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SHORT COMMUNICATION

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A chemical view of the most ancient metazoa – biomarker chemotaxonomy of hexactinellid sponges

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Abstract Hexactinellid sponges are often considered to be the most ancient metazoans. Lipid biomarkers from 23 species were studied for information on their phylogenetic properties, particularly their disputed relation to the two other sponge classes (Demospongiae, Calcarea). The most prominent lipid compounds in the Hexactinellida comprise C_{28} to C_{32} polyenoic fatty acids. Their structures parallel the unique patterns found in demosponge membrane fatty acids ('demospongic acids') and strongly support a close phylogenetic association of the Demospongiae and the Hexactinellida. Both taxa also show unusual mid-chain methylated fatty acids (C_{15}–C_{25}) and irregular C_{25}- and C_{40}-isoprenoid hydrocarbons, tracers for specific eubacteria and Archaea, respectively. These biomarkers indicate a similar, highly conservative symbiont community, although some shift in the abundance of the associated microbiota was observed. The lack of these features in calcareous sponges further contradicts the still common view that Calcarea and Demospongiae are more closely related to each other than either is to the Hexactinellida.

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Introduction

It is now well established that the Porifera (sponges) are true animals. Their basic mode of organization and their biochemical properties place them at the very base of the kingdom Metazoa. A characterization as ancestral organisms seems particularly valid for the 450–500 species of the class Hexactinellida (see Hooper 2000). These sponges are considered to be an early branch within the Porifera, characterized by 'hexactine' siliceous spicules and a unique mode of soft body organization. Much of their tissue consists of multinucleate cytoplasm ('choanosyncytium') comprising collared bodies, sharing a common nucleus and linked together by plasmic bridges (Reiswig 1979; Mackie and Singla 1983).

The general monophyly of the Porifera has been proposed, but their interclass relationships are still open to debate. Reiswig and Mackie (1983) assigned separate subphylum status for the Hexactinellida (*Symplasma*), based on their syncytial organization. Accordingly, the Calcarea (calcareous sponges) and the Demospongiae were grouped into the 'cell-bearing' subphylum *Cellularia* and, due to their common possession of a pinacoderm layer, *Pinacophora* (Reitner and Mehl 1996). Alternatively a close relationship between the Hexactinellida and the Demospongiae has been proposed, according to larval similarities and their homology in chemical spicule composition (Böger 1988).

During the last decade, molecular approaches have contributed significantly to understanding sponge phylogeny, although conflicting data have not resolved early metazoan evolution. Based on amino acid sequence data, Müller (1997) calculated that the Porifera, as the first metazoan phylum, diverged from a common ancestor 0.8 Ga (1 Ga = 10^9 years) ago, thus extending the fossil range of these animals by more than 0.2 Ga. Studies on the composition of insulin receptor polypeptides assigned an age of 1.4 Ga to the Hexactinellida, and 1.3 Ga and 1.1 Ga for the separation of the Demospongiae and the Calcarea (Schütze et al. 1999; Skorokhod et al. 1999). A study of the protein coding gene *Hsp70* (Borchiellini et al. 1998) indicated that Demospongiae form a clade with

the Calcarea, excluding the Hexactinellida. However, recent gene sequence analyses supported a common Demospongiae–Hexactinellida taxon, whereas the Calcarea display closer affinities with the Ctenophora (comb jellies) than to Demospongiae (Cavalier Smith et al. 1996; Van de Peer and De Wachter 1997; Collins 1998; Adams et al. 1999; see also Zrzavy et al. 1998).

To further investigate sponge taxonomy, we conducted a study on lipid biomarkers complementary to the classical morphological and molecular biological approaches. Demosponges possess unusual membrane lipids reflecting specific enzyme systems which control biosynthetic properties such as carbon chain elongation and the introduction of distinctive double bonds. In particular, many investigations revealed the presence of unique long-chain fatty acids (LCFA, $>C_{24}$) in demosponges, the so-called 'demospongic' acids (see Litchfield et al. 1976; Hahn et al. 1988; Garson et al. 1994, also see van Soest and Braekman 1999 for a review on sponge chemosystematics). These compounds provide excellent targets for chemotaxonomic analyses at the class level. However, previous lipid studies have largely focused on demosponges, and only a few studies on a limited number of species and chemical properties exist for the Hexactinellida (Bergquist et al. 1984; Lawson et al. 1984). Our study aimed to fill this gap with a comprehensive chemical view on hexactinellid lipids and their interrelationships with those of the Demospongiae and Calcarea.

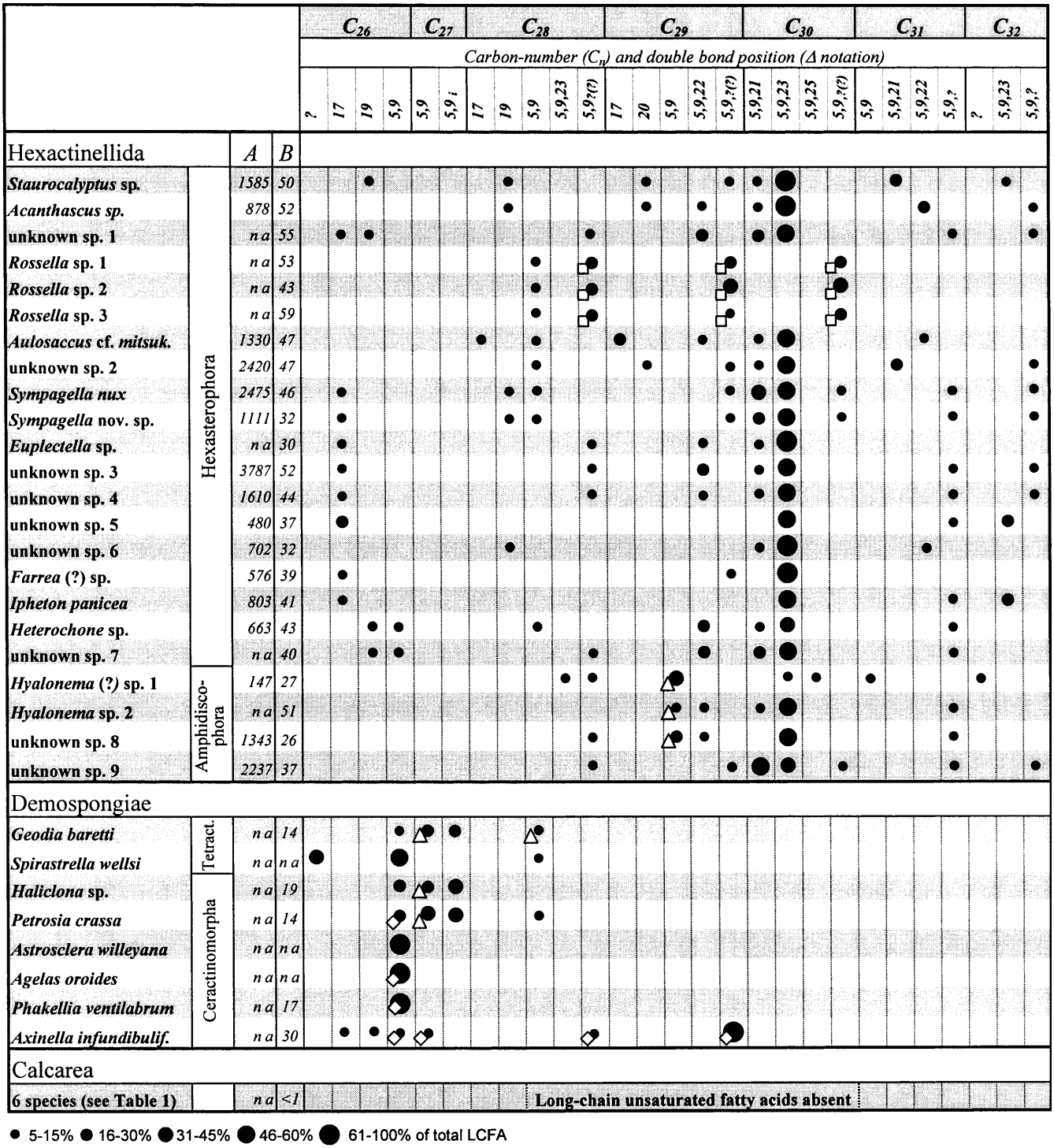
Methods

Twenty-three Hexactinellida, six Calcarea, and eight Demospongiae were studied. For species, taxonomy and sampling locations see Table 1. For sample preparation details, see Table 2 and Fig. 1.

Table 1 Sample list, taxonomy, and sampling locations

Species/Taxonomy			Sample location	Depth (m)
Hexactinellida				
Staurocalyptus sp.	Hexasterophora	'Lyssakinosa' – Rossellidae	Punta Mangle, Galapagos	445
Acanthascus sp.		'Lyssakinosa' – Rossellidae	Punta Vincente, Galapagos	440
Unknown sp. 1		'Lyssakinosa' – Rossellidae	Gulf of Chirqui, Panama	490
Rossella sp. 1		'Lyssakinosa' – Rossellidae	Kapp Norvegia, Eastern Weddell Sea	265
Rossella sp. 2		'Lyssakinosa' – Rossellidae	Kapp Norvegia, Eastern Weddell Sea	265
Rossella sp. 3		'Lyssakinosa' – Rossellidae	Kapp Norvegia, Eastern Weddell Sea	265
Aulosaccus cf. *mitsukuri*		'Lyssakinosa' – Rossellidae	Isla Bartholome, Galapagos	440
Unknown sp. 2		'Lyssakinosa' – Rossellidae (?)	Santa Cruz Island, Galapagos	320
Sympagella nux		'Lyssakinosa' – Rossellidae	Isla Marchena, Galapagos	380
Sympagella *nov.* sp.		'Lyssakinosa' – Rossellidae-(Strobiliplumicoma)	Sula-Ridge, Norway	285
Euplectella sp.		'Lyssakinosa' – Euplectellidae	Isla Marchena, Galapagos	580
Unknown sp. 3		'Lyssakinosa' – Euplectellidae	Plana Cay, Bahamas	860
Unknown sp. 4		'Lyssakinosa' – Euplectellidae	Rocas Gordon, Galapagos	430
Unknown sp. 5		'Lyssakinosa'	Lucea, Jamaica	480
Unknown sp. 6		'Lyssakinosa'	Tenerife, Canary Islands	335
Farrea (?) sp.		Hexactinosida – Farreidae	Charleston Lumps, South Carolina	215
Ipheton panicea		Hexactinosida – Euretidae	Turks & Caicos Islands, Caribbean	480
Heterochone sp.		Hexactinosida – Aphrocallistidae	Isla Marchena, Galapagos	475
Unknown sp. 7		Hexactinosida	Wolf Island, Galapagos	365
Hyalonema (?) sp. 1	Amphidiscophora	Amphidiscosida – Hyalonematidae	Negril, Jamaica	375
Hyalonema sp. 2		Amphidiscosida – Hyalonematidae	Long Island, Bahamas	800
Unknown sp. 8		Amphidiscosida – Hyalonematidae (?)	Negril, Jamaica	825
Unknown sp. 9		(?)	Hierro, Canary Islands	675
Demospongiae				
Geodia barretti	Tetractinomorpha	Astrophorida – Geodiidae	Sula-Ridge, Norway	320
Spirastrella (Acanthochaetetes) wellsi		Hadromerida – Spirastrellidae	Osprey Reef, off Great Barrier Reef	10
Haliclona sp.	Ceractinomorpha	Haplosclerida – Chalinidae	Sula-Ridge, Norway	290
Petrosia crassa		Haplosclerida – Petrosiidae	Sula-Ridge, Norway	320
Astrosclera willeyana		Agelasida – Astroscleridae	Pearl Reef, Great Barrier Reef	15
Agelas oroides		Agelasida – Agelasidae	Banyuls sur Mer, France, Mediterranean Sea	20
Phakellia ventilabrum		Halichondrida – Axinellidae	Sula-Ridge, Norway	290
Axinella infundibuliformis		Halichondrida – Axinellidae	Sula-Ridge, Norway	320
Calcarea				
Grantiopsis sp.	Calcaronea	Leucosoleniida – Grantiidae	Wistari Reef, Great Barrier Reef	5
Sycon sp.		Leucosoleniida – Sycettidae	Northwest Island, Great Barrier Reef	15
Leucaltis (*clathria?*)	Calcinea	Clathrinida – Leucaltidae	Davies Reef, Great Barrier Reef	20
Leucetta sp.		Clathrinida – Leucettidae	Hook Island, Great Barrier Reef	5
Pericharax heteroraphis		Clathrinida – Leucettidae	Myrmidon Reef, Great Barrier Reef	15
Levinella prolifera		Clathrinida – Levinellidae	Wistari Reef, Great Barrier Reef	5

Table 2 Presence and relative abundances of C_{26} to C_{32} long-chain fatty acids (*LCFA*). *Sizes of dots* refer to the relative concentrations with respect to the total amounts of C_{26} to C_{32} LCFA (*see lowermost row*). *Column A* Absolute concentrations ($\mu g/g_{dry\ sponge}$) of the summed C_{30} LCFA. *Column B* Relative proportion of C_{26} to C_{32} LCFA (%) in the total amount of fatty acids and sterols. △ includes isomeric mixtures of mid-chain methylated LCFA; □ tetraenoic LCFA ($\Delta^{5,9,?,?}$); ◇ includes brominated LCFA; *n.a.* not analysed; *i iso* (ω2-methyl) and *anteiso* (ω3-methyl) branched LCFA. Fatty acid methyl esters were obtained by extraction/transesterification of ~200 mg dry sponge matter with trimethylchlorosilane/methanol (1:8; v/v; 2 h, 70°C), re-extraction with *n*-hexane vs H_2O, and filtration of the *n*-hexane layer over silica gel with excess CH_2Cl_2/ethyl acetate (3:1; v/v). Fatty acid methyl esters were separated from the extract by elution over a 500 mg LC-NH_2 aminopropyl (Supelco) solid phase extraction cartridge (rinsing with 1 ml *n*-hexane; 4 ml *n*-hexane/CH_2Cl_2 (3:1; v/v). The presence of linear vs methyl branched fatty acids was checked by comparison of the GC-retention times and mass spectra of the hydrogenated products with those of reference compounds and published data. Double bond positions were determined on *n*-acyl-pyrrolidide derivatives (Lankelma et al. 1983)



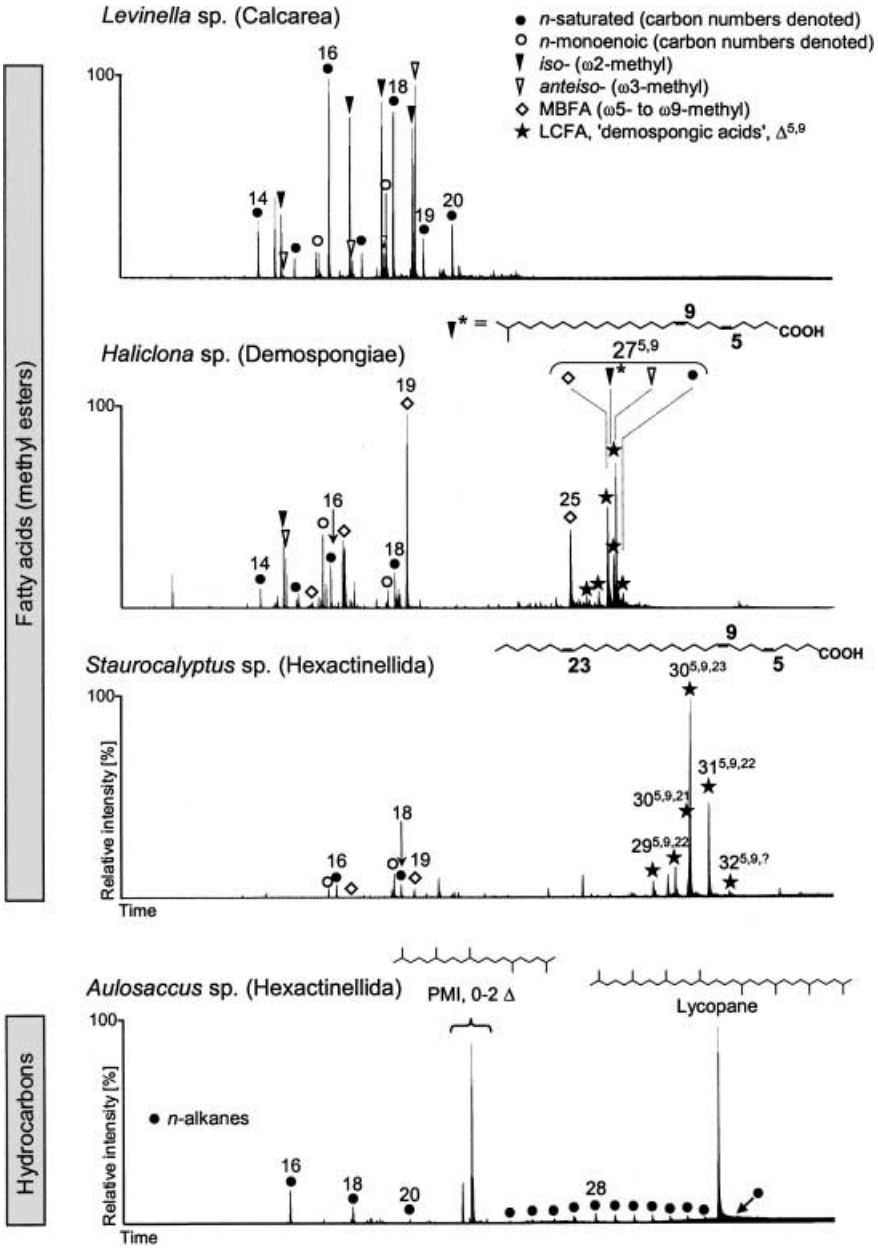


Fig. 1 *Top* Total ion currents (*TIC*) of fatty acids (methyl esters) typical for the Calcarea, Demospongiae, and Hexactinellida. Note the absence of long-chain fatty acids (*LCFA*) in the Calcarea. *Bottom* Hydrocarbons from *Aulosaccus* sp., with 2,6,10,15,19-pentamethylicosane (*PMI*) derivatives and lycopane as main compounds; a common pattern found for hexactinellids. Hydrocarbons were prepared from the CH_2Cl_2:methanol extract (1:1; v/v) by elution with two-column volumes of *n*-hexane over a silica gel 60 column. Biomarkers were examined by gas-chromatography mass spectroscopy (*GC-MS*), using a Micromass Quattro II spectrometer (EI, 70 eV) interfaced to a HP6890 GC with a 30 m fused silica capillary column (DB5-MS, 0.32 mm i.d., 0.25 µm film thickness). Carrier gas: He. Temperature regime: 5 min 80°C; 80°C to 310°C at 4°C min^{-1}; 20 min 310°C; quantification via internal standard compounds

Results

Very long carbon chains from C_{28} to C_{32} characterize the fatty acid moieties of all hexactinellids (Table 2). As a rule, the most prominent compounds are *n*-C_{30} polyunsaturated carbon chains, with triaconta-5,9,23-trienoic acid ($C_{30}^{5,9,23}$) and triaconta-5,9,21-trienoic acid ($C_{30}^{5,9,21}$) being the most abundant. An exception was found for the Antarctic *Rossella* sp., which contain tetraenoic C_{29} and C_{30} LCFA ($\Delta^{5,9,?,?}$) as main compounds. Whereas most demosponges produce dienoic LCFA with 24–28 carbon atoms, the hexactinellid LCFA generally have more C_{30} polyenoic acids (Fig. 1). Furthermore, in contrast to demosponges, most hexactinellids lack methyl branched carbon chains among their LCFA, although a systematic exception from this rule is found in the Hyalonematidae. Brominated LCFA, as often observed in demosponges (Garson et al. 1994), were absent in the hexactinellids studied. No LCFA were observed in the Calcarea investigated (Table 3).

In addition to the linear saturated and C_{16}^{9}, C_{18}^{9} and/or C_{18}^{11} monoenoic fatty acids, significant amounts of mid-chain branched fatty acids (MBFA) occur in both hexactinellids and demosponges (Fig. 1, Table 3). These compounds typically show a distinctive structural peculiarity, as they are characterized by isomeric mixtures of C_{15}–C_{25} homologues (C_{19} predominant), with single methyl groups located between the ω5 and ω9 positions (Thiel et al. 1999). Microscopic analyses revealed a good agreement between MBFA amounts and the overall abundances of associated eubacteria in demosponges. High MBFA contents are observed for *Astrosclera will-*

Table 3 Lipid properties of chemotaxonomic importance. *Filled circles* Significant relative amounts; *open circles* minor relative amounts; *Tr.* trace amounts; *no entry* compound or compound class absent; *n.a.* not analysed. *LCFA* Long-chain fatty acids; *MBFA* mid-chain branched fatty acids; $\Delta^{x,y}$ double bond position(s); *PMI* 2,6,10,15,19-pentamethylicosane; $C_{16}{}^{9}$ *cis*-hexadec-9-enoic acid

		LCFA $\Delta^{5,9}$	LCFA $\Delta^{5,9}$ mid-chain branched	MBFA	LCFA $\Delta^{5,9}$ brominated	$C_{16}\,\Delta^{9}$	PMI, PMIΔ	Lycopane
Hexactinellida								
Staurocalyptus *sp.*	Hexasterophora	●		○		●	Tr.	●
Acanthascus sp.		●		○		●	Tr.	●
Unknown sp. 1		●		○		○	●	●
Rossella sp. 1		●	n.a.	n.a.		●	○	
Rossella sp. 2		●	n.a.	n.a.		n.a.	n.a.	n.a.
Rossella sp. 3		●	n.a.	n.a.		n.a.	n.a.	n.a.
Aulosaccus cf. *mitsuk.*		●		Tr.		○	●	●
Unknown sp. 2		●		○		●	Tr.	●
Sympagella nux		●		○		○	○	●
Sympagella nov. sp.		●		●		●	Tr.	●
Euplectella *sp.*		●		Tr.		●	●	●
Unknown sp. 3		●		○		○	●	●
Unknown sp. 4		●				Tr.	●	●
Unknown sp. 5		●		●		○	●	●
Unknown sp. 6		●		○		○	○	●
Farrea (?) sp.		●				❍	●	●
Ipheton panicea		●		○		○	●	●
Heterochone sp.		●		○		●	●	●
Unknown sp. 7		●		○		●	●	●
Hyalonema (?) sp. 1	Amphidiscophora	●	●	●		●	○	●
Hyalonema sp. 2		●	●	○		○	●	●
Unknown sp. 8		●	●	○		●	n.a.	n.a.
Unknown sp. 9		●		○		○	○	●
Demospongiae								
Geodia barretti	Tetractinomorpha	●	●	●		●		
Spirastrella wellsi		●		Tr.		n.a.		
Haliclona sp.	Ceractinomorpha	●	●	●		●		
Petrosia crassa		●	●	●	●	●	Tr.	
Astrosclera willeyana		●		●		n.a.		
Agelas oroides		●		●	○	n.a.		
Phakellia ventilabrum		●		Tr.	●	●		
Axinella infundibulif		●		○	●	●	Tr.	●
Calcarea								
Grantiopsis (cylindr.?)	Calcaronea					○		
Sycon gelatinosum					○			
Leucaltis (*clathria*?)	Calcinea					○		
Leucetta sp.					○			
Pericharax heteroraph.					Tr.			
Levinella prolifera					●			

eyana (17.5% of the total fatty acids) and *Geodia barretti* (28%) which both contain dense bacterial populations. Only small amounts of MBFA were found in *Spirastrella* ('*Acantochaetetes*') *wellsi* (1.2%) and in *Phakellia ventilabrum* (0.3%) which both possess only minor quantities of eubacteria. In the Hexactinellida, isomeric MBFA are present in many species, although in smaller amounts than in most demosponges (<5%). MBFA were not observed in the Calcarea studied.

A further remarkable feature of many sponge lipid fractions are acyclic, tail-to-tail linked isoprenoid hydrocarbons with 2,6,10,15,19-pentamethylicosane (PMI, C_{25}) skeletons. PMI and several unsaturated derivatives thereof were observed in all hexactinellids and in particular demosponges (Table 3). All hexactinellids containing the C_{25} isoprenoids also show the presence of the structurally related C_{40} homologue lycopane (2,6,10,14,19,23,27,31-octamethyldotriacontane) in even higher amounts. Neither PMI, lycopane nor any other irregular isoprenoid has as yet been found in the Calcarea.

Discussion

The very long carbon chains of hexactinellid LCFA and the unusual $\Delta^{5,9}$-unsaturation are patterns identical to those found in the so-called 'demospongic acids' which are classically considered unique to the Demospongiae. Incorporation studies revealed that demosponges possess a very active fatty acid chain elongation system and synthesize these LCFA by C_2 elongation of short-chain precursors followed by $\Delta^{5,9}$ desaturation (see Hahn et al.

1988). From our results, a similar mechanism can be proposed to operate in the Hexactinellida. Potential precursors of their most prominent $C_{30}^{5,9,23}$ and $C_{30}^{5,9,21}$ fatty acids are the monoenoic, short-chain homologues C_{16}^{9}, C_{18}^{9} and/or C_{18}^{11}. These compounds most probably originate from bacterial lipids (Hahn et al. 1988) and occur in significant amounts in all hexactinellids. The co-occurrence of principal LCFA with unique unsaturation patterns implies very similar or identical enzyme systems for their biosynthesis, and provides a convincing argument for a close phylogenetic association of the Demospongiae and the Hexactinellida.

As for the linear compounds, short-chain MBFA can be considered as plausible precursors of mid-chain methylated sponge LCFA. These compounds frequently occur in demosponges, but among the hexactinellids studied, they were restricted to the Hyalonematidae, a phylogenetically 'diverged' taxon of the subclass Amphidiscophorida (Tabachnik and Menshenina 1999). MBFA are common compounds in many eubacteria and their presence in sponges has thus been attributed to non-phototrophic, associated microorganisms rather than to direct synthesis by the host sponge (Gillan et al. 1988; Thiel et al. 1999). However, the complex isomeric MBFA mixtures typically found in sponges have as yet not been reported from other organisms, nor from marine sediments and sea-water. Anticipating a microbial origin, we regard these MBFA as originating from distinctive eubacteria highly adapted to the internal sponge environment.

PMI derivatives are used as specific archaeal biomarkers (Tornabene et al. 1979; Wakeham 1990; Schouten et al. 1997) and thus point at the presence of associated Archaea in the respective sponges. Unlike PMI, direct sources for lycopane have not been reported. Lycopane-type polyenes occur in several halophiles (Langworthy 1985) and were found in a thermophilic Archaeon (Lattuati et al. 1998). Although these compounds are also present in phototrophic, anaerobic eubacteria (Smith 1988), and were even detected in oxic ocean waters (Wakeham 1990), lycopane was plausibly proposed as a sedimentary biomarker for Archaea, when co-occurring with other diagnostic isoprenoid markers (Brassell et al. 1981). For the hexactinellids studied, the co-occurrence with PMI derivatives suggests lycopane as a further indicator for associated Archaea, namely for the deep-water sponges which certainly lack associated -phototrophs.

The presence of archaeal isoprenoids in Hexactinellida and Demospongiae meets a previous discovery of psychrophilic crenarchaeotes as demosponge symbionts (Preston et al. 1996). From our data, we suggest that the Hexactinellida and the Demospongiae, to the exclusion of the Calcarea, share essentially the same common microbial communities, based on distinctive archaeal and eubacterial taxa. Although these yet unknown organisms occur in both classes, a more pronounced presence of isoprenoid biomarkers suggests a more crucial affinity for specific Archaea in the Hexactinellida (Table 3). Eubacteria, as evidenced by MBFA, show a marked presence throughout the demosponges, but a more scattered distribution among the Hexactinellida. Hence it appears that the evolution of both sponge classes may have been accompanied (or controlled?) by a shift in the abundance and/or quality of their associated microbiota. In any case, the occurrence of the biomarker patterns observed independently of the geographical and environmental settings supports the idea that these microbiota are a constituent property of the porifera and have been 'inherited' in the protective environment of their hosts throughout geological times (Wilkinson 1984).

Conclusions

- The membrane fatty acids of the Hexactinellida preserve an ancestral strategy of lipid biosynthesis. It involves the use of bacterial precursor lipids and results in different biomembrane structures and properties than those found in contemporary organisms other than sponges.
- Their unique lipid characteristics put Hexactinellida and Demospongiae into the same phylogenetic group.
- Both classes share a partly similar symbiont distribution, dominated by Archaea in the Hexactinellida and distinctive, sponge-specific eubacteria in the Demospongiae.
- The lipid patterns of the Hexactinellida and the Demospongiae differ from those of the Calcarea, thus a more distant phylogenetic position for the latter.

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Note added in proof Poriferan paraphyly, with elevation of class Calcarea to phylum level ("Calcispongia"), was proposed by Zrzavy et al. (1998) and most recently by Borchiellini et al. (2001). However, a new detailed study using an extended genetic data set (full length 18S and 28S rDNA sequences) could not resolve this issue with sufficient statistical significance (Medina et al., 2001). Hence the proposal of sponge paraphyly is still contentious. Our independent biomarker approach now clearly supports a close genealogical relationship of Demospongiae and Hexactinellida, with a more distant phylogenetic position of Calcarea. However, our data do not necessarily support either poriferan mono- or paraphyly (due to a lack of outgroup comparison, which is currently in progress).