J. Mar. Biol. Ass. U.K. (2007), **87**, 1571–1576
Printed in the United Kingdom
doi: 10.1017/S0025315407058201

The systematics of Raspailiidae (Demospongiae: Poecilosclerida: Microcionina) re-analysed with a ribosomal marker

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We present a 28S rDNA gene tree of selected Raspailiidae, Axinellidae and other demosponges to obtain insight into raspailiid phylogeny and character evolution. The Raspailiidae in our data set cluster in a well-supported clade, distinguished from Axinellidae, Agelasida and Hadromerida. *Raspailia* (*s.s.*), *Eurypon*, *Sollasella*, *Aulospongus* and *Ectyoplasia* form a Raspailiidae clade. Some *Raspailia* subgenera, in particular *R.* (*Parasyringella*), are not retrieved monophyletically. *Trikentrion* falls into the Thrinacophorinae, and not the Cyamoninae as earlier hypothesized. The axinellid genera *Ptilocaulis* and *Reniochalina* also cluster with Raspailiidae, distant from the other Axinellidae. The suitability of particular morphological characters for raspailiid phylogeny is discussed.

INTRODUCTION

Raspailiidae (Demospongiae: Poecilosclerida: Microcionina) is a pivotal taxon in the demosponge systematics. It has played a major role in modern sponge classification because its higher classification has repeatedly been reviewed and changed in the past (e.g. Ridley & Dendy, 1887; Topsent, 1894, 1928; Dendy, 1905; Vosmaer, 1912; Wilson, 1921; Bergquist, 1970; Hooper, 1991, 2002). It was initially included in the order Poecilosclerida (Hentschel, 1923; Topsent, 1928) but subsequently referred to a taxon 'Axinellida' based on combining elements like axial skeleton compression and extra-axial features of many taxa and assumed oviparity of the entire group (e.g. Bergquist, 1970; Hartman, 1982).

'Axinellida' had been erected by Carter (1875) inside the taxon 'Echinonemata' as sister group to (actual) poecilosclerid taxa. It was re-erected by Lévi (1955) as the order 'Axinellida' with nine (assumed) oviparous families: Axinellidae, Bubaridae and Desmoxyidae (all now Halichondrida), Trachycladidae and Hemiasterellidae (all now Hadromerida), Raspailiidae, Rhabderemiidae, 'Euryponidae' and 'Sigmaxinellidae' (all now Poecilosclerida; see Hooper & van Soest, 2002a). 'Axinellida' were temporarily combined with Hadromerida and 'Epipolasida' to the superorder 'Clavaxinellida' assuming their close relationship (Lévi, 1956; see for further details van Soest & Hooper, 2002), before van Soest (1984) independently from Hooper (1984) remarked on inconsistencies in that current classification. This subsequently resulted in redistribution of 'Axinellida' into the present orders Halichondrida, Hadromerida and Poecilosclerida (see also Hooper & van Soest, 2002b).

Indeed, the Raspailiidae studied to date appear oviparous and lack the most important combining character of their order Poecilosclerida: the chelae microscleres. Furthermore, they have a unique ectosomal feature consisting of oxeas or styles forming bouquets surrounding longer choanosomal spicules protruding through the surface. However, Hooper (1990, 1991) observed closer relationships of the family Raspailiidae to the family Microcionidae (Poecilosclerida: Microcionina) than to other axinellid groups, based on morphological and biochemical similarities with Microcionidae, i.e. (plumo-) reticulate skeletons, axial compression, echinating acanthostyles, megasclere category features, carotenoid proteins, protein electrophoresis results and free amino acid patterns (Hooper, 2002).

The controversial classification of Raspailiidae is due to their broad range of morphological features, combined only by the (mostly) shared possession of a 'raspailiid' ectosomal skeleton, consisting of small thin spicules forming bouquets around long styles or oxeas that penetrate the surface, in combination with echinating acanthostyles in the choanosomal skeleton (Hooper, 2002). Due to this morphological plasticity, Hooper proposed in the same publication the erection of the following five Raspailiidae subfamilies, based predominantly on skeletal structure and acanthostyle geometry: (a) Raspailiinae Nardo, 1833 bear a noticeably compressed axial skeleton composed of criss-cross reticulation fibres and/or spicules; their echinating acanthostyles are microcionid-like club-shaped with small granular or erect spines, ranging to club-shaped with strongly recurved or clavulate spines on the basal and distal ends of spicules; (b) Thrinacophorinae Hooper, 2002 lack echinating megascleres altogether and have a more

prominent differentiation between axial and extra-axial skeleton; (c) Cyamoninae Hooper, 2002 have echinating spicules modified to acanthoplagiotriaenes in addition to other shared features; (d) Echinodictyinae Hooper, 2002 bear a regularly reticulate choanosomal skeletal structure, with a vestigial or virtually absent extra-axial skeleton, and all but one species lack ectosomal specialization; echinating megascleres are microcionid-like club-shaped acanthostyles; and finally (e) Plocamioninae Hooper, 2002 have acantho(tylo)strongyles forming the choanosomal skeleton. However, they remain *incertae sedis* and are grouped with the Raspailiidae only by the possession of smooth rhabdostyles.

In this work we test these higher taxa and the affinities between selected species of Raspailiidae in a molecular analysis. We amplified a relatively fast evolving fragment of the 28S rDNA for a number of raspailiid taxa and reconstructed a gene tree for comparison with the present, morphology-based classification. In a former analysis (Erpenbeck et al., 2007a) a more downstream fragment of 28S rDNA, has successfully been recruited to verify morphological hypotheses regarding the Raspailiidae affinity of the genus *Sollasella* (van Soest et al., 2006). In addition, our taxon set comprises several species of the halichondrid family Axinellidae to compare their position with the Raspailiidae. Axinellidae share several (choanosomal) skeleton features with Raspailiidae, but appear clearly distinct by the lack of an ectosomal skeleton (Alvarez & Hooper, 2002), and echinating megascleres.

Furthermore, our molecular data aims to provide insight into the internal classification of the type genus *Raspailia*. Its more than one hundred described species (van Soest et al., 2005) display a broad distribution of character states on growth forms, skeletal structures, structural megascleres, ectosomal structure and the geometry of echinating spicules (Hooper, 2002). Currently, the classification of the genus has (morphologically) been extended by division into the following subgenera: (a) *Raspailia* (*Raspailia*) Nardo, 1833 with microcionid-like, myxillid-like or thin vestigial acanthostyles; (b) *Raspailia* (*Raspaxilla*) Topsent, with echinating rhabdostyles geometrically very different from the usually longer choanosomal extra-axial styles forming a radial skeleton perpendicular to the axis and well differentiated axial and extra-axial skeletons; (c) *Raspailia* (*Clathriodendron*) Lendenfeld, 1888 lacking any axial compression or any differentiation between axial and extra-axial skeletons, but retaining two or more forms of choanosomal structural megascleres; (d) *Raspailia* (*Parasyringella*) Topsent, 1928, which have secondarily lost their echinating megascleres; and (e) *Raspailia* (*Hymeraphiopsis*) Hooper, 1991 having acanthostyles with smooth and strongly swollen tylote bases (Hooper, 2002).

MATERIALS AND METHODS

Samples newly sequenced for this analysis were taken from collection material of the Queensland Museum, where all vouchers for this investigation are kept. Their collection numbers are given in Figure 1. Total DNA was extracted from the choanosome to reduce the chance of amplifying non-sponge DNA. For the extraction we used a commercial DNA extraction kit (Qiamp DNA Mini Kit, Qiagen) and followed the manufacturer's suggestions. The polymerase chain reaction (PCR) reaction volume was 25 µl and contained 3 mM $MgCl_2$, 1 unit HotMaster Polymerase (Eppendorf) with recommended amounts of reaction buffer, 2 mM dNTPs (Gibco), 0.03 mg BSA (Sigma), 10 ng DNA template and 4 pmol of each primer. PCR primers for the 28S C1–D1 fragment were the following: 28S-C2F: GAA AAG AAC TTT GRA RAG AGA GT; 28S-D2R: TCC GTG TTT CAA GAC GGG (temperature regime: 94°C for 2 min; followed by 35 cycles of 94°C for 30 s; 50°C for 20 s; 65°C for 60 s; followed by 65°C for 10 min). PCR products were labelled with ABI BigDye terminator v. 1.1 or 3.1 (ABI Biosystems) and directly sequenced on a capillary sequencer. All sequences are submitted to GenBank (www.ncbi.nlm.nih.gov, Accession numbers: EU146396–ED146439). Additional sequences were taken from GenBank. Their accession numbers are given with their taxon labels in Figure 1. Sequences were handled with MacClade v. 4.06 (Maddison & Maddison, 1992), aligned with MUSCLE v. 3.6 (Edgar, 2004) under default settings and optimized by eye. We attempted to identify homologous positions of the variable parts via alignment to secondary structures to subsequently apply secondary structure-specific models for improved phylogenetic reconstruction as suggested in the literature (Dohrmann et al., 2006; Erpenbeck et al., 2007b). However, the structural variability of the C1–D1 region permitted several equally likely structures, in which paired and unpaired sites could not be identified unambiguously. Therefore we used a rather suboptimal approach by applying one single model to the entire alignment, after filtering positions unsuitable for the phylogenetic reconstruction using GBLOCKS v. 0.91b (Castresana, 2000, with 'half gap positions allowed' and otherwise default settings). Bayesian analyses were run on the parallel version of MrBayes (Altekar et al., 2004) on a Linux cluster at the Gesellschaft für Wissenschaftliche Datenverarbeitung Göttingen (GWDG), Germany (http://www.gwdg.de) with one processor assigned to each Markov chain. Each Bayesian analysis comprised at least two simultaneous runs of four Metropolis-coupled Markov-chains at the default temperature (0.2). Analyses were terminated either after a maximum of 10,000,000 generations, or after a maximum wall-time of 48 hours, or after the chains converged significantly as indicated by an average standard deviation of split frequencies <0.01. For comparison, maximum likelihood bootstrap analyses were conducted using GARLI v. 0.95 (Zwickl, 2006) using a heuristic search with the default option, i.e. under the GTR+G+I model of nucleotide substitution and 100 bootstrap replicates.

RESULTS AND DISCUSSION

The gene tree that resulted from our analysis is provided in Figure 1. It does not support any of the current classifications of Raspailiidae subfamilies and genera, but provides interesting insight into the importance of morphological characters for Raspailiidae systematics. A well-supported Raspailiidae clade is separated from the remaining taxa, which comprise Axinellidae, Agelasida, Hadromerida and some further Poecilosclerida. *Raspailia* (*Raspailia*) is represented here by three species: *R. vestigifera*

https://doi.org/10.1017/S0025315407058201 Published online by Cambridge University Press

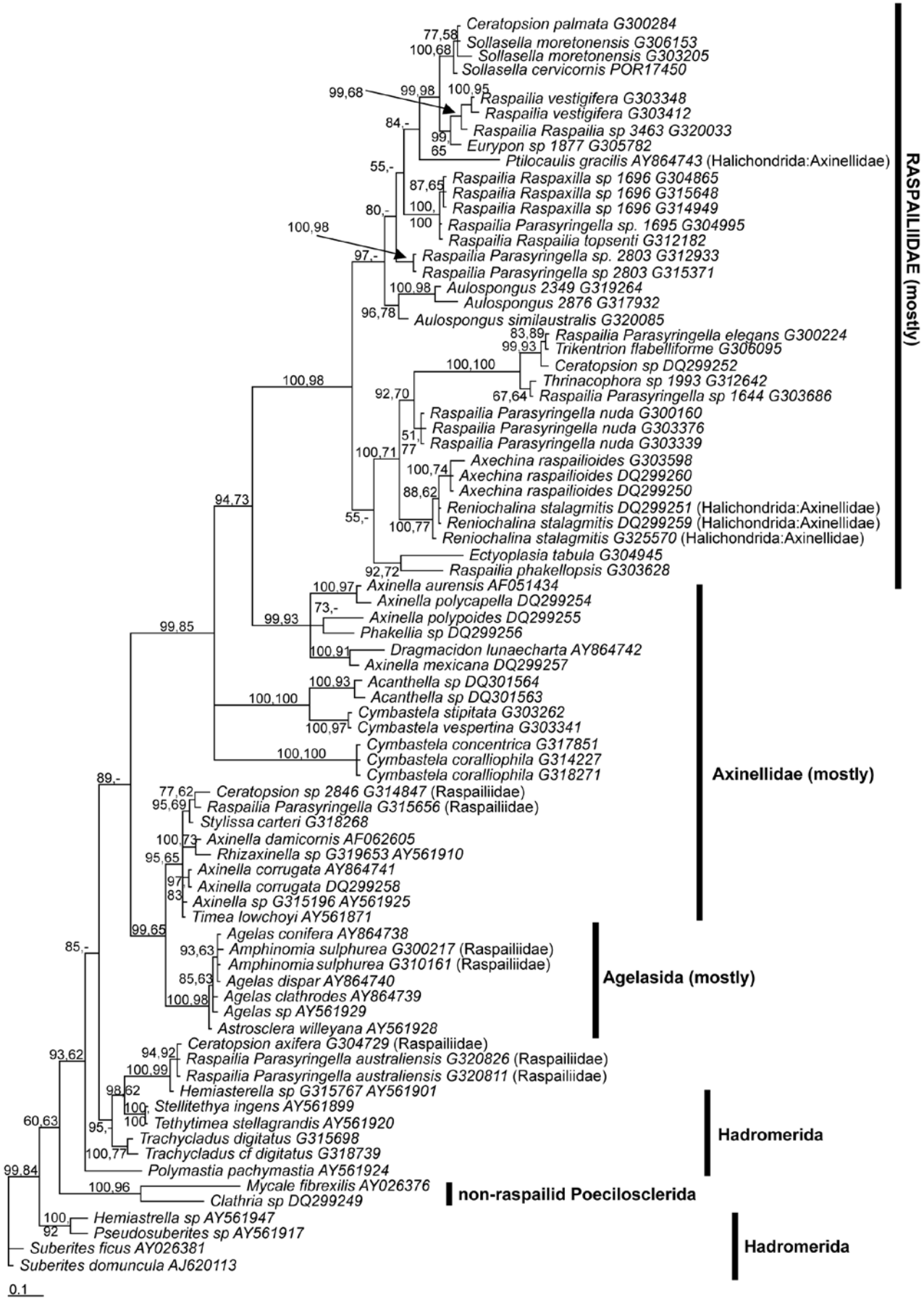

Figure 1. Bayesian inference tree of the 28S rDNA data set. The maximum likelihood tree is congruent. The numbers at the branches refer to posterior probabilities (in %; left or above) followed by the maximum-likelihood bootstrap values (right or below). Branches with support lower than 50 or incongruent are denoted with a dash. The numbers at the end of the taxon names indicate the Queensland Museum (QM) collection number of the voucher (G3.....) or GenBank Accession numbers.

https://doi.org/10.1017/S0025315407058201 Published online by Cambridge University Press

Dendy, 1896; another still undescribed species, which differs from *R. vestigifera* by live coloration (brown, not black), texture (velvety, compressible, not harsh and hispid), and echinating spicules (abundant and homogeneously distributed, not rare and restricted to the junction of axial and extra-axial skeletons, respectively); and *R. phakellopsis* Hooper, 1991. The latter does not cluster with the other *R.* (*Raspailia*) due to major sequence differences. Instead, it forms a sister-group with *Ectyoplasia tabula* (Lamarck, 1814), the type species of *Ectyoplasia* Topsent, 1930, which is in congruence to their shared placement in the subfamily Raspailiinae. Hooper (2002) noted that many morphological features overlap between the two genera and the remaining distinguishing features between the genera are only based on acanthostyle morphology (geometric modifications to acanthostyles) and the genus lacks specialized subectosomal megascleres. The relatively long genetic distances between the specimens may indicate their distinction, but additional *Ectyoplasia* should be investigated molecularly for better support.

Species of three further Raspailiinae genera complete the formation of a Raspailiinae clade:

(a) *Eurypon* Gray, 1867, represented in this data set by an as yet undescribed species, forms a sister-group to *Raspailia* (*Raspailia*). Its genus definition has been considerably broadened to accommodate species with different spicule morphologies (Hooper, 1991). However, some *Eurypon* species differ from *Raspailia* in having only up to three categories of megascleres (instead of four in *Raspailia*) and basal rather than axial compression of the fibre skeleton, which are not always reliable diagnostic characters. Therefore, a merging of some *Eurypon* species with *Raspailia* would be justifiable (while other *Eurypon* species rather resemble *Clathria* spp., Hooper, 2002). The short genetic distance between the analysed *Eurypon* and *Raspailia* (*Raspailia*) supports this view.

(b) *Sollasella* Lendenfeld, 1888 forms the sister-group to the *Raspailia* (*Raspailia*)/*Eurypon* clade. This genus has recently been transferred from Hadromerida to the Raspailiidae based on morphological (van Soest et al., 2006) and molecular evidence (Erpenbeck et al., 2007a). In the latter molecular analysis that recruited a downstream (helices D1–D17) fragment of 28S rDNA, *Sollasella* clustered with Raspailiidae, but could not be distinguished on the basis of molecular features from (the same specimen of) *Eurypon*. Here, the present gene tree based on the further upstream C1–D1 fragment now indicates the monophyly of *Sollasella*.

(c) *Aulospongus* Norman, 1878 is represented by *Aulospongus similaustralis* Hooper et al. (in press) and two (as yet undescibed) species in the current taxon set. Diagnostic for *Aulospongus* are its rhabdostyles, which are present in two size-categories. Further, rhabdostyles in Raspailiidae are found in *Raspailia* of the subgenus *Raspaxilla* Topsent, 1913 (as distinguishing character from other *Raspailia* subgenera) and the genus *Cantabrina* Ferrer Hernández, 1914 (currently *incertae sedis*, Hooper, 2002). *Aulospongus* clearly differs in its rhabdostyle geometry and its rhabdostyle-cored fibres, while *Raspailia* (*Raspaxilla*) fibres are cored by non-rhabdose spicules as in most raspailiids (Hooper et al., 1999). All the *Raspailia* (*Raspaxilla*) of our data set (i.e. *R. topsenti* Dendy, 1924 and one as yet undescribed species) share a close relationship to *Aulospongus* in the present gene tree, indicating the good suitability of rhablostyles as combining characters for raspailid taxa.

Several sequences of the *Raspailia* subgenus *Parasyringella* Topsent, 1928 cluster at various positions in the present gene tree. These sequences comprise the species *R.* (*P.*) *elegans* (Lendenfeld, 1887), *R.* (*P.*) *nuda* Hentschel, 1911, *R.* (*P.*) *australiensis* Ridley, 1884 and three other, yet undescribed species. As the type species, *Raspailia* (*Parasyringella*) *falcifera* Topsent, 1892, could not be included, no conclusion on the phylogenetic position of *Parasyringella* can be drawn. However, this subgenus is in its present form obviously an assemblage of unrelated species. *Parasyringella* has been used as a convenient subgenus to accommodate *Raspailia* species lacking echinating megascleres (Hooper, 2002). Such apomorphic loss of a morphological character is difficult to predict and the chance of combining species with homoplastic lack of spicules is high, because loss of a character is certainly easier than its gain. *Raspailia* (*P.*) *australiensis* is regarded as representative for its subgenus. Its position far from the other *Raspailia* in the gene tree suggests that a revision of *Parasyringella* is necessary.

The sister group to the Raspailiinae clade in the gene tree is formed around Thrinacophorinae taxa. *Thrinacophora* Ridley, 1885 and *Ceratopsion* Strand, 1925 cluster with several *Raspailia* (*Parasyringella*) species that would match the Thrinacophorinae definition by their lack of echinating megascleres. *Ceratopsion*, however, is not retrieved monophyletically from this data set. Several of its sequences cluster polyphyletically and indicate the need for a more detailed analysis of this genus.

The *Trikentrion flabelliforme* Carter, 1882 position with Thrinacophorinae is morphologically difficult to explain. Thrinacophorinae lack echinating megascleres altogether and have a more prominent axial and extra-axial skeleton. Conversely, *Trikentrion* Ehlers, 1870 was placed in the subfamily Cyamoninae on the basis that it possesses characteristic echinating acanthoplagiotriaene spicules and lacks a markedly differentiated axial and extra-axial skeleton (especially *Trikentrion*). However, as another Cyamoninae genus, *Waltherarndtia* De Laubenfels, 1936, has presumably lost its acanthoplagiotriaene spicules and retains most other features common to Cyamoninae (Hooper, 2002), the presence of echinating acanthoplagiotriaenes may not necessarily be diagnostic. However, a molecular verification of *Waltherarndtia* as a Cyamoninae is still to come, as well as further verification of *Trikentrion* with additional sequences.

The third Thrinacophorinae genus, *Axechina* Hentschel, 1912, clusters furthermore together with *Reniochalina* Lendenfeld, 1888 (Axinellidae: Halichondrida), which corroborates earlier published gene trees (Holmes & Blanch, 2007). A similarity in the axial skeletal architecture and the possession of megascleres with spined terminations is present in both genera (Hooper, 2002), but as in the current understanding of demosponge character evolution these features were regarded as relatively superficial, both taxa were allocated to separate orders. *Axechina* spiculation

resembles that of *Ceratopsion* and its partial reticulation of spicules without obvious fibres resembles *Thrinacophora*, which justifies their placement with Thrinacophorinae raspailiids, in congruence with the molecular results. However *Reniochalina*'s axinellid identity is not well founded because the monophyly of the Axinellidae (*sensu* Alvarez & Hooper, 2002) is not supported with molecular data (Erpenbeck et al., 2005; Nichols, 2005). It appears likely that some genera of the Axinellidae will be reclassified in the near future following a molecular study of this heterogeneous family (Alvarez et al., in preparation).

Additionally, we have to remark on another axinellid genus, *Ptilocaulis* Carter, 1883, that apparently belongs to Raspailiidae based on molecular data. *Ptilocaulis* is regarded as very closely related to *Reniochalina* based on morphological and molecular data (Alvarez et al., 2000), and clusters like *Reniochalina* with Raspailiidae rather than with Axinellidae *s.s.* Furthermore, an alternative gene tree, based on the independent mitochondrial cytochrome oxidase 1, corroborates this finding with a *Ptilocaulis–Ectyoplasia* sister group (Erpenbeck et al., this volume, but there are no further Axinellidae in the data set). Neither *Reniochalina* nor *Ptilocaulis* contains the raspailiid apomorphies of echinating acanthostyles and specialized ectosomal bouquets, so it is presently impossible to compare these molecular data with their morphologically-based allocations.

Further results of this gene tree comprise heterogeneity of *Axinella* and *Cymbastela*, and the close relationship of *Cymbastela* and *Acanthella*, in congruence with earlier published analyses (e.g. Alvarez et al., 2000; Erpenbeck et al., 2005).

The raspailiid (Echinodictyinae) genus *Amphinomia* clusters well-supported with the Agelasida in our data set, *Agelas* and *Astrosclera*. This result overlaps with ongoing morphological and molecular analyses concerning these genera and will be discussed in detail elsewhere (De Voogd et al., in preparation).

Finally, at the base of the gene tree, Suberitidae do not cluster with the other hadromerid species, nor do *Clathria* and *Mycale* form a monophyletic Poecilosclerida clade with the Raspailiidae. While this data set is clearly not suitable to resolve deeper demosponge divergences such as those at the ordinal level, both these results are in congruence with earlier analyses (e.g. Nichols, 2005; Erpenbeck et al., this volume). The non-monophyetic clustering of the two *Hemiasterella* species cannot be analysed further as the voucher of the GenBank specimen AY561947 is not in our collection.

We wish to remark that the gene tree presented here is only a phylogenetic hypothesis (like every gene tree) and it can only provide some insight into the phylogenetic relationships of raspailiid taxa. Only a subset of all relevant taxa has been included here and it would require the sequences of many more genera to make solid statements on raspailid phylogeny and classification, due to the simple demosponge sponge morphological features, which are very prone to homoplasies.

However, our analysis also allows us to draw conclusions on the significance of particular morphological characters for classification and phylogenetic reconstruction. Molecular approaches repeatedly identified cases in which morphological classification was misleading, and only robust gene trees can provide insight into the quality of assumed apomorphies (see Erpenbeck et al., 2006 for examples). This gene tree supports earlier assessments (e.g. Hooper, 1991) of the homoplasic nature of axially compressed and extra-axially plumo-reticulate skeletal structures, which are present (among others) in Axinellidae, Raspailiidae and Hadromerida (e.g. *Trachycladus*, as included in the data set). There may be no support for a synapomorphic quality of this character at higher taxonomic level, including genus level (assuming the correct placement of *Trikentrion*), although occasionally the compressed axial skeletal architecture truly combines two genera, *viz* in *Reniochalina* and *Axechina*. Consequently, out of that seemingly close relationship of *Reniochalina* and *Axechina*, we can conclude that other assumed homoplastic characters again (like spined spicule tips) might in fact be valid synapomorphies.

Taxon definitions based on the assumed loss of characters, which to date is the case for many sponge taxa, rarely find support when tested with molecular alternative data sets (*viz* Dictyonellidae, Erpenbeck et al., 2005, or *Raspailia* (*Parasyringella*), this study). Unfortunately, the depauperate suite of complex characters in many demosponge groups cannot facilitate any morphological classification based entirely on positive presence of characters. It is evident that molecular data should be recruited for the verification of the apo- or plesiomorphic nature of shared features.

The authors thank Oliver Voigt, two anonymous referees, the super-computing facility of the University of Göttingen (GWDG) and Jessica Worthington-Wilmer from the Molecular Identities Laboratory (MIL) of the Queensland Museum. D.E. acknowledges financial support of the European Union under a Marie-Curie outgoing fellowship (MOIF-CT-2004 contract no. 2882). G.W. acknowledges funding by Deutsche Forschungsgemeinschaft (DFG) projects Wo896/3 and Wo896/6 (Priority Programme SPP1174 'Deep Metazoan Phylogeny').

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Submitted 30 April 2007. Accepted 14 August 2007.