the orders Astrophorida and Spirophorida (CHOMBARD *et al.,* 1998), with strong molecular and morphological support. The loss of the diagnostic tetraxonid spicules is not exceptional in tetractinellids. It is generally admitted that it occurred in several Astrophorida (genera of the family Ancorinidae) and Spirophorida (family Spirasigmidae) (HOOPER & VAN SOEST, 2002b; URIZ, 2002). More exceptional is the fact that this tetractinellid sponge has no sign of radial architecture and, more importantly, has acquired a viviparous mode of reproduction, which furthermore occurs through a larva type unique in the Porifera.

Within the clade Tetractinellida, the affinities of *Alectona* with the other representatives remain unresolved. The presence of asteroid microscleres rather than sigmaspires strongly advocates for affinities with the order Astrophorida rather than Spirophorida. This is also supported by the discotriaenes of the hoplitomella larva, as these derivatives from triaenes are unknown in the Spirophorida. The molecular evidence, however, is equivocal for its classification in the Astrophorida. The presence in the order Spirophorida of *Samus anonymus* Gray, 1867 (family Samidae), an excavating sponge with small amphitriaenes somewhat resembling the amphiasters of *Thoosa* and *Alectona* (VAN SOEST & HOOPER, 2002), could also indicate affinities with Spirophorida, since excavating sponge are unknown in Astrophorida. The affinities of the family Samidae, however, are doubtful. The peculiarities in spiculation and embryology, the absence of the radial architecture shared by all the undisputed tetractinellids, also suggest that Alectona may represent a clade different from both Astrophorida and Spirophorida and thus would have to be allocated to a special order Alectonida. Pending further investigation, we propose to temporarily classify *Alectona* in the order Astrophorida in a special family, Alectonidae or Thoosidae, as already suggested by ALANDER (1942).

In the absence of molecular data, the interpretation of *Thoosa* remains uncertain. We do not follow the recent proposition of RÜTZLER (2002), who separates *Thoosa* from *Alectona*, the former being classified in the Clionaidae on the basis of the presence of tylostyles, and the second in the Alectonidae, without taking in consideration the uniqueness of reproduction characters in both genera. There are four main reasons for supporting our position. Firstly, the presence of hoplitomella larvae in the two genera is a shared synapomorphy with an important phylogenetic meaning, although these hoplitomella differ by the tetraxonid or monaxonid nature of the plates of the armor. The possibility that the hoplitomella larva derived independently from tetractinellid and hadromerid sponges, developing plates from triaenes in the first case and from monaxonid spicules in the second, cannot be excluded, but appears highly unlikely considering the unique characters of this type of larva. On the other hand, there are several examples in tetractinellids of discs in which the tetraxonid character has presumably been lost, for instance in the lithistid family Neopeltidae (PISERA & LÉVI, 2002) or in *Discodermia dubia* (VACELET & VASSEUR, 1971). The derivation of monaxonial plates from tetraxonial discotriaenes in the hoplitomella of *Thoosa* is thus possible. Secondly, the presence of tylostyles in *Thoosa* is highly uncertain. Their first reports derived from the fact that the type species of *Cliothosa*, *Cliothosa hancocki* (Topsent, 1888) was first described in *Thoosa*.

Most of the authors having described true *Thoosa* representatives did not report the presence of megascleres, or interpreted them as foreign or as being the hoplitomella styles. Thirdly, the spicule complement of several species of *Thoosa* includes pseudotoxes, very likely derived from euasters, which are also found in some Astrophorida of the family Geodiidae (for instances *Caminus apiarium* Schmidt, 1870, and several species of *Erylus*). Fourthly, the pit structure of the excavations displays some similarities in both genera, although the radiating lines are less visible in *Thoosa* than in *Alectona*. We thus propose to maintain this genus with *Alectona* in a family whose correct name should be Thoosidae (ROSELL & URIZ, 1997), to be included in the order Astrophorida as discussed above. The family Thoosidae would also include *Dotona* and *Delectona*, as suggested by RÜTZLER (2002). These genera, however, are still poorly known, and their reproduction pattern, in particular, needs to be investigated. The interpretation of *Scolopes* Sollas, 1888 and *Neamphius* Laubenfels, 1953, also classified in the Alectonidae by RÜTZLER (2002), remains uncertain.

These results demonstrate that the Tetractinellida have a wider diversity than previously thought. They have been able to develop an excavating habit, previously reported in this clade only in the poorly known *Samus*, and a unique reproductive pattern with an incubated hoplitomella.

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