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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 ('demospongiic 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.

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

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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, >C₂₄) in demosponges, the so-called ‘demospongiac’ 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			
<i>Staurocalyptus</i> sp.	Hexasterophora	‘Lyssakinosa’ – Rossellidae	Punta Mangle, Galapagos 445
<i>Acanthascus</i> sp.		‘Lyssakinosa’ – Rossellidae	Punta Vincente, Galapagos 440
Unknown sp. 1		‘Lyssakinosa’ – Rossellidae	Gulf of Chirqui, Panama 490
<i>Rossella</i> sp. 1		‘Lyssakinosa’ – Rossellidae	Kapp Norvegia, Eastern Weddell Sea 265
<i>Rossella</i> sp. 2		‘Lyssakinosa’ – Rossellidae	Kapp Norvegia, Eastern Weddell Sea 265
<i>Rossella</i> sp. 3		‘Lyssakinosa’ – Rossellidae	Kapp Norvegia, Eastern Weddell Sea 265
<i>Aulosaccus</i> cf. <i>mitsukuri</i>		‘Lyssakinosa’ – Rossellidae	Isla Bartholome, Galapagos 440
Unknown sp. 2		‘Lyssakinosa’ – Rossellidae (?)	Santa Cruz Island, Galapagos 320
<i>Sympagella nux</i>		‘Lyssakinosa’ – Rossellidae	Isla Marchena, Galapagos 380
<i>Sympagella</i> nov. sp.		‘Lyssakinosa’ – Rossellidae- (Strobiliplumicoma)	Sula-Ridge, Norway 285
<i>Euplectella</i> 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
<i>Farrea</i> (?) sp.		Hexactinosida – Farreidae	Charleston Lumps, South Carolina 215
<i>Ipheton panicea</i>		Hexactinosida – Euretidae	Turks & Caicos Islands, Caribbean 480
<i>Heterochone</i> sp.		Hexactinosida – Aphrocallistidae	Isla Marchena, Galapagos 475
Unknown sp. 7		Hexactinosida	Wolf Island, Galapagos 365
<i>Hyalonema</i> (?) sp. 1	Amphidiscophora	Amphidiscosida – Hyalonematidae	Negril, Jamaica 375
<i>Hyalonema</i> sp. 2		Amphidiscosida – Hyalonematidae	Long Island, Bahamas 800
Unknown sp. 8		Amphidiscosida – Hyalonematidae (?)	Negril, Jamaica 825
Unknown sp. 9		(?)	Hierro, Canary Islands 675
Demospongiae			
<i>Geodia barretti</i>	Tetractinomorpha	Astrosporida – Geodiidae	Sula-Ridge, Norway 320
<i>Spirastrella</i> (<i>Acanthochaetetes</i>) <i>wellsi</i>		Hadromerida – Spirastrellidae	Osprey Reef, off Great Barrier Reef 10
<i>Haliclona</i> sp.	Ceractinomorpha	Haplosclerida – Chalinidae	Sula-Ridge, Norway 290
<i>Petrosia crassa</i>		Haplosclerida – Petrosiidae	Sula-Ridge, Norway 320
<i>Astrosclera willeyana</i>		Agelasida – Astroscleridae	Pearl Reef, Great Barrier Reef 15
<i>Agelas oroides</i>		Agelasida – Agelasidae	Banyuls sur Mer, France, Mediterranean Sea 20
<i>Phakellia ventilabrum</i>		Halichondrida – Axinellidae	Sula-Ridge, Norway 290
<i>Axinella infundibuliformis</i>		Halichondrida – Axinellidae	Sula-Ridge, Norway 320
Calcarea			
<i>Grantiopsis</i> sp.	Calcaronea	Leucosoleniida – Grantiidae	Wistari Reef, Great Barrier Reef 5
<i>Sycon</i> sp.		Leucosoleniida – Sycettidae	Northwest Island, Great Barrier Reef 15
<i>Leucaltis (clathria?)</i>	Calcinea	Clathrinida – Leucaltidae	Davies Reef, Great Barrier Reef 20
<i>Leucetta</i> sp.		Clathrinida – Leucettidae	Hook Island, Great Barrier Reef 5
<i>Pericharax heteroraphis</i>		Clathrinida – Leucettidae	Myrmidon Reef, Great Barrier Reef 15
<i>Levinella prolifera</i>		Clathrinida – Levinellidae	Wistari Reef, Great Barrier Reef 5

Table 2 Presence and relative abundances of C₂₆ to C₃₂ long-chain fatty acids (LCFA). Sizes of dots refer to the relative concentrations with respect to the total amounts of C₂₆ to C₃₂ LCFA (see lowest row). Column A Absolute concentrations (µg/g dry sponge) of the summed C₃₀ LCFA. Column B Relative proportion of C₂₆ to C₃₂ LCFA (%) in the total amount of fatty acids and sterols. Δ includes isomeric mixtures of mid-chain methylated LCFA; □ tetraenoic LCFA (Δ^{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 trimethylchlorosi-

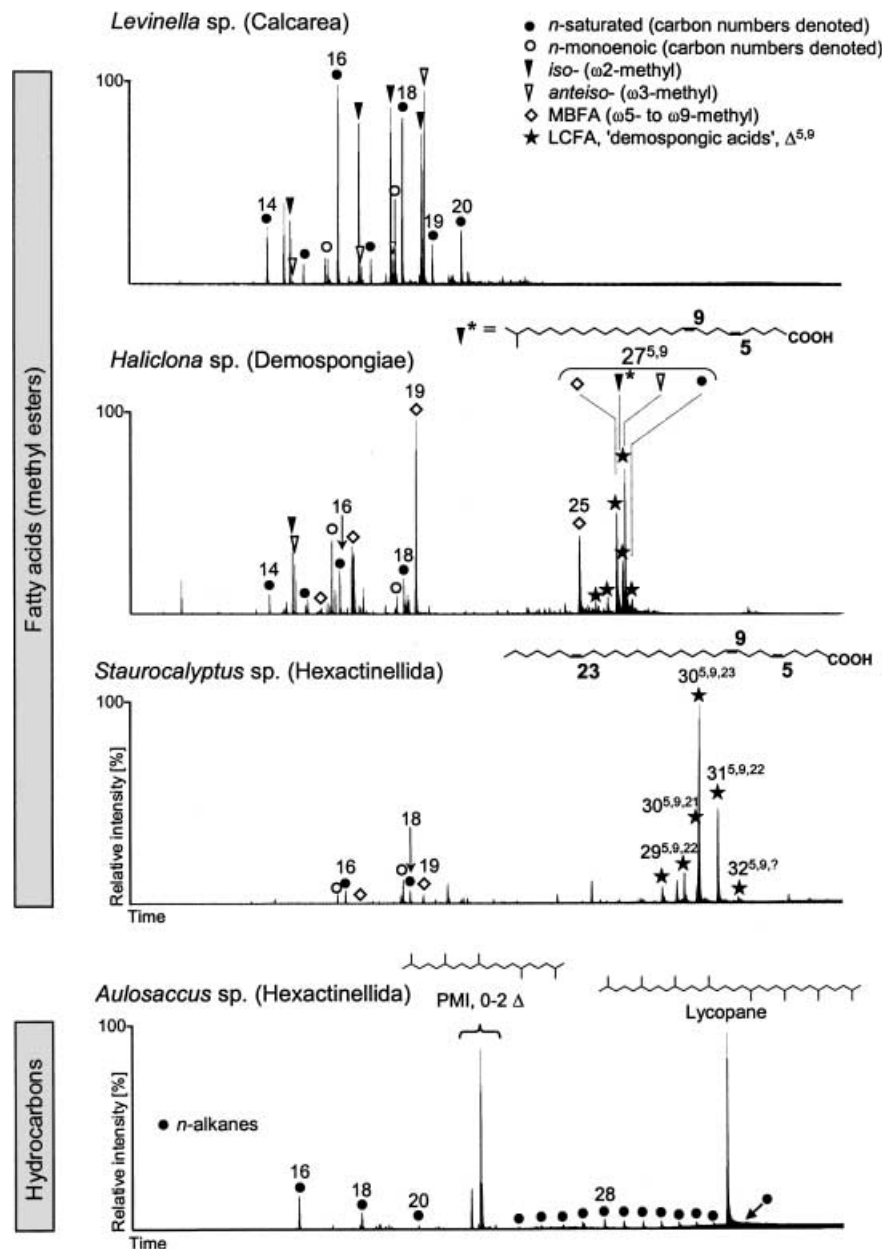
lane/methanol (1:8; v/v; 2 h, 70°C), re-extraction with n-hexane vs H₂O, and filtration of the n-hexane layer over silica gel with excess CH₂Cl₂/ethyl acetate (3:1; v/v). Fatty acid methyl esters were separated from the extract by elution over a 500 mg LC-NH₂ aminopropyl (Supelco) solid phase extraction cartridge (rinsing with 1 ml n-hexane; 4 ml n-hexane/CH₂Cl₂ (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)

				C ₂₆		C ₂₇		C ₂₈			C ₂₉			C ₃₀			C ₃₁		C ₃₂																		
				Carbon-number (C _n) and double bond position (Δ notation)																																	
		A	B	?	17	19	5,9	5,9	5,9,1	17	19	5,9	5,9,23	5,9,?()	17	20	5,9	5,9,22	5,9,?()	5,9,21	5,9,23	5,9,25	5,9,?()	5,9	5,9,21	5,9,22	5,9,?	?	5,9,23	5,9,?							
Hexactinellida																																					
		A	B																																		
<i>Staurocalyptus</i> sp.	Hexasterophora	1585	50				●										●															●					
<i>Acanthascus</i> sp.		878	52								●						●						●														
unknown sp. 1		n.a.	55		●		●								●																			●			
<i>Rossella</i> sp. 1		n.a.	53									●																									
<i>Rossella</i> sp. 2		n.a.	43									●																									
<i>Rossella</i> sp. 3		n.a.	59									●																									
<i>Aulosaccus</i> cf. <i>mitsuk.</i>		1330	47								●																										
unknown sp. 2		2420	47																																		
<i>Sympagella nux</i>		2475	46		●							●																									
<i>Sympagella</i> nov. sp.		1111	32		●							●																									
<i>Euplectella</i> sp.		n.a.	30		●											●																					
unknown sp. 3		3787	52		●											●																					
unknown sp. 4		1610	44		●											●																					
unknown sp. 5		480	37		●																																
unknown sp. 6		702	32		●							●																									
<i>Farrea</i> (?) sp.		576	39		●																																
<i>Ipheton panicea</i>		803	41		●																																
<i>Heterochone</i> sp.		663	43				●		●																												
unknown sp. 7		n.a.	40				●		●																												
<i>Hyalonema</i> (?) sp. 1		Amphidisco-phora	147	27																																	
<i>Hyalonema</i> sp. 2			n.a.	51																																	
unknown sp. 8			1343	26																																	
unknown sp. 9			2237	37																																	
Demospungiaie																																					
			A	B																																	
<i>Geodia baretii</i>		Tetract.	n.a.	14																																	
<i>Spirastrella wellsii</i>	n.a.		n.a.		●																																
<i>Haliclona</i> sp.	Ceractinomorpha	n.a.	19																																		
<i>Petrosia crassa</i>		n.a.	14																																		
<i>Astrosclera willeyana</i>		n.a.	n.a.																																		
<i>Agelas oroides</i>		n.a.	n.a.																																		
<i>Phakellia ventilabrum</i>		n.a.	17																																		
<i>Axinella infundibulif.</i>		n.a.	30		●		●		◇																												
Calcarea																																					
6 species (see Table 1)		n.a.	<1																																		

● 5-15% ● 16-30% ● 31-45% ● 46-60% ● 61-100% of total LCFA

Long-chain unsaturated fatty acids absent

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-pentamethylcosane (PMI) derivatives and lycopane as main compounds; a common pattern found for hexactinellids. Hydrocarbons were prepared from the CH₂Cl₂: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 spectrometry (GC-MS), using a Micro-mass 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 C₂₈ to C₃₂ characterize the fatty acid moieties of all hexactinellids (Table 2). As a rule, the most prominent compounds are *n*-C₃₀ polyunsaturated carbon chains, with triaconta-5,9,23-trienoic acid (C₃₀^{5,9,23}) and triaconta-5,9,21-trienoic acid (C₃₀^{5,9,21}) being the most abundant. An exception was found for the Antarctic *Rossella* sp., which contain tetraenoic C₂₉ and C₃₀ LCFA (Δ^{5,9,?,?}) as main compounds. Whereas most demosponges produce dienoic LCFA with 24–28 carbon atoms, the hexactinellid LCFA generally have more C₃₀ 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 C₁₆⁹, C₁₈⁹ and/or C₁₈¹¹ 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 C₁₅–C₂₅ homologues (C₁₉ 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 *Astrosclella 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-pentamethylcosane; *C₁₆⁹* *cis*-hexadec-9-enoic acid

		LCFA $\Delta^{5,9}$	LCFA $\Delta^{5,9}$ mid-chain branched	MBFA	LCFA $\Delta^{5,9}$ brominated	C ₁₆ ⁹	PMI, PMI Δ	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* ('*Acanthochaetetes*') *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-pentamethylcosane (PMI, C₂₅) skeletons. PMI and several unsaturated derivatives thereof were observed in all hexactinellids and in particular demosponges (Table 3). All hexactinellids containing the C₂₅ isoprenoids also show the presence

of the structurally related C₄₀ 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 'demospongiac 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 C₂ 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 pres-

ence 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 Zrzavý 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).