Morphological, chemical and biochemical characterization of a new species of sponge without skeleton (Porifera, Demospongiae) from the Mediterranean Sea

Jean VACELET
Carole BORCHIELLINI
Thierry PEREZ
Centre d'Océanologie de Marseille
(CNRS-Université de la Méditerranée, UMR 6540 DIMAR),
Station marine d'Endoume, F-13007 Marseille (France)
jvacelet@com.univ-mrs.fr

Valérie BULTEL-PONCÉ
Jean-Paul BROUARD
Michèle GUYOT
Laboratoire de Chimie du Muséum national d'Histoire naturelle,
associé au CNRS, 63 rue Buffon, F-75231 Paris (France)
guyot@mnhn.fr

Vacelet J. et *al.* 2000. — Morphological, chemical and biochemical characterization of a new species of sponge without skeleton (Porifera, Demospongiae) from the Mediterranean Sea. *Zoosystema* 22 (2) : 313-326.

New nucleotide sequence data reported in this paper will be available in the GenBank database under the accession numbers AF268620-AF268622. Alignment will be available on request to the authors.

ABSTRACT
A new species of sponge without skeleton, *Thymosiopsis conglomerans*, is described from the Mediterranean. It differs from the other species of the genus, *T. cuticulatus* Vacelet & Perez, which lives in close sympatry although under different ecological conditions, in its morphology and in sterol composition. The new species is massive and incorporates large amounts of foreign materials. Δ^7 sterols are present in both species of *Thymosiopsis*, but they differ significantly in the side-chains. The 28S rRNA sequences clearly confirm the close affinities of *Thymosiopsis* with *Thymosia*, a North-East Atlantic genus which is distinctive in having a skeleton made up of nodulose spongin fibres. The rRNA sequences also support the allocation of these two genera to the family Chondrillidae. The cytology of the new species is compared with that of *T. cuticulatus* and of *Thymosia guernei* from Portugal using transmission electron microscopy.

KEY WORDS
Porifera,
taxonomy,
sterol chemistry,
rRNA sequences,
Chondrosida.

RÉSUMÉ

Caractérisation morphologique, chimique et biochimique d'une nouvelle espèce d'éponge sans squelette (Porifera, Demospongiae) de la Méditerranée.

Une nouvelle espèce d'éponge sans squelette, *Thymosiopsis conglomerans*, est décrite de la Méditerranée. Elle diffère de l'autre espèce du genre, *T. cuticulatus* Vacelet & Perez, qui vit en étroite sympatrie bien que dans des conditions écologiques différentes, par sa morphologie et sa composition en stérols. La nouvelle espèce est de forme massive et incorpore de grandes quantités de corps étrangers. Des stérols Δ^7 sont présents dans les deux espèces de *Thymosiopsis*, mais diffèrent nettement par leur chaîne latérale. Les séquences d'ARNr 28S confirment clairement les affinités de *Thymosiopsis* avec *Thymosia*, un genre de l'Atlantique Nord-Est qui diffère par la possession d'un squelette très original de fibres de spongine noduleuses. Les séquences d'ARNr confirment aussi l'attribution de ces deux genres à la famille Chondrillidae. La cytologie de la nouvelle espèce est comparée en microscopie électronique à transmission à celle de *T. cuticulatus* et de *Thymosia guernei* du Portugal.

MOTS CLÉS
Porifera,
taxonomie,
chimie des stérols,
séquences de ARNr,
Chondrosida.

INTRODUCTION

A recently described monospecific genus of Mediterranean sponges, *Thymosiopsis* Vacelet & Perez, 1998, is known from a single locality, an unusual cave which, due to similarities in sea water temperatures with the deep Mediterranean, shelters an unusually large number of bathyal invertebrates (Harmelin 1997; Vacelet & Boury-Esnault 1995; Vacelet & Perez 1998; Vacelet *et al.* 1994). This sponge has no skeleton, neither spicule nor spongin fibre. In the absence of these conventional taxonomic characters the classification of such sponges is difficult. Without skeletal information, their taxonomy and the establishment of their phylogenetic relationships rely mostly on subtle characters, of anatomy, cytology, micro-ecology or reproduction, as demonstrated in exemplary fashion for the genus *Halisarca* Dujardin, 1838 in Lévi's pioneering work (Lévi 1956). In the case of *Thymosiopsis*, the type species of the genus, *T. cuticulatus* Vacelet & Perez, 1998, has anatomical and cytological similarities with *Thymosia* Topsent, 1895, a genus tentatively allocated to the family Chondrillidae on the basis of anatomical resemblances (Boury-Esnault & Lopès 1985; Rosell 1988). *Thymosiopsis* was consequently classified in the family Chondrillidae. In this family, the two genera differ in the skeleton, absent in *Thymosiopsis* and made up in *Thymosia* of unique nodulose fibres which are quite different from the diverse fibre types found in keratose sponges (orders Dictyoceratida, Dendroceratida and Verongida).

We have found outside the same cave, thus under very different ecological conditions although only a few metres distant from the place where *T. cuticulatus* is living, a sponge devoid of skeleton which clearly differs from *T. cuticulatus* in its gross morphology. Ultrastructural cytology, sterol chemistry and 28S rRNA sequences, however indicate clear affinities with *T. cuticulatus*. These characters, which have been rarely used in conventional descriptions, provide a basis for allocating the sponge to a new species of the genus *Thymosiopsis* and for specifying the taxonomic allocation of *Thymosia* and *Thymosiopsis*, thus proving again to be significant for the taxonomic characterization of sponges without skeleton and with variable morphological characters.

MATERIAL AND METHODS

FIELD OBSERVATIONS AND SAMPLING

The sponge was observed and collected by means of Scuba diving. The type specimens were fixed in formalin and stored in alcohol. Although large

fragments of the three known individuals were collected, the specimens healed and regenerated, and their survival was checked in March 1999.
Specimens of *Thymosia guernei* Topsent, 1895 were collected from Portugal and preserved either for electron microscope study (cave entrance, Punta da Piedade, 5 m, 20.VIII.1985, coll. J. Vacelet) or in alcohol (cave entrance, Portinho da Arrabida, 7 m, 20.VIII.1997, coll. P. Rios and F. J. Cristobo). Specimens of *Thymosiopsis cuticulatus* were collected from the type locality (3PP cave).
The sea water temperatures were recorded by Seamon® mini Hugrún thermographs over a one year cycle.

Cytology

For light and transmission electron microscopy (TEM), the specimens were fixed in glutaraldehyde 2.5% in a mixture of 0.4 M cacodylate buffer and sea water (4 vol.: 5 vol.). They were maintained in the fixative for 24 hours and post-fixed two hours in 2% osmium tetroxide in sea water. Specimens were decalcified in 10% RDO (Du Page Kinetic Lab) in sea water in order to remove part of the numerous debris included in the tissue, dehydrated through an alcohol series, and embedded in Araldite. Semi-thin sections were stained with toluidine blue. Thin sections, contrasted with uranyl acetate and lead citrate, were observed under a Zeiss EM 912 transmission electron microscope. Thick polished sections were obtained by sawing undecalcified specimens preserved in formalin and embedded in Araldite with a low speed saw using a diamond wafering blade, and wet-ground with abrasive paper.

Chemistry

Pieces of specimens were preserved in methanol. The methanol was removed and specimens extracted with methanol-dichloromethane, then dichloromethane. The solvents were evaporated under vacuum and the residue was partitioned between water and methanol-dichloromethane 2/8. The organic layer was evaporated and the residue purified on a Silicagel column eluting with dichloromethane-acetone 95:5, then a preparative thin-layer chromatography (dichloromethane-acetone 95:5) gave the sterol fraction. GC-MS analysis of the mixture was performed with a CPSi18 column, Chrompack, 100 °C to 290 °C, 10 °C/min, helium flow rate 1ml/min. Free sterols or silylated sterols were analysed on a Nermag R 10-10 mass spectrometer. ^{1}H and ^{13}C NMR and spectra were obtained on a Bruker AC 300 spectrometer with standard pulse sequences operating at 300.13 and 75.28 MHz respectively. The chemical shift values are reported as ppm units and the coupling constants in Hz.

DNA processing

A small tissue sample was taken from the choanosome in order to avoid contamination by any surface algae and invertebrates, and preserved in 80% ethanol. The sample was dehydrated and frozen, then ground to powder in a precooled mortar with liquid nitrogen. The powder was incubated for one hour at 37 °C in 500 µl lysis buffer (10 mm Tris-HCl [pH 8], 0.1 M EDTA [pH 8], 20 µg/ml RNAse DNAse free, 0.5% SDS). After digestion by Proteinase K (100 µg/ml) for three hours at 50 °C, 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 3 M ammonium acetate (pH 7) and 2.5 volumes of absolute ethanol, and resuspended in sterile water at 1 µg/µl after measurement at 260/280 nm.
The 28S rRNA was isolated using PCR. The first PCR was performed using specific primers of 5' (5'ACC CGC TGA ATT TAA GCA T < 3') and 3' (5' > AT(GT) CG(CT) TTC CCT CC(CT) AAC GG < 3'). The reaction mixture was denatured at 94 °C for 5 min before amplification, which was undertaken in a DNA thermal cycler (Perkins Elmer Celtus) in a final volume of 50 µl in the presence of 10 ng of genomic DNA, 5 µl of 10 X *Taq* DNA polymerase buffer, 8 µl of 1.25 mm dNTP mix (Pharmacia), 2.5 µl of each primer (20 µm) and 1.25 U of *Taq* DNA polymerase (Promega). Samples were amplified for 30 cycles under the following regime: denaturation at 94 °C for 1 min, primer annealing for 1 min at 57 °C, extension for 1 min at 72 °C. PCR amplification products were extracted from agarose gel using the Giasex II gel extraction kit (Qiagen) according to the manufacturer's instructions. The 400 base

region was cloned into pTZBlue T-Vector (Tebu) and sequenced by dideoxy-nucleotide chain termination (Sanger *et al.* 1977).

SEQUENCE MANAGEMENT AND ALIGNMENT

Sequences were aligned using the GeneWork program and also manually for correction of any obvious misalignments. Trees were derived from molecular data using the distance matrix neighbour-joining method (Saitou & Nei 1987) and the maximum-parsimony algorithms in the PHYLIP package (Felsenstein 1992) in the PHYLO-WIN package (Galtier *et al.* 1996). Distance matrices were calculated using the observed divergence method. The validity of the results was ascertained by bootstrapping (Felsenstein 1992).

The sequences were deposited in the GenBank database. The accession numbers are: *Thymosia guernei,* AF268620; *Thymosiopsis cuticulatus,* AF268621; *Thymosiopsis conglomerans,* AF268622.

SYSTEMATICS

Order CHONDROSIDA Boury-Esnault & Lopes, 1985

Family CHONDRILLIDAE Gray, 1872

Genus *Thymosiopsis* Vacelet & Perez, 1998

TYPE SPECIES. — *Thymosiopsis cuticulatus* Vacelet & Perez, 1998 by monotypy.

DIAGNOSIS (EMENDED). — Chondrillidae with a general organization similar to that of the genus *Thymosia*, having a smooth surface, a cortex enriched with fibrillar collagen, but lacking spongin fibres. A superficial cuticle and pore-sieves may be present.

ORIGINAL DIAGNOSIS. — "Encrusting Chondrillidae. General organization similar to that of the genus *Thymosia*, having a smooth surface, a superficial cuticle and pore-sieves, a marked cortex enriched with fibrillar collagen, but lacking spongin fibres" has to be slightly modified in order to include the characters of the new species.

Thymosiopsis conglomerans n. sp.

TYPE MATERIAL. — Holotype: **La Ciotat.** Northwestern Mediterranean, near opening of 3PP cave, 17 m, 26.I.1998, one fragment (MNHN D JV 64). Paratype: same locality, 22.X.1998 (MNHN D JV 65).

ETYMOLOGY. — From *conglomer*, Latin, pertaining to the collecting properties of the sponge.

LOCALITY AND HABITAT. — The sponge is known only from the vicinity of the entrance of 3PP cave, 1.2 km South-West of La Ciotat on the French Mediterranean coast (43°09.47'N, 05°36.01'E). Three specimens have been observed and partly collected, 17-18 m deep on the floor of cliff recesses, 0.5 m in length. Two specimens live in the same recess, the third is a few metres distant. Despite its large size, the sponge was long overlooked numerous dives in this area, due to its subspherical shape similar to that of the surrounding pebbles and to its surface cover by algae, invertebrates and various debris. Contrary to what is observed in the homeotherm zone within the cave (Harmelin 1997), the sea water temperature in this place follows the normal seasonal variations (in 1998 from 13 to 23.5 °C at 18 m in depth instead of 13 to 14.5 °C within the cave at the same depth).

MORPHOLOGY

The type specimen (Fig. 1A) is massive, subspherical, 25 cm in maximum diameter and 15 cm high, with a large apical depression, approximately 4 to 6.5 cm in diameter, corresponding to an atrium in which most of the oscules are located. The two other specimens are somewhat smaller (25/15/10 cm), without a well-defined atrium. The color is whitish gray in surface and in the choanosome.

The surface is almost entirely covered by epizoic algae, invertebrates and a large variety of debris. Irregular low conules are determined by foreign material included in the sponge body. In the rare places that are free from debris or epizoic organisms, the surface is either smooth or bears low ridges connecting the conules (Fig. 1B). A cuticle is not macroscopically visible. Pore-sieves seem to be absent, but this is difficult to ascertain as they may be concealed by the extensive debris on the surface. The ostia have not been observed, probably due to the contracted state of the specimens. The oscules, 12 mm in maximum diameter, are mostly located inside the large apical depression, although a few are scattered on the lateral surface. Composite canals are visible within the oscules.

The texture is firm, but fleshy. The choanosome is dense, compact with a small number of canals. A proper skeleton is absent. Both the surface and the body of the sponge contain a large amount of foreign material, such as skeletons of calcareous

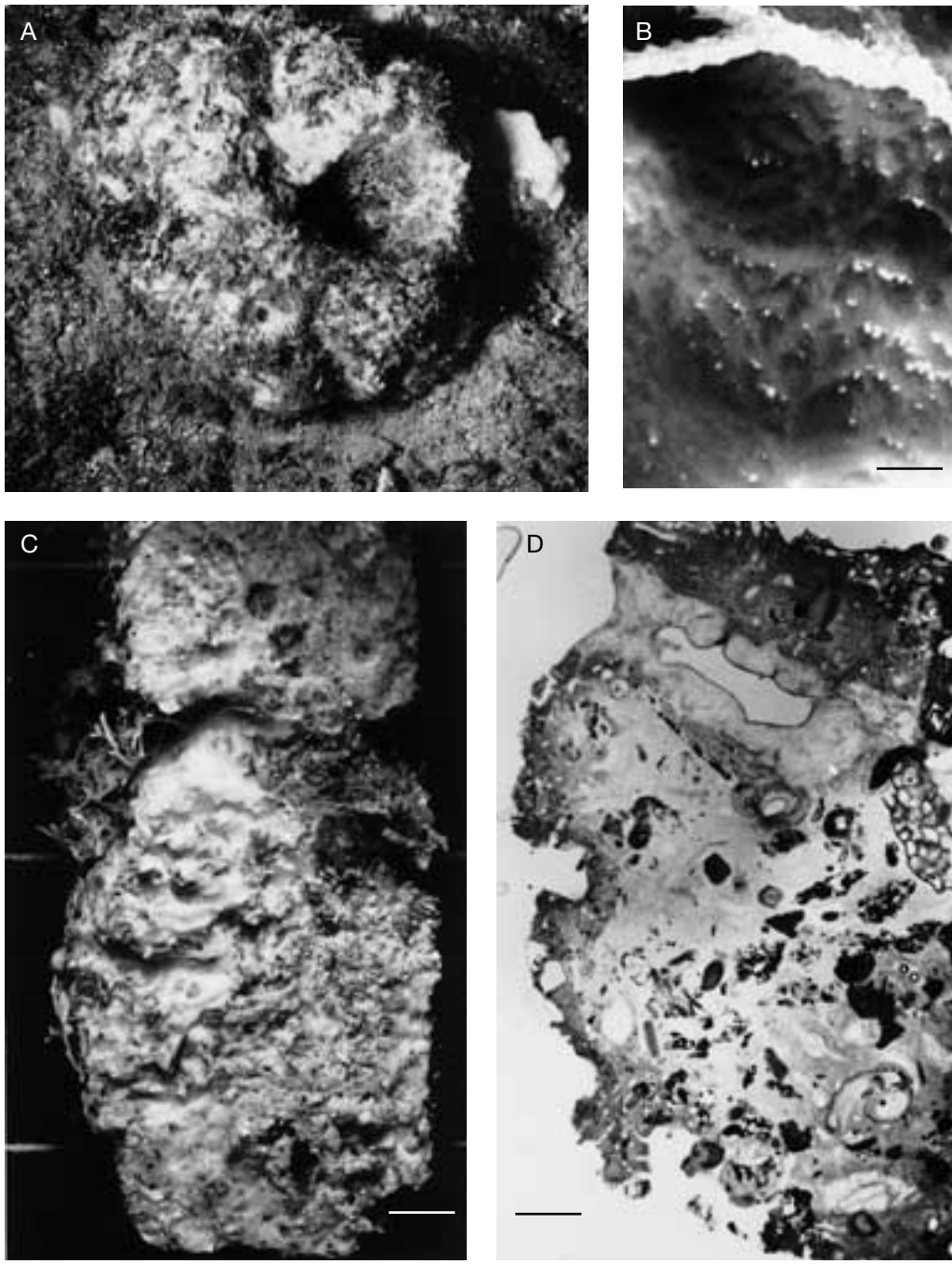

FIG. 1. — *Thymosiopsis conglomerans* n. sp.; **A**, holotype *in situ*, 17 m depth; **B**, view of the surface of the holotype, with reticulation of low ridges and epizoic bryozoan (*Scrupocellaria* sp.); **C**, section through the paratype (surface of the sponge on the left); **D**, thin polished section in the paratype, with the ectosome on the left, canals, and numerous debris of foreign origin. Scale bars: B, 1 mm; C, 14.3 mm; D, 1.4 mm.

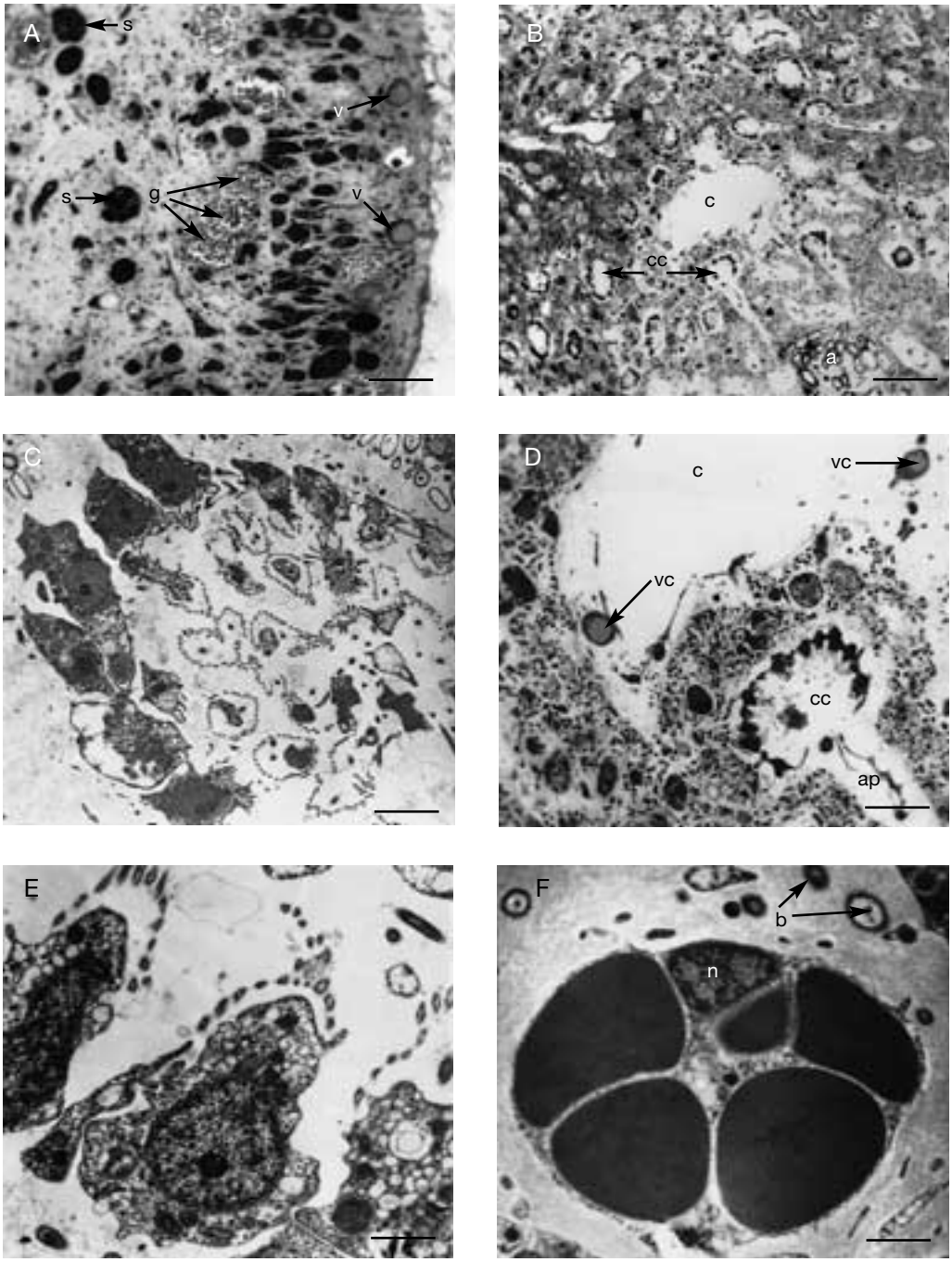

FIG. 2. — *Thymosiopsis conglomerans* n. sp.; **A**, semi-thin section through the ectosome; **B**, semi-thin section through the choanosome; **C**, TEM view of a choanocyte chamber; **D**, enlargement of a part of semi-thin section B; **E**, choanocyte; **F**, spherulous cell. Abbreviations: **a**, foreign body (calcareous alga); **ap**, aphodus; **b**, bacteria; **c**, canal; **cc**, choanocyte chamber; **g**, granular cell; **n**, nucleus; **s**, spherulous cell; **v**, vacuolar cell; **vc,** vacuolar cells near the pinacocyte layer of the canal. Scale bars: A, D, 16 µm; B, 58 µm; C, 3.2 µm; E, F, 1 µm.

algae, bryozoans, mollusc shells, *Posidonia* leaf fibres, sand grains, etc. (Fig. 1C, D).

An ectosomal layer from 50 to 400 µm thick is distinct from the choanosome in its low cell density and relative reinforcement by collagen fibrils (Fig. 2A). This layer, however, is not a resistant, well-defined cortex such as in *Chondrosia* or *Chondrilla*. The ectosome is outwardly lined either by an extremely thin (1 µm) cuticle or by a thin pinacoderm with T-shaped exopinacocytes. The pinacoderm appears to be present mostly in the areas bearing a reticulation of fine ridges. A few vacuolar cells are present near the surface, some of which have a short process extending toward the surface (Fig. 2A). The ectosome includes most cell types other than choanocytes, but in lower density than in the choanosome. A layer of elongate cells parallel to the surface, embedded in a moderately dense matrix of collagen fibrils with the same orientation, is often visible near the choanosomal boundary. Collagen bundles are poorly developed.

The choanosome has a higher cell density than the ectosome (Fig. 2B, D). Most of the choanosome volume is occupied by granular cells, which are closely pressed together in places. The choanocyte chambers are spherical or slightly ovoid, 24-40 µm in diameter, and belong to the aphodal type. The aphodus is up to 60 µm long and 12 µm in diameter. Numerous symbiotic bacteria are present in the intercellular spaces. The canals are moderately developed. Their walls are lined by vacuolar cells, which may lie under the endopinacocyte layer or within the lumen of the canals.

No stage of reproduction has been observed in the specimens, which were observed in January, August and October.

Cytology

The choanocytes are quite irregular in shape, 3.7 to 4 µm in size (Fig. 2C, E). The nucleus, 1.8 to 2 µm in diameter, is most often nucleolated (0.5-0.6 µm). There is no periflagellar sleeve. The cell body often displays lateral pseudopodia arising from below the collar and connecting neighboring cells (Fig. 2C, D). The collar is made up of 30 to 34 microvilli. Apopylar cells were not recognized. Although a few cells have been observed in the lumen of the choanocyte chambers, they are not definitely identified as central cells, due to the irregularity of the shape of the choanocyte layer. The choanocyte base generally emits short pseudopodia anchoring the cell in the underlying mesohyle. The aphodus is lined with endopinacocytes which are usually detached from the wall, probably a fixation artifact.

Four distinct types of cells with inclusions have been observed:

Spherulous cells (Fig. 2F), 6-14 µm in diameter, contain about 10 homogeneous, dense spherules, 2-4 µm in diameter, which occupy most of the cytoplasm volume. The anucleolate nucleus is 2 µm in diameter. Probably less mature stages have a higher volume of cytoplasm free from inclusion and a higher number of spherules irregular in size, some of them as small as 1 µm. Spherulous cells are dispersed in the ectosome and the choanosome.

Granular cells (Fig. 3A, B, F), 10-18 µm, with a small anucleolate nucleus 1.8 µm in diameter, contain numerous dense granules with an irregular outline, 0.5-1 µm in diameter, enclosed in clear vesicles delimited by a thin sheet of cytoplasm. The smaller cells are denser than the large ones, which have a clear cytoplasm in which the dense regular sized granules are conspicuous. Degenerative stages of the granules have not been observed. Granular cells are very abundant in most regions and may occur in dense clusters.

Vacuolar cells (Fig. 3C), 8-10 µm in diameter, usually display a single vacuole, 6 µm in diameter, containing a large spherical inclusion with homogeneous, dense contents, lined by a thin empty space. The vacuole is surrounded by a cytoplasm layer 0.1-0.7 µm in thickness, with a small nucleus (less than 1 µm) and mitochondria. A few cells have two or more vacuoles. The inclusion stains green with toluidine blue. The vacuolar cells, rare in the choanosomal tissue, are mostly found around the canals, with some of them migrating through the pinacocyte layer towards the canal lumen (Figs 2D; 3C). A few of them have been observed in the most superficial layer of the ectosome, with an extension of the cell, including the vacuole, toward the surface (Fig. 2A). Such cells are reminiscent of the "flask-shaped cells" described in the ectosome of dictyoceratids (Bidder 1892; Burck 1909).

Microgranular cells (Fig. 3D), 5 µm in maximum size, with an anucleolate nucleus 1.8 µm in diam-

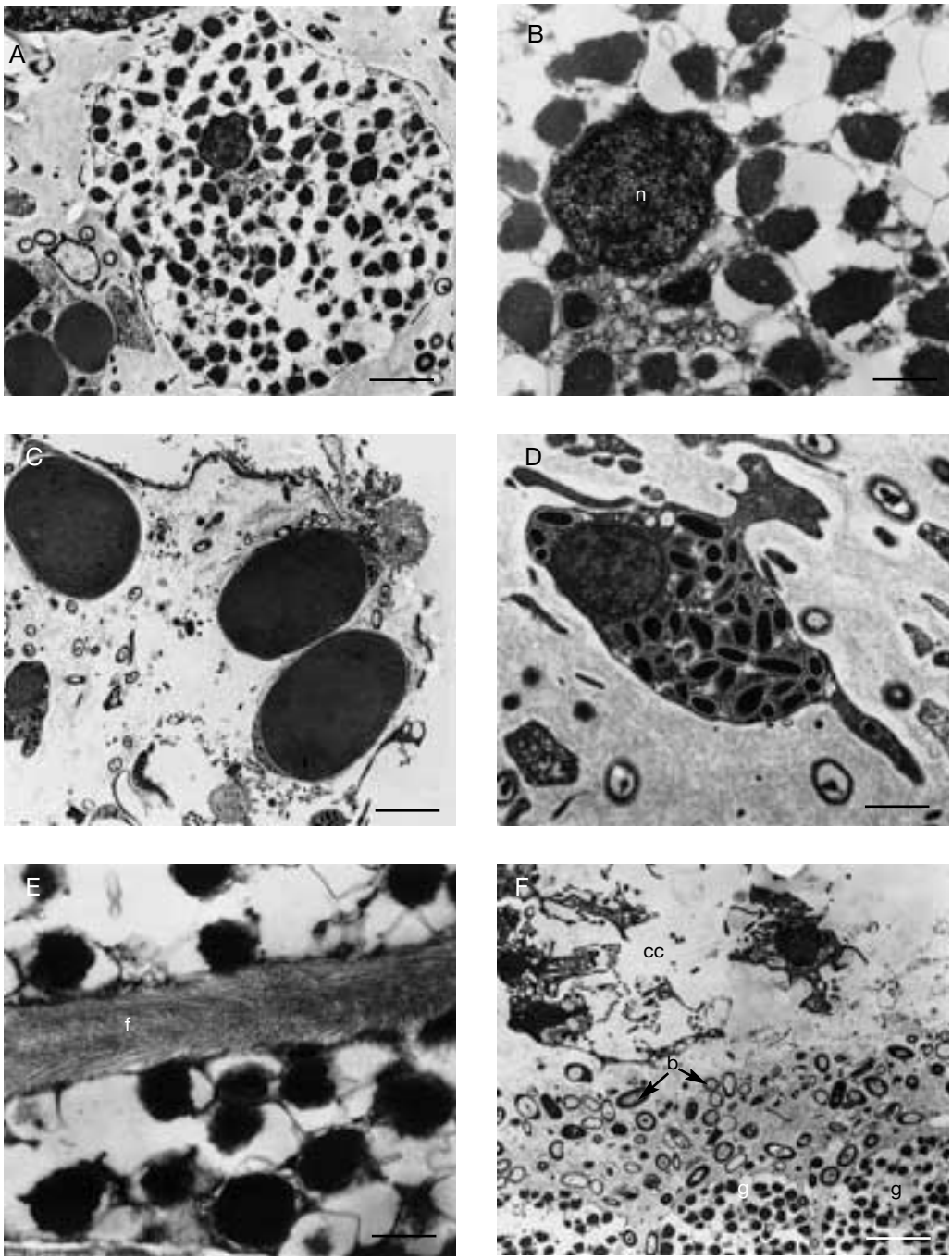

FIG. 3. — *Thymosiopsis conglomerans* n. sp.; **A**, granular cell in the ectosome, with a spherulous cell bottom left; **B**, enlargement of the nucleus and granules of the cell seen in A; **C**, three vacuolar cells near the pinacocyte layer of a canal; **D**, microgranular cell; **E**, collagen fibrils in the mesohyl between two granular cells; **F**, symbiotic bacteria in the mesohyle, near a choanocyte chamber and granular cells. Abbreviations: **b**, bacteria; **cc**, choanocyte chamber; **f**, collagen fibrils; **g**, granular cell; **n**, nucleus. Scale bars: A, 2 µm; B, 0.6 µm; C, F, 3.2 µm; D, 1 µm; E, 0.5 µm.

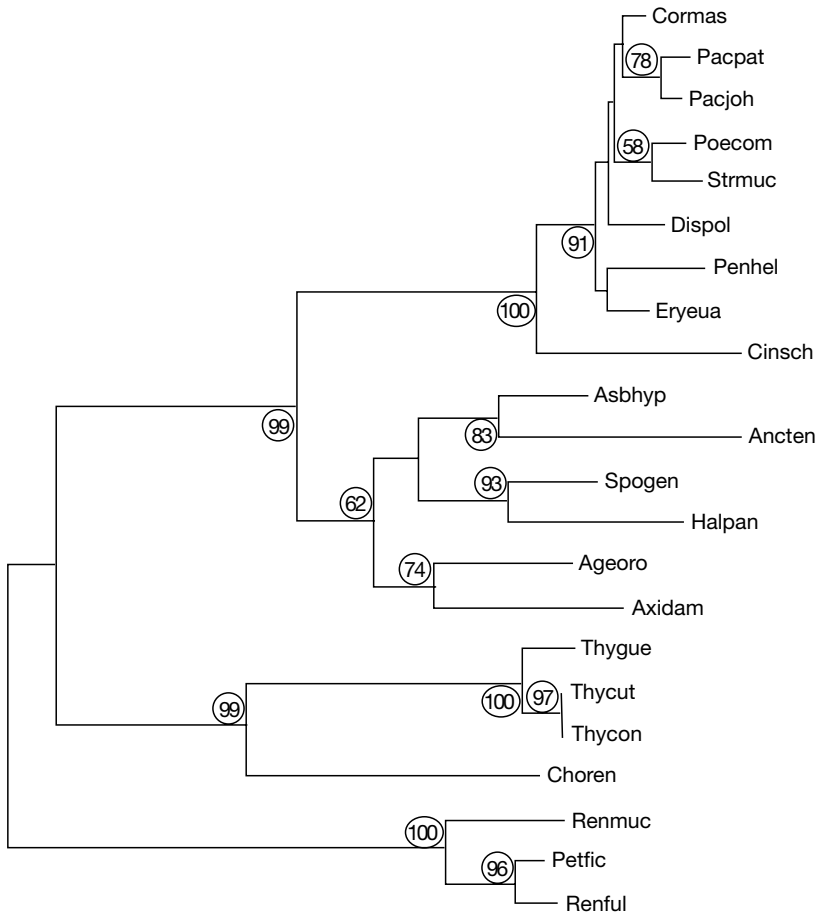

FIG. 4. — Molecular phylogenetic tree based on a comparison of 28S rRNA sequences from 22 species. The topology is a consensus bootstrap neighbour-joining tree obtained after 500 bootstrap replicates with Haplosclerida as outgroup. Bootstrap proportions are shown above internal branches. Distances were calculated using Kimura method. Abbreviations : **Ageoro**, *Agelas oroides*; **Ancten**, *Anchinoe tenacior*; **Asbhyp**, *Asbestopluma hypogea*; **Axidam**, *Axinella damicornis*; **Choren**, *Chondrosia reniformis*; **Cinsch**, *Cinachyrella schulzei*; **Cormas**, *Corallistes masoni*; **Dispol**, *Discodermia polydiscus*; **Eryeua**, *Erylus euastrum*; **Halpan**, *Halichondria panicea*; **Pacpat**, *Pachastrissa pathologica*; **Pacjoh**, *Pachymatisma johnstoni*; **Penhel**, *Penares helleri*; **Petfic**, *Petrosia ficiformis*; **Poecom**, *Poecillastra compressa*; **Renful**, *Reniera fulva*; **Renmuc**, *Reniera mucosa*; **Spogen**, *Spongosorites genitrix*; **Strmuc**, *Stryphnus mucronatus*; **Thycon**, *Thymosiopsis conglomerans*; **Thycut**, *Thymosiopsis cuticulatus*; **Thygue**, *Thymosia guernei*.

eter and with long pseudopodia, contain very dense, ovoid inclusions, 0.2 µm/0.8 µm, in the cytoplasm. Microgranular cells are dispersed throughout the sponge tissue.

Collagen fibrils (Fig. 3E) are loosely organized in thin bundles which are especially visible in a narrow zone between ectosome and choanosome. There is no disjunction between the collagen bundles and a granulo-fibrillar matrix such as is observed in some species of *Halisarca* or Chondrillidae (Vacelet & Donadey 1987). The fibrils are unusually thin, approximately 9 nm in diameter, with a faint periodic striation, and resemble the smooth type of collagen fibrils (Garrone 1978).

Symbiotic bacteria, rare and dispersed in the ectosome, are numerous in the choanosome (Figs 2D; 3F). They are extracellular and display the high morphological diversity usually found in most massive Demospongiae, varying in size (on sections) from 0.2-0.4 µm in diameter to 0.7-2.4 µm in length.

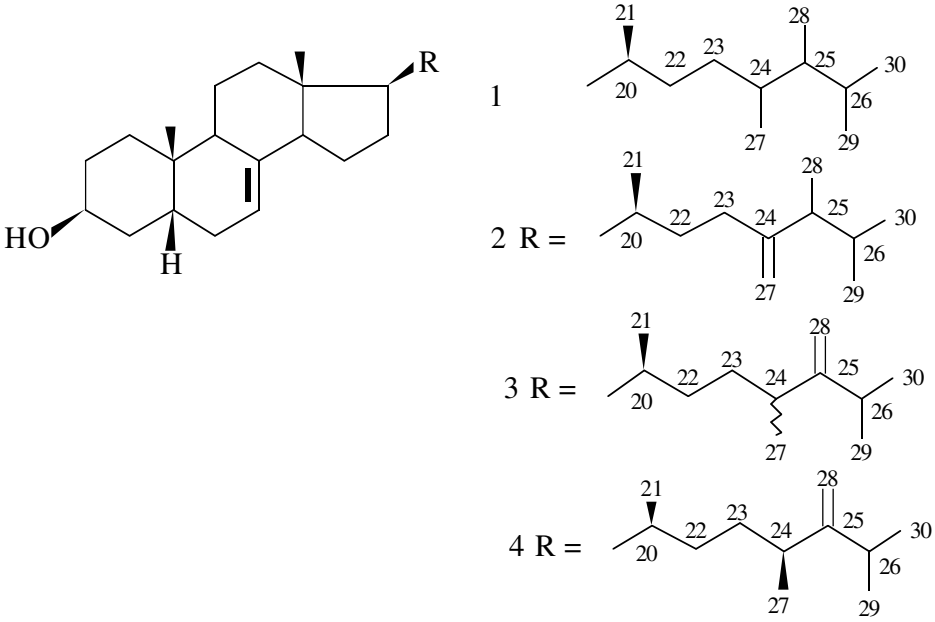

FIG. 5. — **1**, Thymosiosterol; **2**, Δ^{24} thymosiosterol; **3**, main sterol of *T. conglomerans*; **4**, pulchrasterol.

SEQUENCE COMPARISON

The sequences of 28S rRNA of *Thymosiopsis conglomerans, T. cuticulatus* and *Thymosia guernei* were compared with existing sequences from several representatives of sponges extracted from GenBank. Phylogenetic trees were constructed by both neighbour-joining (Fig. 4) and parsimony (data not shown). The two methods yielded identical branching orders.

These methods are not well suited to the distinction of taxa at the species level and the differences between the two species of *Thymosiopsis* appear slight and could be considered as insignificant. The genus *Thymosiopsis* forms a clade with *Thymosia* with a high degree of reliability, i.e. in 100% of the bootstraps. The two genera *Thymosia* and *Thymosiopsis* form a clade with the representative of family Chondrillidae, *Chondrosia reniformis*, with Haplosclerida as an outgroup. This clade is supported by a high bootstrap value (98%).

STEROLS

The new species *T. conglomerans* was shown to contain two new Δ^7 sterols, thymosiosterol and Δ^{24} thymosiosterol, bearing unusual side-chains (Bultel-Poncé *et al.* 1999).

Thymosiopsis cuticulatus also contains unusual Δ^7 sterols. GC-MS analysis revealed the presence of a major sterol (95%), (RT 48.2) m/z 426 (M^+) (29), 411 (13), 393 (2), 314 (5), 299 (6), 271 (100), 255 (8), 246 (6), 231 (13), 213 (22), accompanied by sterols having M^+ 426 (RT 49.9, 4%), M^+ 440 (RT 52.8, 2%). Due to the paucity of the available material, NMR data were recorded from the crude sterol fraction. ^{1}H NMR data displayed a broad doublet at δ 5.13 ppm (J = 1.3 Hz) characteristic of a Δ^7 vinyl proton, in addition to signals at δ 4.72 and 4.66 (two singlets) indicative of a terminal methylene group. Three doublets centered at δ 1.00 ppm (6H), 0.99 (3H), 0.89 (3H) ppm respectively indicate the presence of only methyl groups as substituents. The two singlets at δ 0.77 and 0.50 ppm assigned to C-19 and C-18 protons confirm the Δ^7 unsaturation. All these data are reminiscent of those described for pulchrasterol (Crist *et al.* 1983) (Fig. 5).

Table 1 gives the chemical shifts of the methyl groups of the main sterols of *T. cuticulatus*, 3, in comparison with those of sterols of *T. conglomerans* 1 and 2 and pulchrasterol 4. A COSY experiment allowed confirmation of the chemical shifts

TABLE 1. — ^{1}H NMR data (300 MHz) of the methyl groups of **1**, **2** (*T. conglomerans*), **3** (*T. cuticulatus* major sterol) and **4*** (pulchrasterol). **4*** from Crist *et al.* 1983.

	C-18	*C-19*	*C-21*	*C-27*	*C-28*	*C-29-C-30*
1	0.51	0.77	0.88	0.82	0.73	0.80-0.85
2	0.51	0.77	0.92	4.66, 4.69	0.9	0.81-0.82
3	0.50	0.77	0.89	0.99	4.66, 4.72	1.00-1.00
4*	0.52	0.79	0.91	1.00	4.68, 4.75	1.02-1.02

of CH_3-29 and 30 at δ 1.00 (d, 6H), coupled with H-26 at δ 2.17 and CH_3-27 at d 0.99 (δ, 3H) coupled to H-24 at δ 1.97 ppm. HMBC experiment furnished correlations between CH_3-27, 29, 30 protons, H-26 and H-24 with C-25 (δ 163.2), confirming the position of the substituents. Combination of COSY and heteronuclear correlations (HMBC) experiments shows that the main sterol of *T. cuticulatus* was 24,26,26-trimethylcholesta-7,25(27)-dien-3β-ol. The minor sterols (less than 5% of the total sterol content) were not identified.

Thymosia guernei (specimens from Portugal) contains cholestanol as the sole sterol: EIMS: m/z 388 (M^+). ^{1}H NMR data were in agreement with literature data.

CYTOLOGY OF *THYMOSIA GUERNEI*

Some observations have been made on the cytology of *Thymosia guernei* using specimens from Portugal (Fig. 6), redescribed by Rosell (1988). This species shares some cytological characters with both species of *Thymosiopsis*, although it lacks the diagnostic granular cells. A few vacuolar cells with the same characters as in *Thymosiopsis* are present. The spherulous cells are more numerous, with homogeneous spherules which can be significantly larger than those of *Thymosiopsis* (up to 8 µm). The choanocyte chambers and choanocytes are similar in shape to those of *Thymosiopsis*.

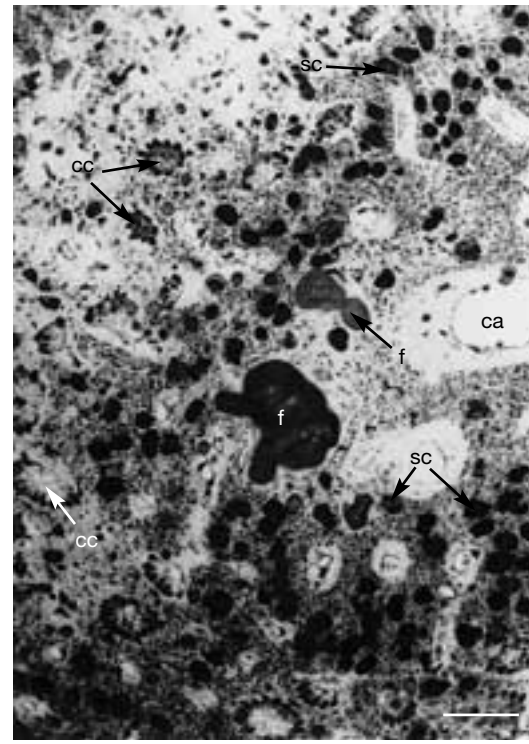

FIG. 6. — *Thymosia guernei* Topsent, specimen from Portugal. Semi-thin section through the choanosome. Abbreviations: **ca**, canal; **cc**, choanocyte chamber; **f**, spongin fibre; **sc**, spherulous cell. Scale bar: 34 µm.

DISCUSSION

In the absence of skeleton, the new sponge is very difficult to classify on the basis of its morphological characters. A comparison is necessary with *Hyrtios collectrix* (Schulze, 1879) because this Mediterranean dictyoceratid, which also incorporates a high amount of extraneous detritus has a reduced fibre skeleton that may be easily overlooked (Schulze 1879; Vacelet 1959). The absence of spongin fibres appears to be complete in *Thymosiopsis*, and the two sponges differ in shape, consistency, color, and surface morphology. However, it would be interesting to investigate the cytology of *H. collectrix*, which is poorly known.

Among the sponge genera devoid of skeleton, namely *Oscarella* Vosmaer, 1884 and *Pseudocorticium* Boury-Esnault *et al.*, 1995 (Homosclerophorida, Plakinidae); *Hexadella* Topsent, 1896 (Dendroceratida, Darwinellidae); *Halisarca* Dujardin, 1838 (Halisarcida, Halisarcidae); *Myceliospongia* Vacelet & Perez, 1998 (Demospongiae *incertae sedis*); *Chondrosia* Nardo, 1847 and *Thymosiopsis*, Vacelet & Perez, 1998 (Chondrosida, Chondrillidae), the new sponge has possible affinities only with representatives of the Chondrillidae. Representatives of *Chondrosia* may include foreign materials, as shown for *C. reniformis* Nardo (Bavestrello *et al.* 1996) and for *Chondrosia plebeja* Schmidt, 1878 (Topsent 1918), but they are well characterized by a highly differentiated cortex containing thick strands of collagen bundles, a structure which is absent in *T. conglomerans*. The recently described genus *Thymosiopsis*, known from a single species, *T. cuticulatus*, has a different morphology, being encrusting rather than massive like the new species, having clearly defined pore-sieves and lacking incorporated detritus.

However, the anatomy of the new sponge is not very different from that of *T. cuticulatus* and furthermore its cytology is so remarkably similar that the two sponges appear to be very close relatives. The differences in surface structure, with the pore-sieves and the extremely thin cuticle not very conspicuous in the new sponge, could be ascribed to the abundance of foreign material which makes it difficult to observe, and also may suppress the development of surface structures. The size and shape of the choanocyte chambers are similar, with choanocytes having pseudopodia below the collar. The presence of an aphodus and the absence of cells sending lamellipodia into the chamber lumen may represent differences between species. The canal system is more developed in *T. conglomerans*, possibly attributable to the massive versus encrusting shape. There were few differences in cytology. The cells with inclusions are similar, particularly the granular cells which do not however display the aspect interpreted as degenerative in *T. cuticulatus*. The most important difference is the presence of vacuolar cells in *T. conglomerans*. These were not originally described in *T. cuticulatus*, but a restudy has shown that vacuolar cells are actually present, but rare. This is possibly a result of their localization around the canals, which are poorly developed in the encrusting *T. cuticulatus*. The cytology of both species has some similarities to that of *Thymosia guernei*; a significant difference is the absence of the diagnostic granular cells in *Thymosia*.

These striking similarities in cytology between *T. conglomerans* and *T. cuticulatus* suggest that it is either a new species of *Thymosiopsis* or a morphological variation of *T. cuticulatus* in a different environment. Although the two sponges occur only about a hundred metres apart, the environmental conditions in which they live are widely different and presumably sufficient to modify their gross morphology. *Thymosiopsis cuticulatus* is living on the wall of a dark cave in a trapped body of cold water, an oligotrophic environment with water movements slower than 2 cm/sec and with water temperature varying from 13 to 14.5 °C (Harmelin 1997). *Thymosiopsis conglomerans* is living in dim light in a nearly open environment subjected to water temperature varying from 13 to 24 °C, in which it is exposed to normal wave and current action.

This typical situation in sponge taxonomy where conventional criteria cannot define whether we are dealing with two species or with two ecomorphs is particularly problematic in the absence of skeletal characters. This is a case where cytology, chemical and biochemical data, which are rarely available for routine taxonomy, may have a crucial role. However, each of these methods is significant at a particular taxonomic level (Borchiellini *et al.* 2000). Allozyme polymorphism, which appears to be the best method for the discrimination between sibling species, was not applicable to the present case where only a small population is available for the two presumed species.

The differences in cytology are too slight to be conclusive, especially as they are possibly influenced by the gross morphology of the two sponges, massive versus encrusting shape, density of the choanosome, number of canals and presence/absence of foreign debris. These differences, however, appear constant in all the specimens examined and no seasonal variation is apparent.

The differences in 28S rRNA are too slight to indicate that the two sponges belong to different species. This molecule has not a high discrimination power for low taxonomic rank and especially for sibling species. However, it is worth noting that similar sequences have been found in *Suberites ficus* and *Suberites domuncula* (Chombard 1998), although they are considered as different species.

Finally, the sterol content argues for the fact that there are two distinct species. Both sponges contain unusual Δ^7 sterols which both have double alkylation at the 26 position. The position of the substituents in the side chain is however clearly different in the two sponges. In addition, two other minor sterols were present in *T. cuticulatus*, having M^+ 426 and M^+ 440, while *T. conglomerans* contains an additional minor sterol having M^+ 414.

Sterols were among the first groups of sponge natural products to be used in chemotaxonomy (Bergmann 1949). It was further shown that the overall sterol content of sponges remains relatively constant over time despite season and location, and sterol composition has frequently been used for tentative chemical classification (Bergquist *et al.* 1980, 1986, 1991; De Rosa *et al.* 1973; Hougaard *et al.* 1991; Rovirosa *et al.* 1990). Results given in the literature indicate that the sterol composition presents a consistent pattern within a genus, and that problems of heterogeneity within a family generally result from misidentification.

Our data on sterol content thus confirm that the two species of *Thymosiopsis* are significantly different, although having close affinities. In contrast to Δ^5 sterols, especially cholesterol, which are by far the main sterols in marine invertebrates, Δ^7 sterols are rather unusual (less than 3% of total sterols in sponges). However Δ^7 sterols are the major components in some species without any taxonomic affinity (Kerr & Baker 1991). For example Δ^7 sterols represent respectively 40% and 61% of the sterol content in *Agelas mauritiana* and *Agelas oroides*, 89% in *Aciculites pulchra* (Crist *et al.* 1983), 94% in *Xestospongia* sp. (Kerr *et al.* 1991) and 14% in *Chondrilla nucula* (Sica *et al.* 1978). Except for *Chondrilla nucula,* these sponges have no taxonomic affinity with *Thymosiopsis*.

At a higher taxonomic level, our data confirm that the genus *Thymosiopsis* has clear affinities with *Thymosia*, and that both genera are to be classified in family Chondrillidae. The 28S rRNA sequences strongly support the monophyly of *Thymosiopsis* and *Thymosia* with the representative of the family Chondrillidae, *Chondrosia reniformis* (Fig. 4). Cytology provides additional evidence for the affinities between *Thymosiopsis* and *Thymosia*, although it does not reinforce clearly the affinity with *Chondrosia*. The sterol composition, however, is less informative at this taxonomic level, as *Thymosia guernei* has no Δ^7 sterols, but contains cholestanol as the sole sterol. The data from sterols as well as from cytology thus do not provide evidence as clear as the 28S rRNA sequences for the classification at the family level.

In this study, the biochemical and chemical work was not undertaken for taxonomic purposes, and the methods used may not be the best known for the discrimination of sibling species or ecomorphs. They nevertheless prove interesting for routine taxonomy in such a difficult case.

Acknowledgements

We gratefully acknowledge the assistance of C. Bézac for preparation for TEM studies and for photography, C. Jalong for diving assistance and C. Marshall for photography. P. Rios and F. Cristobo kindly collected specimens of *Thymosia guernei* in Portugal. This work was supported by "Réseau national de Biosystématique (ACC-SV7)" and the European program MAS3-CT97-0118.

REFERENCES

Bavestrello G., Cerrano C., Cattaneo-Vietti R., Sarà M., Calabria F. & Cortesogno L. 1996. — Selective incorporation of foreign material in *Chondrosia reniformis* (Porifera, Demospongiae). *Italian Journal of Zoology* 63: 215-220.

Bergmann W. 1949. — Comparative biochemical studies on the lipids of marine invertebrates, with special reference to the sterols. *Journal of Marine Research* 8: 137-176.

Bergquist P. R., Hofheinz W. & Oesterhelt G. 1980. — Sterol composition and the classification of the Demospongiae. *Biochemical Systematics and Ecology* 8: 423-435.

Bergquist P. R., Lavis A. & Cambie R. C. 1986. — Sterol composition and classification of the Porifera. *Biochemical Systematics and Ecology* 14: 105-112.

Bergquist P. R., Karuso P., Cambie R. C. & Smith D. J. 1991. — Sterol composition and classification of the Porifera. *Biochemical Systematics and Ecology* 19: 17-24.

Bidder G. 1892. — On the flask-shaped ectoderm and spongoblasts in one of the Keratosa. *Proceedings of the Royal Society* 52: 135-139.

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

Boury-Esnault N. & Lopès M. T. 1985. — Les Démosponges littorales de l'archipel des Açores. *Annales de l'Institut océanographique* 61: 149-225.

Bultel-Poncé V., Brouard J.- P., Vacelet J. & Guyot M. 1999. — Thymosiosterol and Δ^{24} Thymosiosterol, new sterols from the sponge *Thymosiopsis* sp. *Tetrahedron Letters* 40: 2955-2956.

Burck C. 1909. — *Zur Kenntnis der Histologie einiger Hornschwämme, sowie Studien über einige Choanoflagellaten*. Inaugural Dissertation, University of Heidelberg, Heidelberg, Germany, 60 p.

Chombard C. 1998. — *Les Demospongiae à asters : essai de phylogénie moléculaire. Homologie du caractère « aster »*. Thèse, Muséum national d'Histoire naturelle, Paris, France, 182 p.

Crist B. V., Li X., Bergquist P. R. & Djerassi C. 1983. — Sterols of marine invertebrates. 44. Isolation, structure elucidation, partial synthesis, and determination of absolute configuration of pulchrasterol. The first example of double bioalkylation of the sterol side chain at position 26. *Journal of Organic Chemistry* 48: 4472-4479.

De Rosa M., Minale L. & Sodano G. 1973. — Metabolism in Porifera II. Distribution of sterols. *Comparative Biochemistry and Physiology* 46: 823-837.

Felsenstein J. 1992. — *Phylip*. University of Washington, Seattle.

Galtier N., Gouy M. & Gautier C. 1996. — SEAVIEW and PHYLO-WIN: two graphic tools for sequence alignment and molecular phylogeny. *Computer Applied Biosciences* 12: 543-548.

Garrone R. 1978. — Phylogenesis of connective tissue. Morphological aspects and biosynthesis of sponge intercellular matrix: 1-250, *in* Robert L. (ed.), *Frontiers of Matrix Biology*. Karger, S, Bâle.

Harmelin J.- G. 1997. — Diversity of bryozoans in a Mediterranean sublittoral cave with bathyal-like conditions: role of dispersal processes and local factors. *Marine Ecology Progress Series* 153: 139-152.

Hougaard L., Christophersen C., Nielsen P. H., Klitgaard A. & Tendal O. 1991. — The chemical composition of species of *Geodia*, *Isops* and *Stryphnus* (Choristida: Demospongia: Porifera). A comparative study with some taxonomical implications. *Biochemical Systematics and Ecology* 19: 223-235.

Kerr G. R. & Baker B. J. 1991. — Marine sterols. *Natural Product Reports* 8: 465-497.

Kerr R. G., Kerr S. L., Pettit G. R., Herald D. L., Groy T. L. & Djerassi C. 1991. — Sterols of marine invertebrates. 63. Isolation and structure elucidation of sutinasterol, the major sterol of the marine sponge *Xestospongia* sp. *Journal of Organic Chemistry* 56: 58-62.

Lévi C. 1956. — Étude des *Halisarca* de Roscoff. Embryologie et systématique des démosponges. *Archives de Zoologie expérimentale et générale* 93: 1-184.

Rosell D. 1988. — Morfologia de *Thymosia guernei* (Porifera, Chondrosiidae), primera cita per a la Península Ibèrica. *Miscellania Zoologica* 12: 353-357.

Rovirosa J., De La Luz Vásquez M. & San Martín A. 1990. — Chemotaxonomic considerations in relation to sponges of the genera *Reniera* and *Haliclona*. *Biochemical Systematics and Ecology* 18: 53-55.

Saitou N. & Nei M. 1987. — The neighbor joining method: a new method of reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Sanger F., Nicklen S. & Coulson A. R. 1977. — DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74: 5463-5467.

Schulze F. E. 1879. — Untersuchungen über den Bau und die Entwicklung der Spongien: Die Gattung *Hircinia* Nardo und *Oligoceras* n. g. *Zeitschrift für Wissenschaftliche Zoologie* 33: 1-38.

Sica D., De Simone F. & Zollo F. 1978. — Sterols of the sponge *Chondrilla nucula*. *Gazzetta chimica Italiana* 108: 575-578.

Topsent E. 1918. — Éponges de San Thomé. Essai sur les genres *Spirastrella*, *Donatia* et *Chondrilla*. *Archives de Zoologie expérimentale et générale* 57: 535-618.

Vacelet J. 1959. — Répartition générale des éponges et systématique des éponges cornées de la région de Marseille et de quelques stations méditerranéennes. *Recueil des Travaux de la Station marine d'Endoume* 16 (26): 39-101.

Vacelet J. & Boury-Esnault N. 1995. — Carnivorous sponges. *Nature* 373: 333-335.

Vacelet J. & Donadey C. 1987. — A new species of *Halisarca* (Porifera, Demospongiae) from the Caribbean, with remarks on the cytology and affinities of the genus: 5-12, *in* Jones W. C. (ed.), *European Contributions to the Taxonomy of Sponges*. Litho Press Co., Midleton.

Vacelet J. & Perez T. 1998. — Two new genera and species of sponges without skeleton (Porifera, Demospongiae) from a Mediterranean cave. *Zoosystema* 20 (1): 5-22.

Vacelet J., Boury-Esnault N. & Harmelin J. G. 1994. — Hexactinellid cave, a unique deep-sea habitat in the scuba zone. *Deep-Sea Research* 41: 965-973.

Submitted on 26 March 1999;
accepted on 6 October 1999.