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Phylogenetic analysis of the order Halichondrida (Porifera, Demospongiae), using 3β-hydroxysterols as chemical characters

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Abstract

To analyze and extract phylogenetic information from chemical characters, the 3β-hydroxysterol composition of 12 sponge species of various families of the Poriferan order Halichondrida (sensu lato), was determined. The current definition of the order is ambiguous, and its classification remains in a state of flux and hence, further chemical characters may aid in stabilizing it. *Axinella corrugata, Pseudaxinella reticulata, P. explicata, Ptilocaulis walpersi, Dragmaxia undata* (family Axinellidae), *Myrmekioderma rea, M. gyroderma* (family Desmoxyidae), *Scopalina ruetzleri* (family Dictyonellidae), *Halichondria lutea, H. magniconulosa, Topsentia ophiraphidites* and *Petromica ciocalyptoides* (family Halichondriidae) were collected in the Santa Marta area, Colombian Caribbean. Cladistic principles were used to advance a hypothesis of genealogical relationship among the studied species and to contrast current classifications at various taxonomic ranks. Sterol identity and various sterol properties (number of carbons, nuclei type, saturations and substitutions of the lateral chain) were coded as discrete characters according to their relative abundance in 4% ranges, for each of the studied species, and for an out group of 41 species of nine orders whose sterol fractions had been published. Of more than 300 codified characters, 74 constituted unique evolutionary novelties within the studied in group. Fifty-one of these characters (including 15 sterols) uniquely defined six species. Twelve sets of novel characters were shared exclusively by two or more species of the studied group and were thus informative in a phylogenetic sense. The cladogram, built by hand, had seven characters that were compatible with each other and were shared by two or more species (i.e. synapomorphies) and five that were incompatible with two or more. However

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few, these seven characters can be used to advance and contrast falsifiable hypotheses of phylogeny. There was not a sterol character unique for the order Halichondrida s.l. At the family level, only Desmoxyidae had a sterol synapomorphy, but it needs confirmation as only species of *Myrmekioderma* were studied. The only clear familial relationship was the existence of a unique synapomorphy for a group formed by the studied Axinellidae and Dictyonellidae, which contradicts relationships hypothesized from morphological characters. At a lower taxonomic rank, there were unique synapomorphies relating the studied species of *Myrmekioderma* and of *Pseudaxinella*. In contrast, *Halichondria* was evidently polyphyletic from sterol data, although three out of four Halichondriidae species were related by a unique sterol property.

Keywords: Halichondrida; Sterols; 3β-hydroxysterols; Classification; Chemo-systematics; Chemical characters; Out-group

1. Introduction

Sponge taxonomy and classification is inherently difficult as sponges posses rather few morphological characters; the description of characters and whole specimens is generally vague and ambiguous, and expertise and long experience is usually needed for a good approximation to the problem. The problems of definition and scope are not limited to species, genera and families, but extend to orders and even to classes. Hence, finding new sets of characters, e.g. those of chemical nature, has brought forth new elements to aid in classification and phylogeny of sponges (e.g. Bergquist and Wells, 1983). However, the use of both morphological and chemical characters for classification in a context of similarity (i.e. phenetic or evolutionary taxonomy schools of systematics), has added to the ambiguity.

The definition of the order Halichondrida is a typical example of the history of sponge classification problems. As originally defined based on spicules and skeletal architecture, this order contained, among others, the morphologically somewhat similar families Halichondriidae and Axinellidae. However, with the introduction of reproductive characters for classification, both families were then considered orders of different subclasses: Halichondrida in subclass Ceractinomorpha (viviparous), and Axinellida in subclass Tetractinomorpha (oviparous, axial condensation of the skeleton) (Lévi, 1953; Lévi, 1973; Bergquist, 1978; for an account see van Soest, 1990). Later, many studies proved the lack of consistency of reproductive characters at the subclass and ordinal level (see van Soest, 1990). Reconsidering the morphological gradation in many of the generic constituents, the families Halichondriidae and Axinellidae were again joined under order Halichondrida sensu lato, together with families Desmoxyidae and Dictyonellidae (van Soest et al., 1990; van Soest, 1990, 1991) and Agelasidae and Ceratoporellidae (van Soest and Braekman, 1999).

These recent changes in classification have occurred mainly in the context of the cladistic or phylogenetic school of systematics, in which primitive characters are discriminated from those that represent an evolutionary novelty (i.e. are derived). In the cladistic school terminology (see Wiley, 1981), primitive characters are called plesiomorphic and derived characters are called apomorphic. A derived character

which is unique to a taxon is called autapomorphic, and one that is shared by two or more taxa is called synapomorphic. In the cladistic school, only the latter types of characters are considered informative for phylogenetic inference. In the case of the newly redefined order Halichondrida, the occurrence throughout the full sponge body of a combination of spicules of the type oxea and styles and modification of these was used as the shared derived character defining the group (van Soest et al., 1990). The possession of isocyanoterpene compounds in some species was also suggested as a possible synapomorphy for the group (van Soest, 1991), but may rather represent a derived (but not universally present) character for the Axinellidae and Halichondriidae families only (van Soest and Braekman, 1999). Secondary metabolite profiles of some species of the order were also explored in search for supporting evidence (Pomponi et al., 1991), but since these were treated in an overall similarity context, the results were not phylogenetically useful. Due to the ambiguity in the definition of the proposed morphological synapomorphies for the order and some families, there has been wide disagreement with van Soest's and collaborators proposal, and the allocation, definition and generic composition of the various families has since changed (e.g. Hooper and Bergquist, 1992; Hooper and Lévi, 1993; Carballo et al., 1996; van Soest and Lehnert, 1997; Hooper et al., 1997). Hence, the taxonomy of the Halichondrida sensu lato remains in a state of flux (see also van Soest and Braekman, 1999). Recently, parallel studies are approaching the problem from a molecular (i.e. DNA sequencing) point of view, and have rejected the monophyletic nature of Halichondrida s.l. (e.g. Boury-Esnault and Solé-Cava, 2002; Erpenbeck et al., 2002).

3β-Hydroxysterols (sterols for short) have been used as tools for sponge classification under a phenetic-evolutionary taxonomy scheme (Bergmann, 1962; Bergquist and Wells, 1983; Kerr and Baker, 1991; Fromont et al., 1994; Duque et al., 1994). However, under a recent cladistic scrutiny of the general occurrence of sterol types and structural properties, with a few exceptions, they have been disregarded as useful in drawing phylogenetic information in high rank groups of sponges because of their wide distribution within and outside Porifera (van Soest and Braekman, 1999). Nevertheless, we felt that a more detailed analysis of sterols as characters at various taxonomic ranks was needed before rejecting their usefulness. We used sponges of the order Halichondrida s.l. to find if sterols could aid in resolving the current classification conflicts.

2. Materials and methods

2.1. Sponge material

Twelve sponges species representing the four families of the order Halichondrida as defined by van Soest et al. (1990), i.e. Axinellidae, Desmoxyidae, Dictyonellidae and Halichondriidae, were sampled in the Santa Marta Area, Caribbean coast of Colombia (Table 1). Although a wider scope of the order Halichondrida includes the families Agelasidae and Ceratoporellidae (van Soest and Braekman, 1999), we

Table 1
Classification and depth of collection of studies species (material collected between January 1997 and December 1998)

Family	Species	Depth (m)	Voucher (INV-POR)	Local taxonomic reference
Axinellidae[a]	*Axinella corrugata* (George and Wilson)	24–25	326	Zea (1987), as *Teichaxinella burtoni*
	Pseudaxinella reticulata (Ridley and Dendy)	15–22	525	Zea (1987), as *P. lunaecharta*
	Pseudaxinella explicata (Wiedenmayer)[b]	15	526	
	Ptilocaulis walpersi (Duchassaing and Michelotti)	15–18	322	Zea (1987); Alvarez et al. (1998)
	Dragmaxia undata (Alvarez et al.)	10–23	527	Wintermann-Kilian and Kilian (1984), as *D. variabilis*
Desmoxyidae[c]	*Myrmekioderma rea* (de Laubenfels)[d]	22	528	
	Myrmekioderma gyroderma (Alcolado)[e]	20	529	
Dictyonellidae	*Scopalina ruetzleri* (Wiedenmayer)	3–25	333	Zea (1987), as '*Ulosa ruetzleri*'
Halichondriidae	*Halichondria lutea* (Alcolado)	24	530	
	Halichondria magniconulosa (Hechtel)	0.2–0.5	531	
	Topsentia ophiraphidites (de Laubenfels)	18–25	532	
	Petromica ciocalyptoides (van Soest and Zea)	9–12	443	van Soest and Zea (1986), as *Monanthus*

[a] According to Alvarez et al. (1998).

[b] Erroneously synonimized to *P. reticulata* by Alvarez et al. (1998), but found to be a valid species by S. Zea (unpublished); both species live sympatrically at Santa Marta.

[c] According to van Soest and Lehnert, 1997 (see also Hooper and Lévi, 1993).

[d] Corresponds to the original *Myrmekioderma styx* (de Laubenfels), but not to the reefal *M. styx* of most Caribbean contemporary authors. It has a spicule complement of slender styles and oxea, plus the usual smaller acanthoxea. It is a predominantly psammophyllic sponge.

[e] Corresponds to the typical reefal *M. styx* of most Caribbean authors. It has stout oxea and no styles in addition to the smaller acanthoxea.

used the more restricted representation of van Soest et al. (1990) as its monophyletic nature was more clearly stated. Sponges were collected, frozen and identified by S.Z. at the Instituto de Investigaciones Marinas y Costeras—INVEMAR, where voucher specimens are deposited (INV-POR, see Table 1). Sponges were identified following Zea (1987); Díaz et al. (1993) and Alvarez et al. (1998) with some amendments (see footnotes in Table 1).

2.2. Isolation and identification of sterols

After air-shipment to the Natural Products Laboratory of the Universidad Nacional de Colombia in Bogotá, sponge material was submitted to MeOH extraction for 24 h, followed by another 24 h extraction in $CHCl_3$. The chloroform extract was dried in Na_2SO_4 and concentrated. Then, it was submitted to silica gel chromatography under vacuum in a discontinuous gradient of solvents. From the fraction eluted by benzene:EtOAc (5:1) the crude sterol mixture was obtained. This fraction was then purified by repetitive column chromatography on silica gel employing benzene:EtOAc (5:1) as isocratic eluent to obtain a purified sterol mixture showing a Rf similar to cholesterol (although the sterols of *Axinella corrugata* had a slightly higher Rf than cholesterol, characteristic of A-norstanols). Finally, the sterol mixture was submitted to fractionation by reverse phase preparative High Performance Liquid Chromatography (HPLC) to obtain pure sterols or enriched mixtures of them, which were then analyzed by high resolution gas chromatography coupled to mass spectrometry (HRGC-MS).

2.2.1. Analytical methods

HPLC was carried out using a Merck-Hitachi L-6000A chromatograph with a Merck LaChrom refraction index detector L-7490 equipped with a Lichrochart RP-18 (125 × 4 mm) column, with MeOH as eluent at 0.7 ml min^{-1}.

HRGC-MS was performed in a Shimadzu GC-17A CGMS-QP5050A gas chromatograph equipped with an HP-1 fused silica column (25 m × 0.2 mm i.d; film thickness, 0.33 μm) maintained at 270 °C, and He as carrier gas at a 1 ml min^{-1}. Split injection 1:10 and 1 μl injection volumes were used. Temperatures of injector and interface were fixed at 300 °C. For the electronic impact mass spectrometry the ion source was held at 70 eV and the filament at 60 μA.

2.2.2. Structural assignment

3β-Hydroxysterols isolated from the studied sponges were identified by the careful analysis of their mass spectra and by the comparison of the spectral data and retention times relative to cholesterol both in HRGC and HPLC with authentic samples or with those reported in the literature. Quantitative data for sterols (percent of total sterol mixture) in each sponge were obtained by normalizing areas in both HRGC and HPLC.

2.3. Chemo-systematic analysis

As characters for the analysis, the following classes of sterol properties were taken into account: identity (85 sterols), number of carbons (seven types), nuclei type (eight types), position of unsaturations in the side chain (six types), and substitution patterns in the side chain (10 types). The relative abundance of each type of structure within each class was calculated as its percentage of the total sterol mixture. For further cladistic analysis, to treat these abundances as discrete instead of continuous characters, they were coded with letters from **a** to **y** in 4% ranges, following the frequency method devised for polymorphic characters by Wiens (1995). Additionally, as the absence of a property (relative abundance of 0%) cannot be considered a character, and as resolution for trace sterols vary with methods and equipment used by the various workers, sterols or properties with abundances below 1% were coded together with those absent.

Although other authors had shown that sterol composition remains more or less constant within a species irrespective of geographical locality or season of collection (Bergquist et al., 1980; Fromont et al., 1994), we also undertook the comparison of the sterol composition in samples of some species collected in different dates. This procedure was also necessary to test if the within-species variation was less than the 4% of relative abundance ranges used for the codification process. The studied species were *Scopalina ruetzleri* ($n = 6$ dates, all individuals from a single date pooled), *Halichondria magniconulosa* ($n = 3$ dates, all individuals from a single date pooled), *Myrmekioderma gyroderma* ($n = 4$ individuals treated separately, three dates), and *Pseudaxinella reticulata* ($n = 9$ samples, three dates, two of those with four individuals each, treated separately). The coefficient of variation (CV) of the relative abundance of each sterol among individuals and dates within each of the above species were calculated. CVs $>20\%$ were found in sterols occurring in abundances lower than 2%, while CVs $<3.5\%$ were found for sterols in abundances greater than 8% (see Castellanos, 2000 for details). Hence, it was decided to combine the first two 4% ranges in a single one (1–8%), and relative abundances were coded as **a1** ($<1\%$, including absences or non-detections), **b** (1–8%), and from **c** to **y** in 4% intervals up to 100%. The relative constancy of sterol proportions across individuals and times confirmed they are fixed in a species-specific fashion and could be used for systematic purposes.

Of the total 116 properties and their relative abundance codes, more than 300 discrete characters were drawn. Each was assumed to be an independent character or state. For example, character '30 carbon sterols' in abundance **b** was considered independent of character '30 carbon sterols' in abundance **y**. Thus, all possible evolutionary changes from one particular abundance to another (e.g. **a1** to **b**, or **a1** to **y**, or **b** to **y**) within the same sterol property were assumed equally likely. This is not necessarily a realistic assumption, but had to be taken due to the lack of detailed information on biosynthetic pathways of most of the sterols found in the studied sponges. The presence of each sterol character was determined in the studied group (i.e. the in group) and in an external group (i.e. the out group) conformed by 41 species of the sponge orders Dendroceratida, Nepheliospongida, Verongida,

Dictyoceratida, Haplosclerida, Poecilosclerida, Hadromerida, Spirophorida and Choristida, whose sterol composition had been published (Bergquist et al., 1980). The in group was assumed to be monophyletic following the proposal of van Soest (1990). Species of *Agelas* were excluded from the dataset as there has been much controversy regarding the relationship of this genus with those of the Halichondria s.l. families. If a sterol character was present in both the in group and the out group it was considered primitive; if it was present only in the in group, it was considered derived. Different characters which had the same distribution within the in group (i.e. were present and with the same abundance in the same species) were considered redundant and pooled in a single character. This step had to be taken because of the lack of definitive knowledge of their independence. Among the derived characters, those present in a single species (i.e. autapomorphic) were noted. A single cladogram was built manually step by step with the remaining characters (derived ones shared by two or more species) selecting between alternative branches with the least number of incompatible characters (cf. Lynch, 1997). The generated cladogram was advanced as the least-rejected hypothesis of the phylogeny of the studied group of species, to be contrasted to other hypotheses. The characters supporting the branching of the cladogram (i.e. the synapomorphies) were considered evidence of evolutionary relationship among the species comprising the branch, i.e. that they conform a monophyletic group. The incompatible characters were considered evidence against the relationship. It should be noted that the least-rejected cladogram obtained may be different from the more parsimonious one (i.e. that with the least number of character changes).

3. Results

3.1. Sterol composition of the studied group of species

The sterol composition expressed in relative percentages, of each one of the studied sponge species of the order Halichondrida s.l. is presented in Table 2. The table shows the identity of the sterols (nucleus and side chain), its molecular weight, and relative retention times in both HRGC and HPLC. As shown, general sterol composition differs greatly among many of the studied sponges. However, there was a great similarity, qualitatively as well as quantitatively in the sterol contents of the studied species within the genera *Pseudaxinella* and *Myrmekioderma*. This was not the case for the two species of the genus *Halichondria*, both with very different sterol compositions. It is also remarkable that there were species with a unique sterol in a high proportion, as was the case for 24-isopropyl-22-dihydrocholesterol (sterol 66) both in *Halichondria lutea* and *Petromica ciocalyptoides*.

Regarding the distribution of nuclei types, most species had a single predominant type. For example, in *Halichondria magniconulosa* most sterols had a Δ°-3β-hydroxiandrostane (71%), while in *Axinella corrugata* sterols with 3β-(hydroxymethyl)-A-norsterane were dominant, although there were also other more common nuclei.

Table 2
3β-Hydroxysterol composition of the studies species (see Castellanos, 2000, for details)

No	M^+	STEROL	RtT HR-GC[a]	RtT HPLC[a]	Relative abundance (%) (t = traces)											
					A. corrugata	*D. undata*	*H. lutea*	*H. magni-conulosa*	*M. rea*	*M. gyroderma*	*P. ciocalyp-toides*	*P. explicata*	*P. reticulata*	*P. walpersi*	*T. ophira-phidites*	*S. ruetzleri*
1	330	C1	0.49	0.50										t		
2	368	E2	1.01	0.64		t										
3	370	C3	0.71	0.64	t	t		t	t	t		2.4	2.6	t		t
4	370	D3	0.78	0.65		t		t								
5	372	A3	0.73	0.73		t		t						t		
6	372	F3	0,69[b]	0,64[b]	t											
7	384	C4	0.92	0.78	0.6	8.8	t	t	t	2.4		0.7	1.6	1.5	t	7.7
8	384	C5	0.89	0.71	t	1.3	t	t	0.7	0.9		2.6	2.6	1.3	t	2.6
9	384	D4	1.02	0.82	t	t		0.8		0.6		0.7		0.9		0.9
10	384	D5′	0.98	0.79	t	t										
11	386	A4	0.96	0.90		t		2.1			t					
12	386	C6	1.00	1.00	t	33.7	0.9	4.5	2.5	7.8	1.1	12.8	13.6	7.9	1.1	18.4
13	386	D6	1.09	0.99		4.8		6.9							t	
14	386	F5′	0,88[b]	0,75[b]	1.7											
15	386	F4	0,90[b]	0,80[b]	7.4											
16	388	A6	1.02	1.10		3.8	t	62.5		t	t		t	t	0.8	0.6
17	388	B6	0.93	0.78		0.6										
18	388	F6	1,00[b]	1,00[b]	24.8											
19	396	C7	1.08	0.67							3.0	2.6				
20	398	C8	1.20	0.82	t	8.5		t	0.3	2.3		t	t	0.8	t	15.0
21	398	C9	1.09	0.84	0.9	4.8	0.5	2.4	6.4	5.1	t	3.3	3.6	4.5	0.9	9.4
22	398	C10	1.09	0.90	2.2	8.7	0.5	2.8	9.4	8.9	t	16.9	14.3	9.3	0.9	14.8
23	398	C11	1.16	0.78								t				
24	398	D9	1.22	0.87	t			1.0								
25	398	D8	1.37	0.81				t								

(*continued on next page*)

Table 2 (*continued*)

No	M^+	STEROL	RtT HR-GC[a]	RtT HPLC[a]	Relative abundance (%) (t = traces)											
					A. corrugata	*D. undata*	*H. lutea*	*H. magni-conulosa*	*M. rea*	*M. gyroderma*	*P. ciocalyp-toides*	*P. explicata*	*P. reticulata*	*P. walpersi*	*T. ophira-phidites*	*S. ruetzleri*
26	398	D10	1.22	0.92	t			0.8								
27	398	C12	1.04	0.85										t		
28	400	H6	1.34	1.10							t					
29	400	A9	1.12	0.88				1.7								
30	400	A8	1.23	0.98				1.9								
31	400	C13′	1.22	1.09	0.6	3.0	t		3.7	4.1	t	5.1	6.2	8.2		3.2
32	400	D13′	1.34	1.10		t										
33	400	F9	1,10[b]	0,82[b]	11.6											
34	400	F10	1,09[b]	0,89[b]	10.0											
35	400	F8	1,18[b]	0,82[b]	t											
36	400	l6	1.20	<1,11							t					
37	402	A13′	1.24	1.15		t		1.4								
38	402	F13′	1,20[b]	1,05[b]	10.3											
39	410	C14	1.45	0.79					1.4			t				
40	410	C15	1.31	0.78					0.9			4.3	4.8			
41	410	E16	1.36	0.92		t										
42	412	C16	1.31	1.04	1.0	4.9	0.6	3.2	13.9	5.9	t	18.0	18.7	12.0	1.0	7.7
43	412	C17	1.48	1.00				t		1.3		2.7	1.2			1.4
44	412	C18	1.44	0.94				t	t	t		1.8	1.1			8.7
45	412	C19	1.39	1.03		0.6		t	2.8	t		1.7	2.3	t		t
46	412	C20	1.55	0.96								t				
47	412	D16	1.42	1.07		2.2										
48	412	D17′	1.44	1.00						1.3						
49	412	C21	1.41	0.89						0.3						
50	412	C-$C_{10}H_{19}$ Δ^{22}	1.27	1.06								1.2	1.9	2.8		
51	414	F17	1,48[b]	0,95[b]	t											

Table 2 (*continued*)

No	M^+	STEROL	RtT HR-GC[a]	RtT HPLC[a]	Relative abundance (%) (t = traces)											
					A. corrugata	*D. undata*	*H. lutea*	*H. magni-conulosa*	*M. rea*	*M. gyroderma*	*P. ciocalyp-toides*	*P. explicata*	*P. reticulata*	*P. walpersi*	*T. ophira-phidites*	*S. ruetzleri*
52	414	F18	1,44[b]	1,00[b]	t											
53	414	A16	1.31	1.13		t										
54	414	F19′	1,26[b]	1,10[b]	4.2											
55	414	C22	1.47	1.15	2.3	10.8	1.1	4.5	56.3	57.2	0.6	19.5	18.1	48.8	0.6	7.7
56	414	C23′	1.50	1.19									t			
57	414	F16	1,30[b]	1,00[b]	7.4											
58	414	A17′	1.60	1.05	t											
59	416	A22	1.48	1.28				1.3		t	t			t		
60	416	F22	1,44[b]	1,09[b]	10.8											
61	416	F23′	1,49[b]	1,24[b]	t											
62	416	G13	1.52	1.50							t					
63	416	A23	1.57	1.35					t							
64	424	C-$C_{11}H_{19}$ $\Delta^{22,25(26)}$	1.50	0.82											2.1	
65	424	C-$C_{11}H_{19}$	2.24	<0,7											t	
66	426	C24	1.54	1.16		1.4	96.4			0.6	96.3				28.5	
67	426	C25	1.67	1.04							t	0.9	0.6			
68	426	C26	1.60	1.02								1	0.8		1.5	
69	426	C27	1.67	0.98						t						
70	426	C28	1.74	1.05								1.0	0.8			
71	426	C29	1.89	1.05									0.8	1.1		
72	426	C-$C_{11}H_{19}$	1.86	1.70												1.0
73	426	C30	1.72	1.10											49.0	
74	426	C31	1.81	1.05											0.9	
75	428	A24	1.58	1.46			t				t				0.9	
76	428	C32	1.72	1.35			t				t		t		1.3	
77	428	F25′	1,69[b]	1,10[b]	1.3											

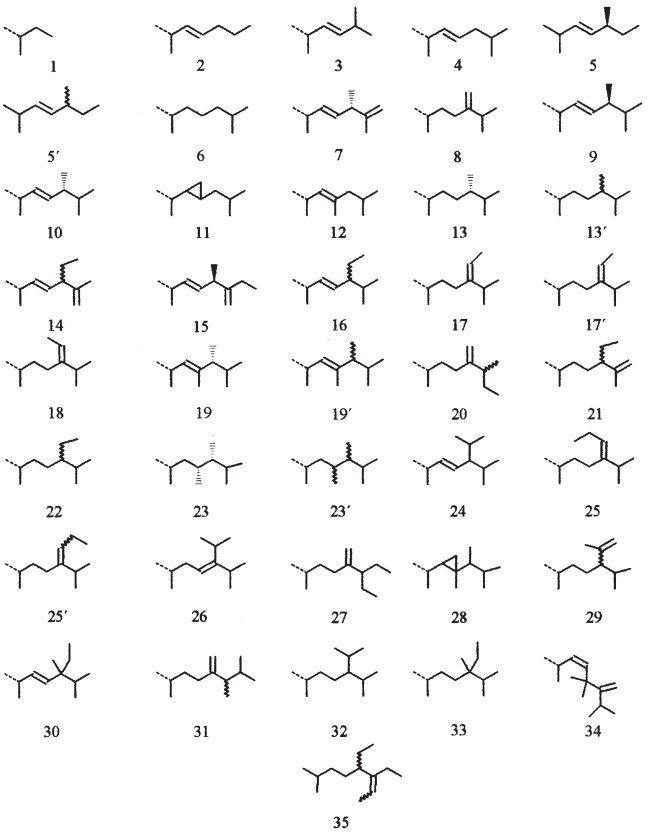
Table 2 (*continued*)

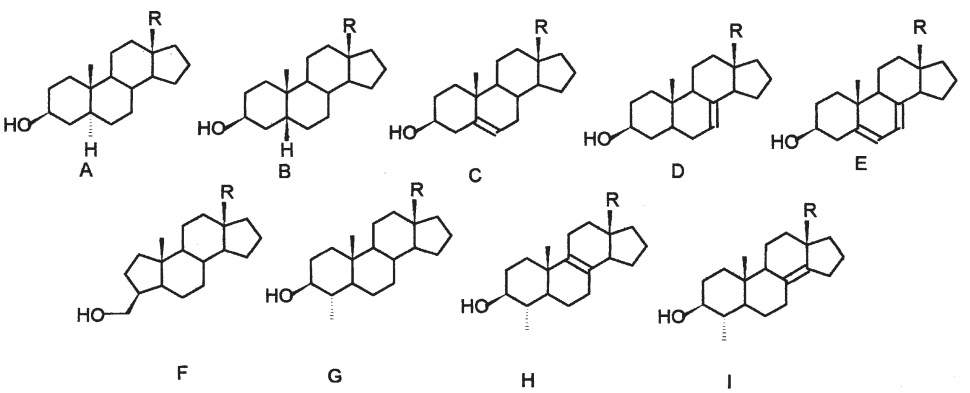
No	M^+	STEROL	RtT HR-GC[a]	RtT HPLC[a]	Relative abundance (%) (t = traces)											
					A. corrugata	*D. undata*	*H. lutea*	*H. magni-conulosa*	*M. rea*	*M. gyroderma*	*P. ciocalyp-toides*	*P. explicata*	*P. reticulata*	*P. walpersi*	*T. ophira-phidites*	*S. ruetzleri*
78	428	122	1.79	1.15		t										
79	428	C33	1.95	1.25											1.2	
80	430	A32	1.81	1.58							t					
81	438	C34	1.77	0.95											7.2	
82	438	??	2.02	1.00											t	
83	440	C-$C_{12}H_{23}$ Δ^{22}	2.07	1.20									0.6			
84	440	?-$C_{12}H_{23}$ Δ^{22}	1.87	0.90											0.8	
85	440	C35	1.99	1.30											t	

(side chains and nuclei on next page)

[a] Relative to cholesterol.
[b] Relative to A-norcholestanol.

(Side chains and nuclei for Table 2)





In half of the studied species sterols with saturated side chains were dominant, while in the other half-dominated sterols with an unsaturation in C-22. *Pseudaxinella reticulata, P. explicata* and *Topsentia ophiraphidites* had significant abundances of diunsaturated side chains. On the other hand, there was no clear pattern of distribution by number of carbons within the in group, excepting perhaps a somewhat large proportion (8.3%) of C_{31}-sterols in *Topsentia ophiraphidites*. This species also had the novel sterol 73 (Castellanos et al., submitted), having a methyl-ethyl disubstitution in C-24, in greater proportion than the usual isopropyl substitution of most of its other sterols.

3.2. Autapomorphic characters

Of the more than 300 defined sterol characters, 74 were found to be derived in the studied in group. Of these, 51 were present in a single species, among which there were 15 particular sterol molecules uniquely present within some of the studied species and altogether absent in the species of the out group (i.e. autapomorphic). These were (proportion code in parenthesis): sterols 14(b), 15(b), 18(g), 33(c), 34(c), 38(c), 54(b), 57(b), 60(c) and 77(b) in *Axinella corrugata*, 48(b) in *Myrmekioderma gyroderma*, 64(b), 73(m), and 81(b) in *Topsentia ophiraphidites* and 72(b) in *Scopalina ruetzleri*. These constitute diagnostic characters for these species.

3.3. Informative characters and cladogram construction

Among the 74 derived characters, 23 were informative; that is, they were shared by two or more species of the in group. However, there were several groups of characters that were redundant, that is, gave the same type of information, and hence had to be treated as a single character. Thus, only 12 characters or groups of characters (Table 3, henceforth called simply characters by their number) were used to build the cladogram (Fig. 1). First, independent and nested branches were successively constructed with six characters that did not contradict each other. The remaining six characters were incompatible and were drawn in the cladogram as evidence against the hypothesis of phylogeny it represented. Among these, characters 6 and 11 were incompatible to each other and generated an unresolved tricotomy for the group *Topsentia ophiraphidites, Halichondria lutea* and *Petromyca ciocalyptoides* (Table 3, Fig. 1). However, within character 6, sterol 66 (Table 2) occurred in this group in a relative abundance of about 96% (coded **y**, vs. up to 35% in other species of the in group and up to 1% in the out group), with an isopropyl substitution which is uncommon in nature, while C_{28}-sterols (character 11) are the most abundant in nature, and there is relatively little change in abundance among the in group species. Hence, we resolved to give more weight to character 6 and considered it as a true synapomorphy for the group formed by the above-mentioned three species. In summary, the cladogram was built by seven synapomorphic characters (1, 2, 4, 5, 6, 9 and 12), and was contradicted by five incompatible characters (3, 7, 8, 10 and 11) (see Table 3 and Fig. 1).

From the sterol data, there was no single sterol character shared by all the species

Table 3
Matrix of informative (with redundant) sterol characters

Code	Character (abundance)	Abundance in out group	Abundance in in group											
			A. cor.	*D. und.*	*H. lut.*	*H. mag.*	*M. rea*	*M. gyr.*	*P. cio.*	*P. exp.*	*P. ret.*	*P. wal.*	*T. oph.*	*S. rue.*
1	Sterol 3 (b)	al	al	al	al	al	al	al	al	**b**	**b**	al	al	al
	Sterol 19 (b)		al	al	al	al	al	al	al	**b**	**b**	al	al	al
	Sterol 40 (b)	al	al	al	al	al	al	al	al	**b**	**b**	al	al	al
	Sterol 42 (e)	b,d	b	b	al	b	d	b	al	**e**	**e**	d	b	b
	Sterol 44 (b)	c	al	al	al	al	al	al	al	**b**	**b**	al	al	c
	C_{29} (m)	al–c,e,g,q	g	e	b	c	s	q	al	**m**	**m**	q	b	g
2	Sterol 8 (b)		al	**b**	al	al	al	al	al	**b**	**b**	**b**	al	**b**
3[a]	Sterol 45 (b)		al	al	al	al	**b**	al	al	**b**	**b**	al	al	al
	Diunsaturated side chain (b)		al	al	al	al	**b**	al	al	**b**	**b**	al	c	al
4	Sterol 50 (b)		al	al	al	al	al	al	al	**b**	**b**	**b**	al	al
5	Sterol 55 (o)	b,e–g,j,k,n,p	b	c	b	b	**o**	**o**	al	e	e	m	al	b
6	Sterol 66 (y)	al	al	b	**y**	al	al	al	**y**	al	al	al	h	al
	C-24 isopropyl substitution (y)	al	al	b	**y**	al	al	al	**y**	al	al	al	i	al
	C-22 unsaturation (y)	b,c,e–k	m	**i**	**y**	e	i	g	**y**	m	m	i	u	h
	C_{30} (y)	al	b	b	**y**	al	al	al	**y**	b	b	b	v	al
7[a]	Sterol 68 with C-23 unsaturation (b)		al	al	al	al	al	al	al	**b**	al	al	**b**	al
8[a]	Δ° nucleus (b)	al,c,e,h,p,u,w,y	al	**b**	al	r	al	al	**b**	al	al	al	**b**	al
9	Saturated side chain (b)	i,j,m–w	m	o	**b**	u	p	r	**b**	j	j	q	**b**	h
10[a]	C-22 unsaturation (m) C-24 methyl substitution (k)	al–c,e–k	**m**	i	y	e	i	g	y	**m**	**m**	i	u	h
		al–g,i	**k**	e	c	c	g	e	al	**k**	**k**	f	b	h
11[a]	C_{28} (b)	c–i,k,l	j	g	**b**	d	e	f	al	h	g	f	**b**	k
12	C_{30} (b)	al	**b**	**b**	y	al	al	al	y	**b**	**b**	**b**	v	**b**

Relative abundance ranges: al, present, but <1%; b, 1–8%, c to y in 4% intervals up to 100%. Blank abundances in out group mean the sterol property has not been recorded. Sterol numbers are those of Table 2. For full name of species examined see Tables 1 and 2. Bold-faced codes indicate the species sharing a particular character.

[a] Incompatible character.

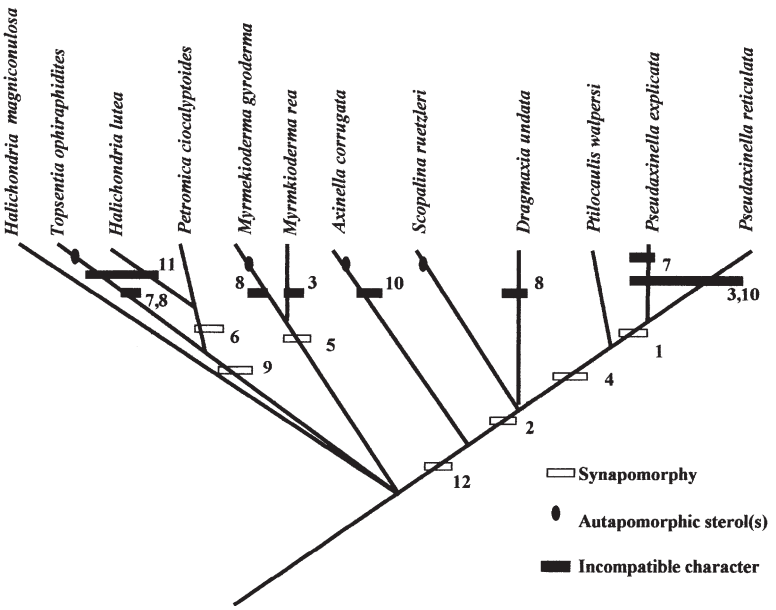


Fig. 1. Cladogram for the species of the order Halichondrida s.1. collected in the Colombian Caribbean Sea. Built from relative abundance data of sterols and sterol properties as continuous characters, codifying relative proportions by the frequency method. Explanation for codes of characters in Table 3. See autapomorphic sterols in the text (Section 3.2).

of the studied group. This means that either 3β-hydroxysterols are not informative at the order level in Halichondrida s.l., or that its current definition and scope are not appropriate and some of the studied species should belong to other orders. Monophyletic groups formed from sterol data were (see Fig. 1 and Table 3): (1) *Topsentia ophiraphidites*, *Halichondria lutea* and *Petromyca ciocalyptoides* supported by character 9, a decrease, in relation to other species of the in group, in the relative abundance (from h or higher to b) of sterols with saturated side chain. (2) The two analyzed species of *Myrmekioderma* which shared an increase (from lower than m to o) in abundance of sterol 55 (character 5). (3) All studied species of family Axinellidae and *Scopalina ruetzleri* (family Dictyonellidae) were related by a decrease in the abundance (from v and y to b) of C_{30}-sterols (character 12). (4) All the latter, excepting *Axinella corrugata*, shared exclusively sterol 8 in abundance b (character 2). (5) *Ptilocaulis walpersi* and the analyzed species of *Pseudaxinella* shared exclusively sterol 50 in abundance b (character 4). (6) A synapomorphy for the two species of *Pseudaxinella* turned out to be the exclusive sharing of various sterols, plus some slight changes in relative abundances of other properties (character 1, see Table 3).

4. Discussion

4.1. Sterol composition

The diversity of sterols found in the studied species of Halichondrida (from 12 to 30 in a given species) confirms the usual pattern in Porifera (e.g. Bergquist et al., 1980). However, there are interesting contrasts in our results that warrant discussion. Our report of 3β-(hydroxymethyl)-A-norstane in high proportion (90.6%) in *Axinella corrugata* contrasts with the report by Bohlin et al. (1981) of dominant Δ^5-3β-hydroxyandrostane in *Teichaxinella morchella*, a species which has been synonymized to the former by Alvarez et al. (1998). Hence, either these species are different, or there is a certain degree of geographical variation in the sterol composition (Santa Marta vs. Gulf of Mexico). It is important to note that the norstane sterols have a restricted distribution in nature, having been found only in 11 sponge species (Aknin et al., 1996).

Also relatively uncommon in Porifera is the presence of significant amounts of sterols with diunsaturated side chains, as was the case found here for *Pseudaxinella reticulata, P. explicata* and *Topsentia ophiraphidites*. Also, the presence of C_{31}-sterols in the latter species, although in somewhat small relative abundance (8.3%), is rather uncommon in Porifera and even in nature (Stoilov et al., 1986; Theobald and Djerassi, 1978; Kerr and Baker, 1991). This fact makes this species an interesting target for biogenetic studies of long-chain and polyalkylated sterols. Additionally, the presence of a dialkyl substitution in C-24 in the novel and major sterol 73 of *T. ophiraphidites*, contrasts with the usual presence of isopropyl substitution in C-24 in most Porifera major sterols (Bergquist et al., 1980; Kerr et al., 1997).

The relative constancy of sterol proportions within species found in this study confirms previous analyses (e.g. Bergquist et al., 1980; Fromont et al., 1994). Hence, sterols as such can be used as chemical characters.

4.2. Phylogenetic analysis

It is clear from our work that of such a large suite of sterols and sterol characters, only a handful of them may turn out to be informative in a phylogenetic context. This underscores the importance of detailed analysis of the distribution of sterols and sterols characters among the various groups of sponges. The publication of the full suite of a given class of chemical compounds, new or known, found in an organism, thus becomes critical (van Soest and Braekman, 1999). Our incorporation in the analysis of relative abundances in a formal codification procedure, allowed the finding of new informative characters. These may shed some light on the phylogenetic relationships within the constituting families of the order Halichondrida s.1. Indeed, most of the 12 groups of informative sterol characters found could represent plausible evolutionary changes. Excepting character 10, and perhaps 11 (Table 3) they either implied the appearance de novo or an increase or a decrease in relative abundance of a particular sterol, or an important change in the proportion of a sterol property. Incompatible characters, although also plausible, were contradicted by two

or more of the compatible characters, indicating that they may represent true additional evolutionary steps (e.g. reversals, convergences, etc.). Nevertheless, they stand as contradictory evidence until further study.

This study revealed the lack of a sterol character to further support the monophyly of Halichondrida s.1. However, this lack does not constitute an argument against it. Nevertheless, the general vagueness of some of the of the proposed morphological synapomorphies for and within the group (non-localized interchangeable styles and oxeas, fleshy ectosome, loss of reticulate skeleton, etc.) and the poor resolution of biochemical ones (scattered presence of isonitriles; similarity in chemical profiles) (van Soest, 1990; van Soest et al., 1990; Pomponi et al., 1991), call for a revision of the scope and monophyly of Halichondrida s.1. and of some of its families, and perhaps for the familial placement of some of the genera and species. This revision is already taking place (see for example, Hooper et al., 1992; Hooper and Lévi, 1993; Alvarez and Crisp, 1994; Alvarez et al., 1998; van Soest and Lehnert, 1997), and molecular phylogenies are also showing the para- or polyphyletic nature of the group (Boury-Esnault and Solé-Cava, 2002; Erpenbeck et al., 2002). In contribution, sterol characters show that three out of four of the Halichondriidae species, all Desmoxyidae species, and the combined Dictyonellidae-Axinellidae species may conform monophyletic groups.

Within defined families, there were sterol characters that grouped some of the genera and species within them, which may serve to provide new, or contradict, or support, the previous hypotheses of phylogenetic relationships. Three of the four studied species of Halichondriidae (*Topsentia ophiraphidites*, *Halichondria lutea*, and *Petromica ciocalyptoides*) appear related by an important reduction in sterols with saturated side chains (character 9). A close relationship between *Topsentia* and *Petromica* had already been drawn from morphology (Díaz et al., 1991) and from similarity in secondary metabolite composition (Pomponi et al., 1991). In contrast, the two studied species of *Halichondria* came out separately in our analysis, not sharing a sterol synapomorphy, further evidencing the fact that this genus has been mainly defined by primitive characters (van Soest et al., 1990; Díaz et al., 1991) and that it is polyphyletic. The hypothesized close relationship between *H. lutea* and *P. ciocalyptoides* from sterol data (character 6) implies that the former may not belong to *Halichondria*, and calls for a reinterpretation of its morphological characters. Morphologically, the nature of the sharp, corrugated projections it has on the surface, and the lack of a clear tangential reticulation of spicule tracts (see Díaz et al., 1993) puts *H. lutea* apart from other species of the genus.

There was a sterol character constituting a synapomorphy for the two studies species of the family Desmoxyidae (increase in abundance of sterol 55), but as these belong to a single genus, *Myrmekioderma*, no definite conclusion can be drawn from sterols at the family level. van Soest et al. (1990) and Díaz et al. (1991), placed *Myrmekioderma* and the morphologically similar *Didiscus* in family Halichondriidae, but both later were included in Desmoxyidae by Hooper and Lévi (1993) and van Soest and Lehnert (1997). Similar biochemical profiles between *Myrmekioderma* and the typical Desmoxyidae genus *Higginsia* (Hooper et al., 1992), may constitute independent evidence to support the monophyly of Desmoxyidae. The polytomy at the

base of our cladogram does not allow for additional evidence to show whether *Myrmekioderma* is more related to Halichondriidae genera or to other Desmoxyidae. Morphological characters showed that *Myrmekioderma* and *Didiscus* are sister groups, but falling among other Halichondriidae genera (van Soest et al., 1990), although they have dissimilar thin layer chromatography (TLC) secondary metabolite profiles (Pomponi et al., 1991). However, this overall dissimilarity does not preclude sharing a single, synapomorphic secondary metabolite. Within *Myrmekioderma* there was an autapomorphy for *M. gyroderma*, further confirming the clear morphological differences between the two species. As additional distinction, *M. rea* produced trace amounts of secondary metabolites in TLC, while *M. gyroderma* (as *M. sytx*) had unique patterns related to depth (Pomponi et al., 1991).

All studied species of Axinellidae and the one species of Dictyonellidae shared character 12 (large decrease in C_{30}-sterols). This contradicts the finding of van Soest et al. (1990), that Halichondriidae and Dictyonellidae are sister groups, more closely related to each other than to Axinellidae by virtue of sharing the secondary loss of reticulate skeleton. *Ptilocaulis* was initially placed in Desmoxyidae (van Soest et al., 1990), but was later moved to Axinellidae (Alvarez and Crisp, 1994; Alvarez et al., 1998; van Soest and Lehnert, 1997). Our study does support this change from some congruent synapomorphies (12, 2 and 4, Table 3). Indeed, although sterol HRGC-MS profiles of *Ptilocaulis* and *Myrmekioderma* were very similar (Castellanos, 2000), these similarities turned out to be of primitive nature (i.e. plesiomorphic). In contrast, a morphological cladogram of Caribbean Axinellidae (Alvarez and Crisp, 1994) was not congruent with our sterol cladogram. While from morphology *Ptilocaulis walpersi* and *Axinella corrugata* were more closely related to each other than to *Pseudaxinella explicata*, from sterols *P. explicata* and *P. walpersi* appeared more closely related.

The two studied species of *Pseudaxinella* share a suite of exclusive sterols, of which sterols 19 and 40 had already been isolated from the E Atlantic *P. lunaecharta*, twin species of *P. reticulata* (Sjöstrand et al., 1981). This is good evidence for the monophyly of these three species of *Pseudaxinella*. However, there is need of studies on other species of the genus to confirm the sterol synapomorphy. Its current morphological definition is based on the lack of typical Axinellid characters, and several of the species are morphologically rather different (Hooper and Lévi, 1993; Alvarez et al., 1998). There were not unique sterols to distinguish between the sympatric *P. reticulata* and *P. explicata* to confirm their clear morphological differences (Zea, unpublished), although sterol proportions vary slightly between these two species (Table 2).

In conclusion, our study shows that detailed analysis of the distribution of sterol properties among sponges, yields phylogenetically informative characters, however few, that may be used to contrast or support hypotheses of phylogeny and proposals of classification at various ranks. In the case of the order Halichondrida s.l., sterol characters neither contradict nor support its monophyly, but call for a closer scrutiny of the scope of some of the families and of the relationship among them. Within families, the polyphyletic nature of genus *Halichondria* is evident from sterols, while

the monophyletic nature of studied species of *Myrmekioderma* and *Pseudaxinella* is supported.

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References

Aknin, M., Gaydou, E.M., Boury-Esnault, N., Costantino, V., Fattorusso, E., Mangoni, A., 1996. Norsterols in *Axinella proliferans*, sponge from the Indian Ocean. Comp. Biochem. Physiol. B 113, 845–848.

Alvarez, B., Crisp, M.D., 1994. A preliminary analysis of the phylogenetic relationships of some axinellid sponges. In: van Soest, R.W.M., van Kempen, T.M.G., Braekman, J.-C. (Eds.), Sponges in time and space. Balkema, Rotterdam, pp. 117–122.

Alvarez, B., van Soest, R.W.M., Rützler, K., 1998. A revision of Axinellidae (Porifera: Demospongiae) of the Central West Atlantic region. Smithson Contrib. Zool. 598, 1–47.

Bergmann, W., 1962. Comprehensive biochemistry. Academic Press, New York.

Bergquist, P.R., 1978. Sponges. Hutchinson, London.

Bergquist, P.R., Hofheinz, W., Oesterhelt, G., 1980. Sterol composition and the classification of the Demospongiae. Biochem. Syst. Ecol. 8, 423–435.

Bergquist, P.R., Wells, R.J., 1983. Chemotaxonomy of the Porifera: The development and current status of the field. In: Scheuer, P.J. (Ed.), Marine natural products. Academic Press, New York, pp. 1–50.

Bohlin, L., Sjöstrand, U., Djerassi, C.W., Sullivan, B., 1981. Minor and trace sterols in marine invertebrates. Part 20. 3β-Hydroxy-methyl-A-nor-patinosterol and 3β-Hydroxy-methyl-A-nor-dinosterol. Two new sterols with modified nucleus and side-chain from sponge *Teichaxinella morchella*. J. Chem. Soc, Perkin. Trans. 1, 1023–1028.

Boury-Esnault, N., Solé-Cava, A., 2002. The present state and future perspectives in molecular natural history of sponges. Boll. Mus. Ist Biol. Univ. Genova 66-67, 33–34.

Carballo, J.L., Uriz, M.J., García-Gómez, J.C., 1996. Halichondrids or axinellids? Some problematic genera of sponges with descriptions of new species from the Strait of Gibraltar (southern Iberian Peninsula). J. Zool. Lond. 238, 725–741.

Castellanos L. Composición esterólica de esponjas del orden Halichondrida (Porifera, Demospongiae) del Caribe colombiano y su significancia como caracter químico de clasificación. M.Sc. Thesis (Chemistry), Universidad Nacional de Colombia, Santafé de Bogotá, 2000.

Castellanos L, Duque C, Echigo S, Fujimoto Y. Isolation of a new 24-ethyl-24-methylsterol from the sponge, *Topsentia ophiraphidites*: C-24 stereochemistry of marine 24-ethyl-24-methylsterols. Tetrahedron (submitted).

Díaz, M.C., van Soest, R.W.M., Pomponi, S.A., 1991. A systematic review of the Central-Atlantic Hal-

ichondrida (Demospongiae, Porifera). Part I: evaluation of characters and diagnosis of general. In: Reitner, J., Keupp, H. (Eds.), Fossil and recent sponges. Springer-Verlag, Heidelberg, pp. 134–149.

Díaz, M.C., Pomponi, S.M., van Soest, R.W.M., 1993. A systematic revision of the central West Atlantic Halichondrida (Demospongiae, Porifera). Part III: description of valid species. Sci. Mar. 57, 283–306.

Duque, C., Castillo, G., Buitrago, S., Osorno, O., Zea, S., 1994. Esteroles libres monohidroxilados de la esponja marina *Agelas schmidti* (Wilson, 1902). Rev. Col. Quím. 23, 63–72.

Erpenbeck, D., Breeuwer, J.A.J., van Soest, R.W.M., 2002. The systematics of halichondrid demosponges—A multiple gene approach. Boll. Mus. Ist Biol. Univ. Genova 65, 66–67.

Fromont, J., Kerr, S., Kerr, R., Riddle, M., Murphy, P., 1994. Chemotaxonomic relationships within, and comparisons between, the orders Haplosclerida and Petrosida (Porifera: Demospongiae) using sterol complements. Biochem. Syst. Ecol. 22, 735–752.

Hooper, J.N.A., Bergquist, P.R., 1992. Cymbastela, a new genus of lamellate coral reef sponges. Mem. Qld. Mus. 32, 99–137.

Hooper, J.N.A., Capon, R.J., Keenan, C.P., Parry, D.L., Smith, N., 1992. Chemotaxonomy of marine sponges: families Microcionidae, Raspailiidae and Axinellidae, and their relationships with other families in the orders Poecilosclerida and Axinellida (Porifera: Demospongiae). Invertebr. Taxon. 6, 261–301.

Hooper, J.N.A., Cook, S.D., Hobbs, L.A., Kennedy, J.A., 1997. Australian Halichondriidae (Porifera: Demospongiae): I. Species from the Beagle Gulf. In: Hanley, J.R., Caswell, G., Megirian, D., Larson, H.K. (Eds.), Proceedings of the sixth international marine biological workshop. The Marine flora and fauna of Darwin Harbour, Northern Territory, Australia, Museums and Art Galleries of the Northern Territory and the Australian Marine Science Associations, Darwin, pp. 1–65.

Hooper, J.N.A., Lévi, C., 1993. Axinellida (Porifera: Demospongiae) from the New Caledonian Lagoon. Invertebr. Taxon. 7, 1395–1472.

Kerr, R.G., Baker, B.J., 1991. Marine sterols. Nat. Prod. Rep. 8, 465–497.

Kerr, R.G., Foss, C., Matsunaga, S., Fusetani, N., 1997. Isolation and structure elucidation of epipolasterol and 22-dihydroepipolasterol from the marine sponge *Epipolasis* sp. Comp. Biochem. Physiol. 117B, 561–563.

Lévi, C., 1953. Sur une nouvelle classification des démosponges. CR. Acad. Sci. Paris 236, 853–855.

Lévi, C., 1973. Systématique de la classe de Demospongia (démosponges). In: Grassé, P.P. (Ed.), Traité de Zoologie. Masson et Cie, Paris, pp. 557–631.

Lynch, J.D., 1997. Intrageneric relationships of mainland *Eleutherodactylus* II. A review of the *Eleutherodactylus sulcatus* group. Rev. Acad. Colomb. Cienc. 21, 353–372.

Pomponi, S., Wright, A.E., Díaz, M.C., van Soest, R.W.M., 1991. A systematic revision of the central Atlantic Halichondrida (Demospongiae, Porifera). Part II. Patterns of distribution of secondary metabolites. In: Reitner, J., Keupp, H. (Eds.), Fossil and recent sponges. Springer-Verlag, Berlin, Heidelberg, pp. 150–158.

Sjöstrand, U., Kornprobst, J.M., Djerassi, C., 1981. Minor and trace sterols from marine invertebrates 29. (22E)-ergosta-5,22,25-trien-3β-ol and (22E, 24R)-24,26-dimethylcholesta-5,22,25(27)-trien-3β-ol. Two new marine sterols from the sponge *Pseudaxinella lunacharta*. Steroids 38, 355–364.

van Soest, R.W.M., 1990. Toward a phylogenetic classification of sponges. In: Rützler, K. (Ed.), New perspectives in sponge biology. Smithsonian Institution Press, Washington, DC, pp. 344–348.

van Soest, R.W.M., 1991. Demosponge higher taxa classification re-examined. In: Reitner, J., Keupp, H. (Eds.), Fossil and recent sponges. Springer-Verlag, Heidelberg, pp. 54–71.

van Soest, R.W.M., Braekman, J.-C., 1999. Chemosystematic of the Porifera: a review. Mem Qld Mus 44, 569–589.

van Soest, R.W.M., Diaz, M.C., Pomponi, S.A., 1990. Phylogenetic classification of the halichondrids (Porifera, Demospongiae). Beaufortia 40, 15–62.

van Soest, R.W.M., Lehnert, H., 1997. The genus *Julavis* de Laubenfels (Porifera: Halichondrida). Proc. Biol. Soc. Washington 110, 502–510.

van Soest, R.W.M., Zea, S., 1986. A new sublithistid sponge, *Monanthus ciocalyptoides* n.sp. (Porifera, Halichondrida), from the West Indian region. Bull. Zool. Mus. Univ. Amsterdam 10, 201–205.

Stoilov, I.L., Thompson, J.E., Djerassi, C., 1986. Biosynthetic studies of marine lipids 7. Experimental

demonstration of a double alkylation at C-28 in the biosynthesis of 24-isopropylcholesterol in a sponge. Tetrahedron 42, 4147–4160.
Theobald, N., Djerassi, C., 1978. Determination of the absolute configuration of stelliferasterol and strongylosterol, two marine sterols with extended side chains. Tetrahedron Lett, 4369–4372.
Wiens, J.J., 1995. Polymorphic characters in phylogenetic systematics. Syst. Biol. 44, 482–500.
Wiley, E.O., 1981. Phylogenetics. The theory and practice of phylogenetic systematics. John Wiley & Sons, New York.
Wintermann-Kilian, G., Kilian, E.F., 1984. Marine sponges of the region of Santa Marta (Colombia). Part II. Homosclerophorida, Choristida, Spirophorida, Hadromerida, Axinellida, Halichondrida, Poecilosclerida. Stud. Neotrop. Fauna Environ. 19, 121–135.
Zea, S., 1987. Esponjas Del Caribe Colombiano. Catálogo Científico, Bogotá.