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Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA

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Abstract

Calcareous sponges (Porifera, Calcarea) play an important role for our understanding of early metazoan evolution, since several molecular studies suggested their closer relationship to Eumetazoa than to the other two sponge 'classes,' Demospongiae and Hexactinellida. The division of Calcarea into the subtaxa Calcinea and Calcaronea is well established by now, but their internal relationships remain largely unresolved. Here, we estimate phylogenetic relationships within Calcarea in a Bayesian framework, using full-length 18S and partial 28S ribosomal DNA sequences. Both genes were analyzed separately and in combination and were further partitioned by stem and loop regions, the former being modelled to take non-independence of paired sites into account. By substantially increasing taxon sampling, we show that most of the traditionally recognized supraspecific taxa within Calcinea and Calcaronea are not monophyletic, challenging the existing classification system, while monophyly of Calcinea and Calcaronea is again highly supported.

Keywords: Porifera; Calcarea; Phylogeny; Ribosomal DNA; Bayesian inference; Bayes factors; Doublet-model; Data-partitioning

1. Introduction

Sponges (Porifera Grant, 1836) are sessile, aquatic filter feeders that are considered to be the earliest branching metazoans (e.g., Ax, 1995). Monophyly of Porifera has been questioned by a number of molecular studies (e.g., Adams et al., 1999; Borchiellini et al., 2001; Cavalier-Smith et al., 1996; Collins, 1998; Kruse et al., 1998; Lafay et al., 1992; Medina et al., 2001; Zrzavy et al., 1998)—albeit usually with low statistical support—with the calcareous sponges (Calcarea Bowerbank, 1864) being more closely related to eumetazoans than to the other two classically recognized major sponge lineages Demospongiae Sollas, 1885 and Hexactinellida Schmidt, 1870, which are commonly grouped together as Silicispongia or Silicea. As this would imply that the last recent common ancestor of (Eu)metazoa was a sponge-like organism or, alternatively, the sponge bauplan evolved twice, Calcarea play an important role in the reconstruction of early animal evolution, making a well-resolved and supported phylogeny of this group clearly desirable.

The calcareous sponges are represented by about 500, exclusively marine species distributed in all oceans (Manuel et al., 2002). While the mineral skeleton of Demospongiae and Hexactinellida consists of intracellularly formed siliceous spicules, Calcarea is characterized by the intercellular formation of spicules composed of calcium carbonate, which is an autapomorphic character of this group (Ax, 1995; Böger, 1988; Manuel, 2006; Manuel et al., 2002). The monophyly of calcareous sponges is also supported by ribosomal DNA (rDNA) data (Borchiellini et al., 2001; Manuel et al., 2003, 2004).

Cytological and embryological characters and features of spicule morphology strongly suggest a division of the

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Calcarea into the subtaxa Calcinea and Calcaronea (Bidder, 1898; Borojevic et al., 1990, 2000; Manuel, 2006; Manuel et al., 2002). Another character distinguishing these two groups is the ratio of different carbon isotopes that are incorporated into the spicules during biomineralisation (Reitner, 1992; Wörheide and Hooper, 1999). Although the Calcinea and Calcaronea are very well characterized by these features, there still remains the possibility that some character states in one of the groups represent symplesiomorphies, rendering the respective group paraphyletic with regard to the other (Manuel et al., 2002; but see Manuel, 2006). As rDNA studies (Borchiellini et al., 2001; Manuel et al., 2003, 2004) do support monophyly of Calcinea and Calcaronea, this scenario seems rather unlikely, however.

In contrast, phylogenetic relationships within Calcinea and Calcaronea remain largely unclear, because the existing classification of calcareous sponges (Borojevic et al., 1990, 2000, 2002a,b,c; Vacelet et al., 2002a,b) is primarily typologic, and a phylogenetic system of this group has not been proposed so far (but see Reitner, 1992). Because of the apparent high level of morphological homoplasy (Manuel et al., 2003), such a system would be difficult or impossible to base on the available morphological data alone. Therefore, molecular data provide the most promising means to resolve this branch of the tree of life.

So far, only two studies (Manuel et al., 2003, 2004) explicitly addressed the question of phylogenetic relationships within Calcarea, applying maximum parsimony (MP) and maximum likelihood (ML) methods to infer trees from 18S and 28S rDNA sequences and morphological character data of 17 calcareous sponge species, representing 15 'genera,' 13 'families' and three out of five 'orders.' An important result of these studies was the placement of *Petrobiona massiliana* Vacelet and Lévi, 1958 in Baerida Borojevic et al., 2000 instead of Lithonida Vacelet, 1981, which is also supported by some spiculation features such as the occurrence of microdiactines and pugioles (dagger-shaped tetractines). Furthermore, monophyly of Leucosolenida Hartman, 1958, Grantiidae Dendy, 1892, and *Sycon* Risso, 1826, was not supported. However, taxon sampling was still too sparse, especially with respect to Calcinea, to make further inferences about higher-level relationships within the two major groups of calcareous sponges.

With this study, we extend the set of available calcarean 18S and 28S rDNA sequences to 44 (mostly Indo-Pacific) species, representing 27 'genera', 18 'families' and all five currently recognized 'orders' of Calcarea. Taxon sampling of Calcinea is increased from four (Manuel et al., 2003, 2004) to 20 species. From 31 species we also sequenced ~750 additional base pairs (bp) of the 28S rRNA gene. We analyzed both genes separately and in combination in a Bayesian framework that accounts for different evolutionary constraints of stem and loop regions and non-independence of paired sites, thereby representing a modelling scheme that is biologically more realistic than standard models commonly applied today and leads to statistically more robust estimations of phylogeny (Telford et al., 2005; Erpenbeck et al., unpublished data). The aims of this study were to evaluate the validity of classically recognized calcinean and calcaronean supraspecific taxa, for most of which no clear statements about potential morphological apomorphies can be found in the literature, and to re-evaluate earlier findings (Manuel et al., 2003, 2004) in the light of substantially increased taxon sampling and a more flexible approach of inferring phylogenies. While distinction of the classically recognized 'subclasses' Calcinea and Calcaronea is highly supported by our analyses, our results suggest that the majority of 'orders' and 'families,' as well as some 'genera,' such as the species-rich *Clathrina* and *Leucandra* are not monophyletic.

2. Materials and methods

Species, collection sites, sample-numbers of the Queensland Museum (QM), South Brisbane (Australia), where most vouchers are deposited, and GenBank accession numbers of the sequences generated in this study, as well as those retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), are given in Table 1; for full nomenclature of ingroup-taxa see Supplementary Table 1.

2.1. DNA-extraction, -amplification, and -sequencing

Genomic DNA was extracted from ethanol-preserved or silica-dried samples with the DNEasy Tissue Kit of Qiagen (Hilden, Germany), following the manufacturer's protocol. To avoid contamination with epibiontic organisms, tissue from the interior of the sponges was used whenever possible. Full-length 18S rDNA was amplified by polymerase chain reaction (PCR) with primers 18S1 and 18S2 (Manuel et al., 2003; see Supplementary Table 2) (2 min/94 °C; 34 cycles [1 min/94 °C; 1 min/50–58 °C; 2 min/72 °C]; 7 min/72 °C). Partial 28S rDNA (domain D2 to helix 36; nomenclature of Michot et al., 1990) was amplified with primers from Medina et al. (2001) and Nichols (2005) (see Supplementary Table 2) (10 min/95 °C; 34 cycles [1 min/95 °C; 1 min/50–58 °C; 1–4 min/72 °C]; 7 min/72 °C). Reaction mixes contained 2.5 µl of 10× NH4 PCR-buffer (Bioline, Luckenwalde, Germany), 1.0–1.5 µl $MgCl_2$ (50 mM), 1 µl of each primer (10 µM), 0.5 µl dNTPs (10 mM each), 0.05 µl *Taq*-DNA-Polymerase (5 u/µl; Bioline, Luckenwalde, Germany) and 0.5–5 µl template. Bands of expected size were cut out from agarose gels and purified following Boyle and Lew (1995). Both strands of the amplicons were sequenced directly with BigDye Terminator 3.1 chemistry and an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Sequencing primers are given in Supplementary Table 2. Intragenomic length variation did not allow direct sequencing of *Eilhardia schulzei* and *Plectroninia neocaledoniense*, so PCR products were cloned with the TOPO Cloning Kit for Sequencing (Invitrogen, Karlsruhe) and up to three clones were sequenced. Because the intragenomic indels appeared in regions that were not included in the phylogenetic analyses (see below), only one

Table 1
Species used in this study with accession numbers of the corresponding sequences, as well as collection sites and QM specimen numbers of the species for which new sequences have been generated

Taxon	Collection site	QM-No.	Acc-No. 18S	Acc-No. 28S
Calcinea				
Clathrina wistariensis (Clathrinida, Clathrinidae)	Wistari Reef (GBR)	G313663	**AM180961**	**AM180990**
Clathrina adusta (Clathrinida, Clathrinidae)	Wistari Reef (GBR)	G313665	**AM180961**	**AM180991**
Clathrina helveola (Clathrinida, Clathrinidae)	Heron Reef (GBR)	G313680	**AM180958**	**AM180987**
Clathrina luteoculcitella (Clathrinida, Clathrinidae)	Heron Island/Wistari Reef	G313684	**AM180959**	**AM180988**
Clathrina sp. (Clathrinida, Clathrinidae)	Yonge Reef (GBR)	G313693	**AM180960**	**AM180989**
Clathrina cerebrum[*] (Clathrinida, Clathrinidae)	—	—	U42452	AY563541
Clathrina aff. '*cerebrum*'[a] (Clathrinida, Clathrinidae)	Hook Reef (GBR)	G313824	**AM180957**	**AM180986**
Guancha sp. (Clathrinida, Clathrinidae)	Rene's Nook (GBR)	G316033	**AM180963**	**AM180992**
Soleneiscus radovani (Clathrinida, Soleneiscidae)	Wistari Reef (GBR)	G313661	AF452017	**AM180982**
Soleneiscus stolonifer (Clathrinida, Soleneiscidae)	Wistari Reef (GBR)	G313668	**AM180955**	**AM180983**
Levinella prolifera (Clathrinida, Levinellidae)	Hook Reef (GBR)	G313818	**AM180956**	**AM180984**
Leucaltis clathria (Clathrinida, Leucaltidae)	DJ's Reef (GBR)	G316022	AF452016	**AM180985**
Leucascus sp. (Clathrinida, Leucascidae)	GBR	G316051	**AM180954**	**AM180981**
Leucetta sp. (Clathrinida, Leucettidae)	Yonge Reef (GBR)	G313691	**AM180964**	**AM180993**
Leucetta chagosensis (Clathrinida, Leucettidae)	Osprey Reef (Coral Sea, Australia)	G316279	AF182190	**AM180994**
Leucetta microraphis (Clathrinida, Leucettidae)	Wistari Reef (GBR)	G313659	**AM180965**	**AM180995**
Leucetta villosa (Clathrinida, Leucettidae)	Wistari Reef (GBR)	G313662	**AM180966**	**AM180996**
Pericharax heteroraphis (Clathrinida, Leucettidae)	Holmes Reef (Coral Sea, Australia)	G316295	**AM180967**	**AM180997**
Murrayona phanolepis (Murrayonida, Murrayonidae)	Bougainville Reef (Coral Sea, Australia)	G316290	—	**AM180998**
Murrayona phanolepis (Murrayonida, Murrayonidae)	Osprey Reef (Coral Sea, Australia)	G313992	**AM180968**	—
Lelapiella incrustans (Murrayonida, Lelapiellidae)	Moto Lava (Vanuatu, SW Pacific)	G313914	**AM180969**	**AM180999**
Calcaronea				
Leucosolenia sp. (Leucosolenida, Leucosoleniidae)	—	—	AF100945	AY026372
Sycon capricorn (Leucosolenida, Sycettidae)	Ribbon Reef (GBR)	G316187	**AM180970**	**AM181000**
Sycon raphanus[*] (Leucosolenida, Sycettidae)	—	—	AF452024	AY563537
Sycon ciliatum[*] (Leucosolenida, Sycettidae)	—	—	L10827	AY563532
Sycon calcaravis[*] (Leucosolenida, Sycettidae)	—	—	D15066	—
Grantia compressa[*] (Leucosolenida, Grantiidae)	—	—	AF452021	AY563538
Ute ampullacea (Leucosolenida, Grantiidae)	Wistari Reef (GBR)	G313669	**AM180972**	**AM181002**
Aphroceras sp. (Leucosolenida, Grantiidae)	Osprey Reef (Coral Sea, Australia)	G316285	**AM180971**	**AM181001**
Leucandra nicolae (Leucosolenida, Grantiidae)	Wistari Reef (GBR)	G313672	**AM180974**	**AM181003**
Leucandra aspera[*] (Leucosolenida, Grantiidae)	—	—	AF452022	AY563535
Leucascandra caveolata (Leucosolenida, Jenkinidae)	Hardline (GBR)	G316057	**AM180973**	**AM181004**
Anamixilla torresi[*] (Leucosolenida, Jenkinidae)	—	—	AF452020	AY563536
Vosmaeropsis sp.[*] (Leucosolenida, Heteropiidae)	—	—	AF452018	AY563531
Syconessa panicula (Leucosolenida, Heteropiidae)	Wistari Reef (GBR)	G313671	**AM180976**	**AM181007**
Sycettusa tenuis (Leucosolenida, Heteropiidae)	Heron Reef (GBR)	G313685	**AM180975**	**AM181006**
Sycettusa sp.[*] (Leucosolenida, Heteropiidae)	—	—	AF452025	AY563530
Paraleucilla magna (Leucosolenida, Amphoriscidae)	South Atlantic	—	—	**AM181005**
Paraleucilla sp.[*] (Leucosolenida, Amphoriscidae)	—	—	AF452023	—
Grantiopsis sp. (Leucosolenida, Lelapiidae)	GBR	G313969	**AM180977**	**AM181008**
Grantiopsis heroni (Leucosolenida, Lelapiidae)	Wistari Reef (GBR)	G313670	**AM180978**	**AM181009**
Leuconia nivea[*] (Baerida, Baeriidae)	—	—	AF182191	AY463534
Eilhardia schulzei (Baerida, Baeriidae)	Mac's Reef (GBR)	G316071	**AM180980**	**AM181010**
Petrobiona massiliana[*] (Baerida, Petrobionidae)	—	—	AF452026	AY563533
Plectroninia neocaledoniense (Lithonida, Minchinellidae)	Holmes Reef (Coral Sea, Australia)	G316300	**AM180979**	**AM181011**
Outgroups				
Suberites ficus (Demospongiae)	—	—	AF100947	AY026381
Mycale fibrexilis (Demospongiae)	—	—	AF100946	AY026376
Acanthascus (Rhabdocalyptus) dawsoni (Hexactinellida)	—	—	AF100949	AY026379
Antipathes galapagensis (Cnidaria, Anthozoa)	—	—	AF100943	AY026365
Atolla vanhoeffeni (Cnidaria, Scyphozoa)	—	—	AF100942	AY026368
Saccharomyces cerevisiae (Fungi, Ascomycota)	—	—	V01335	U53879

Classification of Calcarea after Borojevic et al. (2002a,b,c); Vacelet et al. (2002a,b) and Manuel et al. (2003). GBR, Great Barrier Reef (Australia). Accession numbers of new sequences are given in boldface. Asterisks indicate ingroup-species for which no genomic DNA or complete 28S rDNA sequences from GenBank were available.

[a] Note: The specimen with QM-number G313824 shows clear affinities to *Clathrina cerebrum* and *C. brasiliensis* Solé-Cava et al., 1991, because it shares spines on the apical actines of tetractines with these two species, a trait that is known from no other *Clathrina* species (see Klautau and Valentine, 2003). *C. brasiliensis* was described solely from Brazil, and a cosmopolitan distribution of *C. cerebrum* is not considered valid by Klautau and Valentine (2003, 15–16), who restrict the species to the Mediterranean and Adriatic seas. However, *Clathrina cerebrum* possibly constitutes a complex of morphologically similar species (Klautau and Valentine, 2003, 15), and distinction between *C. cerebrum* and *C. brasiliensis* is mainly based on genetical differences (Klautau and Valentine, 2003; Solé-Cava et al., 1991, 11–12). Because G313824 was collected from the Great Barrier Reef (Australia), we give it here the preliminary name *Clathrina* aff. '*cerebrum*', indicating that it might belong to a putative *C. cerebrum*/*C. brasiliensis* species complex.

sequence of each Species was used. Sequences were assembled and edited with the program CodonCode Aligner (http://www.codoncode.com), and validated via BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1990) against the GenBank nucleotide database.

2.2. Alignments

Published calcarean sequences and outgroup-sequences were downloaded from GenBank (Table 1) and automatically aligned together with our new sequences with ClustalX 1.81 (Thompson et al., 1997), followed by manual adjustment using SeaView (Galtier et al., 1996) and Mac Clade 4.08 (Maddison and Maddison, 2002). For some of the species (indicated by asterisks in Table 1) 28S rDNA sequences deposited in GenBank only ranged from domain D2 to helix 26, and no genomic DNA was available. Manual adjustments were done according to secondary structural information that was used to define partitions and paired bases for phylogenetic analyses (see below). 28S rRNA secondary structure was assessed using Hancock et al. (1988); Michot et al. (1990); Schnare et al. (1996); and Erpenbeck et al. (2004) as references. For domains D2, D6, and D7, no unambiguous predictions of paired sites could be made for a consensus structure, so these regions were effectively treated as loops. Secondary structure predictions for 18S rRNA were developed using information on the structure of *Saccharomyces cerevisiae* from the European ribosomal RNA database (http://www.psb.ugent.be/rRNA/; Wuyts et al., 2002) and the structure suggested by Wuyts et al. (2000). For variable regions of the 18S rRNA, predictions from the secondary structure algorithm implemented in RNAstructure 4.1 (Mathews et al., 2004), as well as compensatory base changes between sequences of closely related taxa, were taken into account.

In regions of the 28S rDNA alignment where ambiguity was caused solely by outgroup taxa, the corresponding nucleotides of these taxa were recoded as missing data, because a large proportion of sites (mainly in the D2 domain) was affected in this way, and total exclusion of these sites would have led to the loss of many phylogenetically informative sites for the ingroup. This approach allowed us to keep as much of the available phylogenetic information as possible in the alignment, while minimizing the potentially misleading effects of uncertain assessments of positional homology. In both the 18S and the 28S rDNA alignment, positions that could not be aligned unambiguously for all taxa, and insertions comprising only one or two species or only outgroup taxa, were excluded from all analyses. For the combined analysis, the 28S rDNA sequence of *Sycon calcaravis*, which was not available, was coded as missing data, and the 18S rDNA sequence of *Paraleucilla* sp. was concatenated with the 28S rDNA sequence of *Paraleucilla magna*, because these two species appeared at the same positions in the topologies of the separate analyses. Alignments and corresponding trees are deposited in TreeBASE (http://www.treebase.org; study number: S1520).

2.3. Phylogenetic analyses

Phylogenies were estimated with MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003) under default priors from the 18S rDNA alignment, the 28S rDNA alignment, and a combined matrix. *S. cerevisiae* was used as the outgroup-taxon. ML tree searches and non-parametric bootstrap analyses (Felsenstein, 1985) were also conducted, using the web server of the heterogeneous distributed computing system MultiPhyl (http://www.cs.nuim.ie/distributed/multiphyl.php; see also Keane et al., 2005) with SPR tree search and 1000 bootstrap replicates. However, because the modelling scheme described in the next section could not be implemented in the ML analyses, the results of the two methods were not directly comparable (see Section 4). Given that bootstrap proportions (BP values) are a conservative measure of clade support (e.g., Hillis and Bull, 1993), and Bayesian posterior probabilities (PP values) might be overestimations (e.g., Suzuki et al., 2002; but see Huelsenbeck and Ronquist, 2005, 200, and Huelsenbeck and Rannala, 2004), PP values >95% and BP values >75% were interpreted as giving strong support to the respective clade.

2.3.1. Partitioning and model choice

Stem and loop regions of folded RNA molecules are subjected to different evolutionary constraints (e.g., Dixon and Hillis, 1993; Wheeler and Honeycutt, 1988), and thus require different models of nucleotide substitution. Furthermore, the assumption of independence of sites is clearly violated when stem regions are analyzed like unpaired characters, because paired sites evolve together in order to maintain secondary structure (Dixon and Hillis, 1993; Hillis and Dixon, 1991). The Bayesian Markov chain Monte Carlo (MCMC) technique (see Huelsenbeck et al., 2002 and references therein) makes it possible to combine different datasets in a single analysis and to partition single datasets into potentially differently evolving subsets, while allowing each partition to be modelled independently (Huelsenbeck and Ronquist, 2005; Ronquist and Huelsenbeck, 2003). In addition, the great computational efficiency of the method (Larget and Simon, 1999) allows large datasets to be analyzed within a reasonable time, even under complex models (e.g., Nylander et al., 2004). Although models have been developed to account for non-independence of nucleotide sites (Jow et al., 2002; Muse, 1995; Schöniger and von Haeseler, 1994; Tillier and Collins, 1995, 1998), it has not yet become common practice to use such models in phylogenetic analyses of rDNA sequences.

In this study, alignments were partitioned into stem and loop regions, and stem regions were analyzed under the Doublet model, which is based on the SH model (see Schöniger and von Haeseler, 1994 and Huelsenbeck and Ronquist, 2005, for details). In both stem and loop regions,

all six substitution types were allowed to have different probabilities (nst = 6), which corresponds to the General Time Reversible model of nucleotide substitution (GTR; Tavaré, 1986). Loop regions and regions where paired sites could not be defined unambiguously (see above) were analyzed under the GTR model alone. This most parameter-rich model of the time reversible family of models (see Swofford et al., 1996) was chosen because Bayesian inference has been shown to be much more robust to over- than to underparameterization (Huelsenbeck and Rannala, 2004; Lemmon and Moriarty, 2004). The partitioned Doublet + GTR approach was also tested against a GTR-only approach (no partitioning into stems and loops, no consideration of paired sites) by use of the Bayes factor (Kass and Raftery, 1995; see below), to assess if the Doublet + GTR model could explain our data significantly better. In all analyses, among-site rate variation was modelled with a Γ-distribution with four rate categories, allowing a proportion of sites to be invariant (I+G; Gu et al., 1995). Values for the individual model parameters were estimated by MrBayes from the data. Data partitions (18S stems, 18S loops, 28S stems, 28S loops) were unlinked for all parameters except topology and branch lengths.

ML model search was performed with MultiPhyl (see above) under the Akaike Information Criterion (AIC; Akaike, 1974) and the Bayesian Information Criterion (BIC; Schwarz, 1978).

2.3.2. MCMC settings

Two independent runs with one cold and seven heated Markov chains each per analysis were performed simultaneously until the average standard deviation of split frequencies between the two runs dropped below 0.005, lowered from the default stop value of 0.01 to improve convergence of chains. Analyses were run twice to check for consistency of results. A longer run of the combined dataset ($>8 \times 10^6$ generations) was also performed to check if running the Markov chains for more generations could additionally improve convergence. To improve mixing, the temperature-values of the heated chains were lowered from the default (0.20) to 0.01. Trees were sampled every 100 generations. Topology and branch-length information was summarized in 50% majority rule consensus trees with the 'sumt' command; samples obtained before stationarity of ln-likelihoods against generations had been reached were discarded as burn-in. Analyses were carried out with the MPI-enabled parallel version of MrBayes (Altekar et al., 2004) on a 64-node Linux cluster at the Gesellschaft für wissenschaftliche Datenverarbeitung Göttingen (GWDG; www.gwdg.de), requesting one processor for each of the sixteen Markov chains per analysis. The longer analysis of the combined matrix was run on an Apple Power Mac G5 Dual computer. Batch files are available upon request.

2.3.3. Testing hypotheses of monophyly

To test whether non-monophyly of traditionally recognized supraspecific taxa was statistically significant, we enforced constraints on the topology-priors, making the affected taxa monophyletic a priori. Phylogenetic analysis of the combined dataset was then repeated for each constraint as described above, and the difference between the harmonic means of the likelihood values sampled by the MCMC procedure of the constrained (null hypothesis, H_0) and the unconstrained (alternative hypothesis, H_1) analysis was calculated. A Bayes factor (B_{10}) is equal to the ratio of the marginal likelihoods of H_1 and H_0; as these are difficult to calculate analytically, one can use the harmonic means as a valid approximation (Newton and Raftery, 1994). Harmonic means were obtained using the 'sump' command; the first 25% of the samples were discarded as burn-in. It is possible that trees sampled during the unconstrained analysis accidentally contain the constraint that was used in the constrained analysis, thereby potentially biasing subsequent calculations. Therefore, we filtered the post-burn-in samples of the unconstrained analysis for those trees, using PAUP* 4.0b10 (Swofford, 2002). If such topologies were present, we corrected the harmonic mean (hm) of the likelihood values of the unconstrained analysis (H_1) by multiplying it with $n/(n+n_{cons})$, where n is the number of trees sampled, and n_{cons} is the number of trees containing the constraint. The formula for calculating Bayes factors then became $2\ln(B_{10}) = \mathrm{hm}(H_1)\,(n/(n+n_{cons})) - \mathrm{hm}(H_0)$. Bayes factors were interpreted according to the table of Kass and Raftery (1995, 777; reproduced in Table 2).

Table 2
Interpretation of Bayes factors according to Kass and Raftery (1995)

$2\ln(B_{10})$	Evidence against H_0
0–2	Not worth more than a bare mention
2–6	Positive
6–10	Strong
>10	Very strong

3. Results

3.1. Model comparison

According to the Bayes factor, the partitioned Doublet + GTR model could explain our data significantly better than the GTR-only approach; evidence against the latter was 'very strong' in both the separate and the combined analyses (Table 3). For the ML analyses, both AIC and BIC chose the Tamura–Nei model (TrN; Tamura and Nei, 1993) with a proportion of invariant sites and a Γ-distribution of the variable sites (I + G).

3.2. 18S rDNA

The two independent Bayesian analyses produced identical topologies, and differences in PP values, where present, were minimal. The tree of the first analysis is shown in Fig. 1 (results of second analysis not shown). Monophyly of Calcarea, Calcinea, Calcaronea, Silicea,

Table 3
Harmonic means (hm) of the sampled likelihood values of phylogenies obtained with two different modelling schemes, and the respective Bayes factors

Model (+I+G)	18S		28S		18S + 28S	
	hm	$2\ln(B_{10})$	hm	$2\ln(B_{10})$	hm	$2\ln(B_{10})$
GTR	−8,403.77	1,887.62	−14,645.45	5,562.30	−23,130.49	7,664.04
Doublet + GTR	−7,459.96		−11,864.30		−19,298.47	

Bayes factors were calculated as $2\ln(B_{10}) = 2(\text{hm}(L_1) - \text{hm}(L_0))$, where L_1, likelihood values of H_1 (i.e., Doublet + GTR; stem/loop partitioned) and L_0, likelihood values of H_0 (GTR only; no stem/loop partitioning). See Table 2 for interpretation.

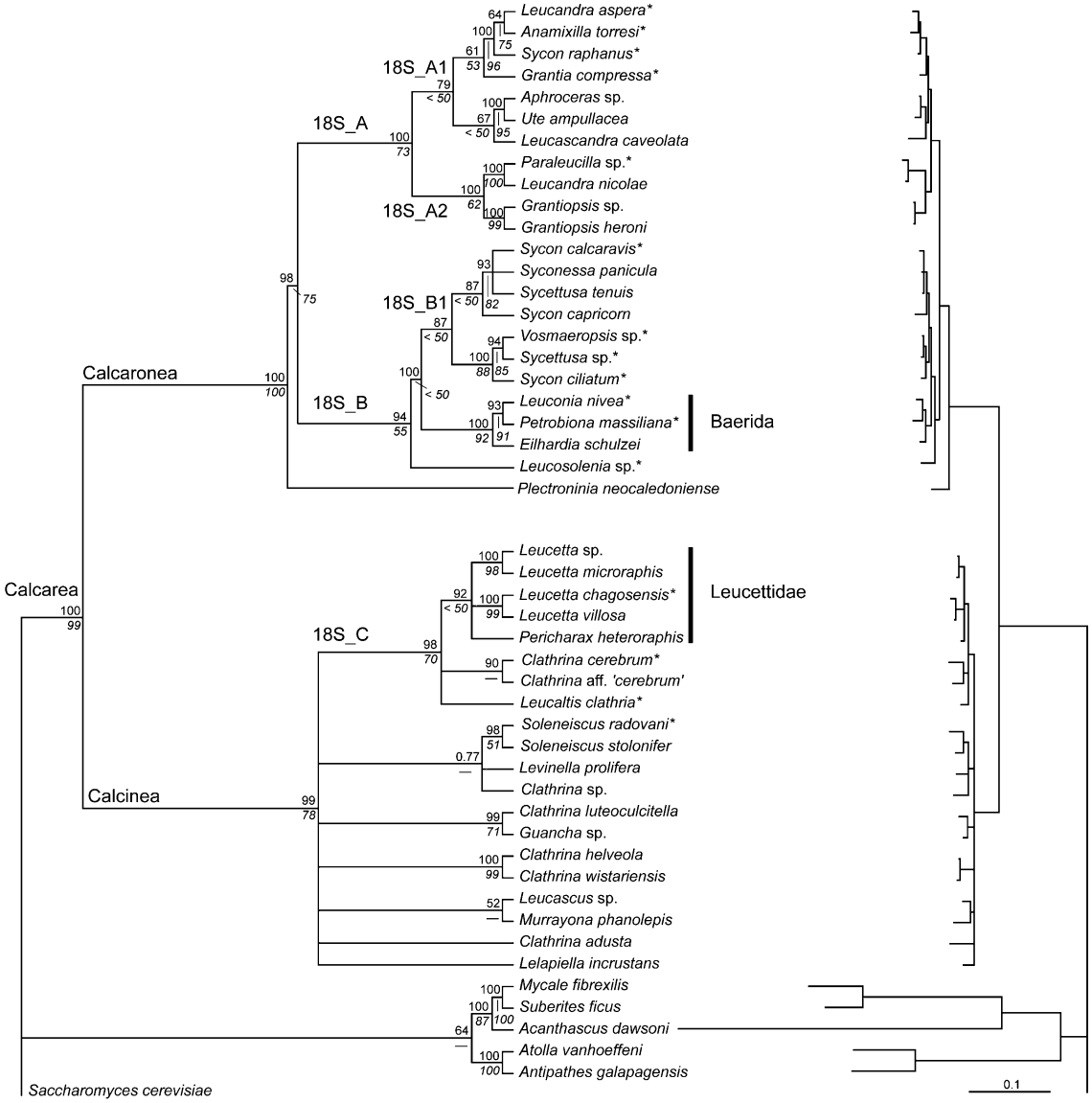

Fig. 1. Bayesian 50% majority rule consensus tree (19,650 trees sampled; burn-in = 1500 trees) inferred from the 18S rDNA alignment under the partitioned Doublet + (GTR+I+G) model. Asterisks indicate previously published ingroup sequences. Bayesian posterior probabilities (%) are given above branches. ML bootstrap proportions (%) calculated under the TrN+I+G model are given below branches (—, clade not included in ML tree). Branch lengths (shown on the right; scale bar, expected number of substitutions per site) are proportional to the mean of the posterior probabilities of the branch lengths of the sampled trees (Huelsenbeck and Ronquist, 2005).

Demospongiae, and Cnidaria was strongly supported. Porifera was recovered as paraphyletic: cnidarians (as representatives of the Eumetazoa) formed a clade with the siliceous sponges; however, with poor support (PP = 64). In the ML tree (Supplementary Fig. 1), Cnidaria weakly grouped with Calcarea (BP < 50). Branches within Calcinea and Calcaronea were extremely short in comparison with those of the outgroup taxa and the branches leading to the Calcarea and its two subclades.

3.2.1. Calcaronea 18S rDNA

Among Calcaronea, *Plectroninia neocaledoniense* (Minchinellidae, Lithonida) was the sister taxon to a well-supported (PP = 98; BP = 75) clade consisting of all other calcaronean species, which split into the subclades named 18S_A and 18S_B in Fig. 1. The Baerida (*Petrobiona massiliana*, *Leuconia nivea*, *Eilhardia schulzei*) were monophyletic but belonged to 18S_B (PP = 94; BP = 55), rendering Leucosolenida paraphyletic. They formed the sister group to 18S_B1 (PP = 87; BP < 50), which contained all members of Heteropiidae (*Sycettusa tenuis*, *Syconessa panicula*, *Vosmaeropsis* sp., *Sycettusa* sp.) and all but one *Sycon* species. Heteropiidae and *Sycettusa*, as well as *Sycon* (and therefore Sycettidae), were not monophyletic. *Leucosolenia* sp. was the sister taxon of 18S_B1/Baerida (PP = 100; BP < 50). 18S_A (PP = 100; BP = 73) contained all members of Grantiidae (*Leucandra aspera*, *L. nicolae*, *Grantia compressa*, *Ute ampullacea*, *Aphroceras* sp.) and Jenkinidae (*Anamixilla torresi*, *Leucascandra caveolata*), as well as *Sycon raphanus*, *Paraleucilla* sp. (Amphoriscidae), and the two *Grantiopsis* species (Lelapiidae). In 18S_A1 (PP = 79; BP < 50), *Ute ampullacea* and *Aphroceras* sp. (both Grantiidae) grouped together and formed a clade with *Leucascandra caveolata* that was the sister taxon to the remaining species of 18S_A1 [(((*L. aspera*/*A. torresi*) *S. raphanus*) *G. compressa*)]. The positions of *L. caveolata* and *Grantia compressa* within 18S_A1 were not well supported. 18S_A2 (PP = 100; BP = 62) consisted of the clade *Paraleucilla* sp. *Leucandra nicolae* and a monophyletic *Grantiopsis*. The topology of 18S_A indicates non-monophyly of Grantiidae, *Leucandra*, *Sycon*, and Jenkinidae.

3.2.2. Calcinea 18S rDNA

The topology of Calcinea was poorly resolved by the 18S rDNA data; it contained only one well-supported clade with more than two species (18S_C in Fig. 1; PP = 98; BP = 70), which included a monophyletic Leucettidae (PP = 92; BP < 50), *Leucaltis clathria* (Leucaltidae), and *Clathrina cerebrum* and *C.* aff. '*cerebrum*.' The latter two species grouped together (as expected; see footnote of Table 1) in the Bayesian tree (Fig. 1), but in the ML tree (Supplementary Fig. 1), they were successive sister groups to Leucettidae. Their position and that of *L. clathria* within 18S_C was not resolved in the Bayesian tree. The same holds true for the position of *Pericharax heteroraphis* within Leucettidae; monophyly of *Leucetta* therefore remained unclear. *Soleneiscus* (Soleneiscidae) was monophyletic (PP = 98; BP = 51); it was associated with *Levinella prolifera* (Levinellidae) and *Clathrina* sp., however with low support. The position of this clade was not resolved, as were the positions of the remaining species. Among these, only a close relationship between *C. luteoculcitella* and *Guancha* sp., and *C. helveola* and *C. wistariensis*, respectively, was inferred. *Leucascus* sp. (Leucascidae) and *Murrayona phanolepis* (Murrayonida) formed a poorly supported clade to the exclusion of *Lelapiella incrustans* (Murrayonida). In the ML tree (Supplementary Fig. 1), *Murrayona* and *Lelapiella* only weakly grouped together (BP < 50). The question of monophyly of Murrayonida and Clathrinida therefore remained open. Monophyly of Leucettidae was relatively well supported by the Bayesian analysis, whereas monophyly of *Clathrina* and Clathrinidae was not recovered by both the Bayesian (Fig. 1) and the ML analysis (Supplementary Fig. 1).

3.3. 28S rDNA

Differences in PP values of the two independent Bayesian analyses were, where present, minimal, and topologies were identical; the tree of the first analysis is shown in Fig. 2 (results of second analysis not shown). Monophyly of Calcarea, Calcinea, and Calcaronea was recovered, but Calcinea received less support (PP = 93; BP < 50) than in the 18S rDNA tree. Silicea, Demospongiae, Porifera, and Cnidaria were also monophyletic, albeit Bayesian support for the latter two was rather low (PP = 66 and 67, respectively). In contrast, bootstrap proportions for Porifera and Cnidaria were relatively high (BP = 80 and 76, respectively). Relative branch lengths were similar to those of the 18S rDNA tree.

3.3.1. Calcaronea 28S rDNA

Like in the 18S rDNA tree, *P. neocaledoniense* was the sister taxon to the rest of the calcaroneans. The remaining topology differed in some respects, however: Although 28S_E in Fig. 2 corresponds to 18S_B1 in Fig. 1, and 28S_D1 corresponds to 18S_A, relationships within these clades were different. In 28S_E, *Sycon capricorn* was the sister taxon to the remaining species; in 28S_D1, *L. caveolata* and *Grantia compressa* grouped together, *Ute ampullacea* and *Aphroceras* sp. were successive sister groups to *Grantiopsis*, and *S. raphanus* (instead of *Anamixilla torresi*) was more closely related to *Leucandra aspera*. Major differences were the placement of Baerida, which was more closely related to 28S_D1 than to 28S_E (compare with Fig. 1), and *Leucosolenia* sp., which was the sister-taxon to 28S_D/28S_E. Implications for (non-) monophyly of supraspecific taxa are the same as in the 18S rDNA analyses.

3.3.2. Calcinea 28S rDNA

Resolution within Calcinea was increased here compared to the 18S rDNA tree. The two *Soleneiscus* species and *L. prolifera* formed a clade that was the sister group of the remaining calcineans. The clade was poorly supported (PP = 66; BP < 50), and relationships between the three species were unclear, however, thereby questioning monophyly of *Soleneiscus*. *Murrayona phanolepis*, *Leucascus* sp., *Lelapiella incrustans*, and a poorly supported clade consisting of *Leucaltis clathria* and *Clathrina* aff. '*cerebrum*' were successive sister groups to Leucettidae (28S_F in Fig. 2; PP = 100; BP = 97). *Leucetta* was recovered as monophyletic by the Bayesian analysis, but with poor support (PP = 59); in the ML tree (Supplementary Fig. 2), *Pericharax heteroraphis* weakly grouped with *Leucetta* sp./*Leucetta microraphis* (BP = 58). 28S_G, the sister group of 28S_F, showed a very

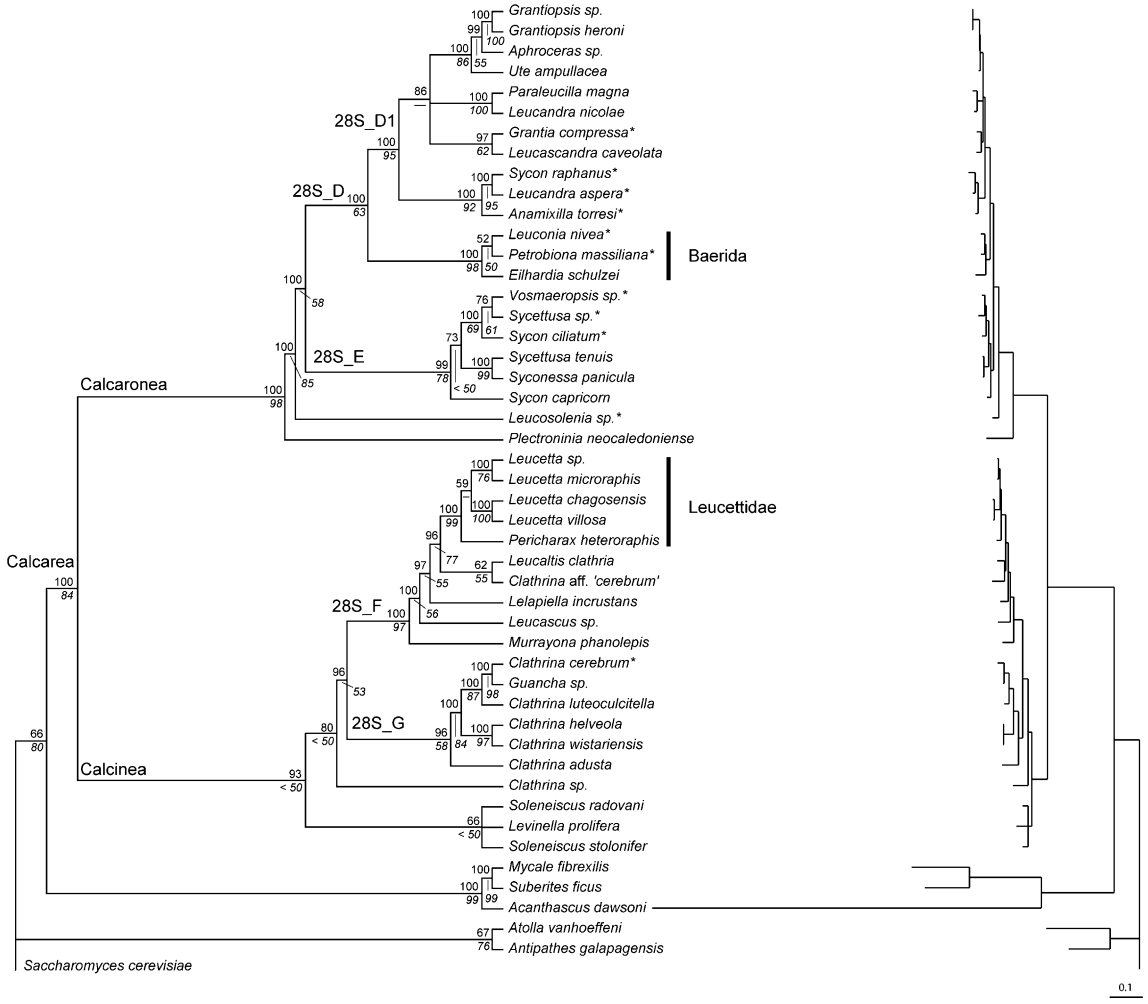

Fig. 2. Bayesian 50% majority rule consensus tree (12,980 trees sampled; burn-in = 600 trees) inferred from the 28S rDNA alignment under the partitioned Doublet + (GTR+I+G) model. Asterisks indicate previously published ingroup sequences. Bayesian posterior probabilities (%) are given above branches. ML bootstrap proportions (%) calculated under the TrN+I+G model are given below branches (—, clade not included in ML tree). Branch lengths (shown on the right; scale bar, expected number of substitutions per site) are proportional to the mean of the posterior probabilities of the branch lengths of the sampled trees (Huelsenbeck and Ronquist, 2005).

well supported topology (except the bootstrap value for inclusion of *Clathrina adusta*; BP = 58). It contained most of the *Clathrina* species, with *Guancha* sp. nested within them. Surprisingly, it also contained *C. cerebrum* (sister-taxon to *Guancha* sp.), thereby questioning a close relationship with *C.* aff. '*cerebrum*' (see above and Fig. 1). *Clathrina* sp. was the sister taxon to 28S_F/28S_G, but this was not well supported (PP = 80; BP < 50). Except the unclear status of *Soleneiscus* and a higher support for Leucettidae (PP = 100; BP = 99; compare with Fig. 1), implications are the same as in the 18S rDNA analyses. However, monophyly of Murrayonida and Clathrinida was clearly rejected (see placement of *Murrayona* and *Lelapiella* in Fig. 2).

3.4. Combined analysis

Differences in PP values of the two shorter independent Bayesian analyses and those of the longer run (burn-in = 20,000 trees; not shown) were, where present, minimal. Topologies were identical, except of an unresolved position of *L. prolifera* within Calcinea in one of the shorter analyses (not shown). The tree of the other analysis is shown in Fig. 3. Monophyly of Calcarea, Calcinea, Calcaronea, Silicea, Demospongiae and Cnidaria was highly supported, but interrelationships of Calcarea, Silicea and Cnidaria (Eumetazoa) remained unclear according to the Bayesian analysis. In the ML topology (Supplementary Fig. 3), Silicea and Calcarea weakly grouped together (BP = 59).

3.4.1. Calcaronea 18S/28S rDNA

Consistent with the results from the single-gene analyses (Figs. 1, 2), *P. neocaledoniense* was the sister taxon to the remaining calcaroneans. The position of *Leucosolenia* sp. was the same as in the 28S rDNA topology. The remaining species were distributed on two clades (Clade_H and Clade_I in Fig. 3). Clade_H corresponds to 18S_A in Fig. 1 and 28S_D1 in Fig. 2. Its topology more closely resembled the 18S rDNA topology, but Clade_H1 and Clade_H2 received less support (PP = 69 and 70, respectively) than 18S_A1 and 2 (see Fig. 1) and were not contained in the ML topology, where the two

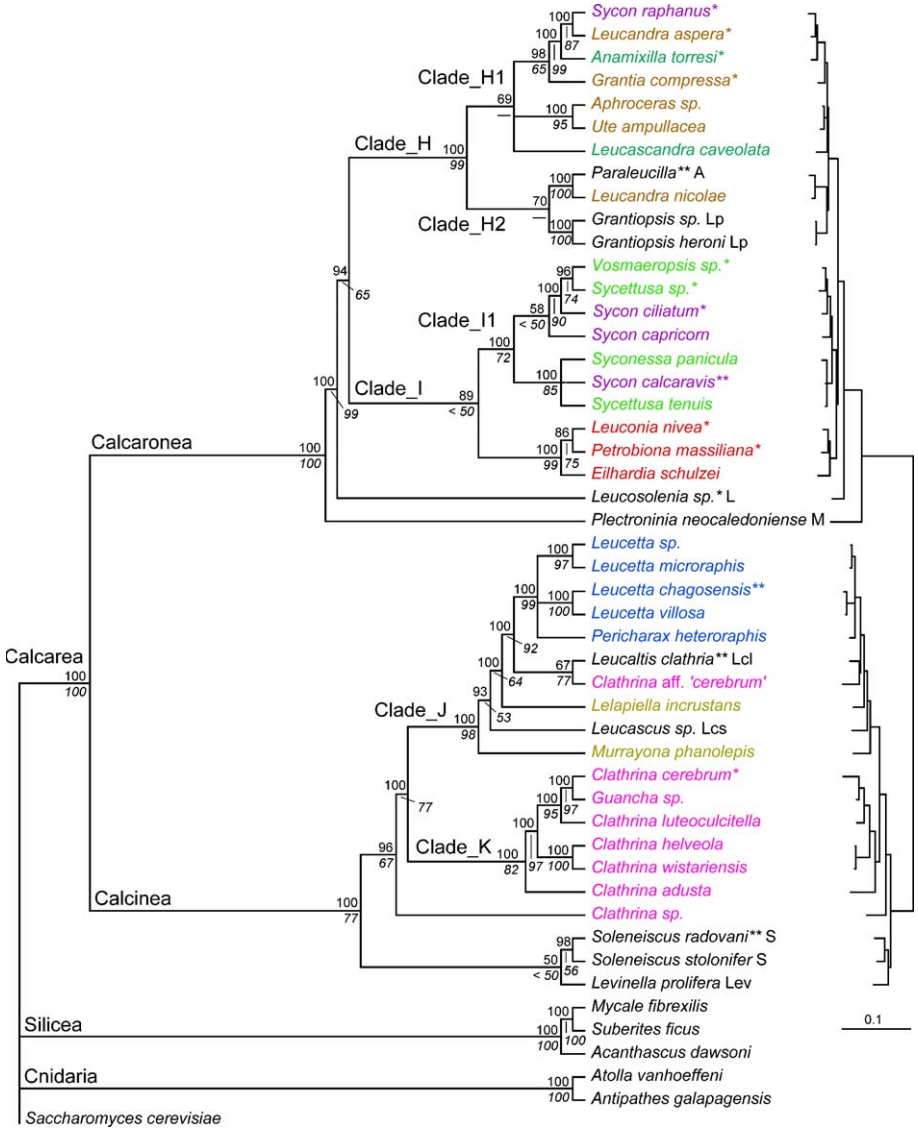

Fig. 3. Bayesian 50% majority rule consensus tree (36,990 trees sampled; burn-in = 1000 trees) inferred from the combined 18S/28S rDNA alignment under the partitioned Doublet + (GTR+I+G)-model. Bayesian posterior probabilities (%) are given above branches. ML bootstrap proportions (%) calculated under the TrN + I+G model are given below branches (—, clade not included in ML tree). Branch lengths (shown on the right; scale bar, expected number of substitutions per site, outgroups omitted for clarity) are proportional to the mean of the posterior probabilities of the branch lengths of the sampled trees (Huelsenbeck and Ronquist, 2005). Selected species are colored according to their assignment to classically recognized supraspecific taxa; 'families' of the other species are given as abbreviations after the species names. Blue, Leucettidae; brown, Grantiidae; green, Heteropiidae; olive, Murrayonida; pink, Clathrinidae; purple, *Sycon*; red, Baerida; and turquoise, Jenkinidae. A, Amphoriscidae; L, Leucosoleniidae; Lcl, Leucaltidae; Lcs, Leucascidae; Lev, Levinellidae; Lp, Lelapiidae; M, Minchinellidae (= Lithonida sensu Manuel et al., 2003); S, Soleneiscidae. * Both sequences from GenBank; **, one sequence from GenBank (see Table 1).

Grantiopsis species grouped with *Ute ampullacea*/*Aphroceras* sp. (Supplementary Fig. 3). The relationships between *S. raphanus*, *L. aspera* and *A. torresi* were identical to those recovered from the 28S rDNA analysis. The topology of Clade_I was almost identical to 18S_B excl. *Leucosolenia* sp., the only difference being the position of *S. capricorn*, which was very poorly supported, however.

3.4.2. Calcinea 18S/28S rDNA

The topology of Calcinea was largely identical to that of the 28S rDNA analysis, but it was generally more robust in terms of clade support. Exceptions were the resolution within Leucettidae and the monophyly of *Soleneiscus*, which correspond to the 18S rDNA tree (Fig. 1).

3.5. Hypothesis testing

Evidence against monophyly of all taxa found in our analysis as non-monophyletic was 'very strong' (Table 4). Trees in the samples of the unconstrained analysis containing the respective constraint were only found in the cases of Murrayonida and Leucosolenida. Given their small numbers (3 and 9, respectively, out of 35,990), correcting for

Table 4
Results of the comparison of constrained analyses vs. the unconstrained analysis of the combined matrix using the Bayes factor (2 ln (B_{10}))

Taxon constrained to be monophyletic	2 ln (B_{10})
Leucosolenida	31.76
Grantiidae	449.30
Heteropiidae	61.44
Jenkinidae	115.82
Sycon	414.94
Leucandra	838.48
Sycettusa	158.14
Clathrinida	160.66
Murrayonida	27.60
Clathrinidae	216.66

See Table 2 for interpretation.

those topologies did not change the outcome of the calculations.

4. Discussion

Calcarea are notorious for being taxonomically difficult. Except from the major split into the two 'subclasses' Calcinea and Calcaronea, phylogenetic relationships of calcareous sponges have remained enigmatic for the most part, and classification schemes currently in use do not rest upon well-supported hypotheses about the underlying phylogeny. Due to limited taxon sampling, the molecular studies conducted so far provided only few detailed insights into relationships within the two 'subclasses.' With the present study, we have substantially increased taxonomic sampling of 18S and 28S rDNA for calcareous sponges and provide a much more comprehensive picture of their phylogeny. Monophyly of Calcarea and its subtaxa Calcaronea and Calcinea was strongly confirmed. In contrast, most of the 'orders', 'families' and 'genera' with more than one species sampled did not represent monophyla. Notable exceptions were the Leucettidae (Calcinea) and the Baerida (Calcaronea), the monophyly of both of which was highly supported.

4.1. Bayesian vs. ML analyses

With some exceptions (e.g. monophyly of Porifera in the 28S rDNA analyses), bootstrap proportions were generally lower than Bayesian posterior probabilities, sometimes considerably so. Especially striking was the very low bootstrap support for monophyly of Calcinea in the 28S rDNA analysis. Also, there were some topological differences, such as the position of *Grantiopsis* in the trees of the combined analyses. However, as already mentioned, outcomes of ML and Bayesian analyses in this study were not directly comparable due to differences in the underlying evolutionary models. When compared to the Bayesian GTR-only trees that we obtained from the model testing (Supplementary Figs. 4, 5, and 6), the differences in clade support and topology were much less striking in most cases. For example, support for monophyly of Calcinea was only 69% in the Bayesian 28S rDNA GTR-only tree (Supplementary Fig. 5). This indicates that the differences between Bayesian and ML analyses in our study were largely due to suboptimal modelling in the latter and did not stem from flaws in one or the other inference method. Therefore, we consider the outcomes of our Bayesian analyses as the more reliable estimates of calcarean phylogeny. For in-depths discussions of posterior probabilities vs. bootstrap proportions, we refer the reader to Alfaro et al. (2003, and references therein) and Huelsenbeck and Rannala (2004).

4.2. Branch-lengths

Branches within Calcinea and Calcaronea were much shorter than branches outside calcareans and branches leading to the two subtaxa. This indicates that they might have undergone a relatively recent radiation, as has been proposed earlier (Borojevic, 1979; Manuel et al., 2003). Alternatively, evolutionary rates might have slowed down in the Calcinea and Calcaronea after the two lineages split. Unfortunately, there is not enough palaeontological data yet to elucidate this issue: the fossil record of modern non-hypercalcified Calcarea is generally very sparse (see Pickett, 2002), and isolated spicules cannot be assigned with certainty to one of the subgroups in most cases (Reitner, 1992).

4.3. Phylogeny of Calcaronea

The most remarkable result concerning the phylogeny of Calcaronea is probably the early-branching position of *Plectroninia neocaledoniense*. This species belongs to the Minchinellidae (Lithonida), a group that is characterized by the formation of a rigid basal skeleton composed of fused spicules (Borojevic et al., 1990; Vacelet et al., 2002b). Calcarea with rigid basal skeletons are often regarded as relicts of otherwise extinct groups of calcareous sponges that survived in cryptic habitats (Reitner, 1992; Vacelet, 1991). Such forms include not only the Minchinellidae, but also *Petrobiona massiliana* (now placed in Baerida; see Introduction) and three species of Calcinea (see next section), of which the basal skeletons are structurally very different, however (Vacelet, 1991). The position of *Plectroninia* in our inferred trees might suggest that a rigid basal skeleton composed of fused spicules is a ground-plan character of Calcaronea that got lost in the lineage leading to the 'Leucosolenida'/Baerida-clade. Alternatively, it might be a highly derived (possibly synapomorphic) character of taxa assigned to Minchinellidae. Decision between these two hypotheses depends primarily on the question whether the Minchinellidae are monophyletic or not, which could not be answered here. Since *Plectroninia* has a leuconoid aquiferous system, its non-nested position also implies that the type of aquiferous system in the most recent common ancestor of Calcarea was not necessarily asconoid, as reconstructed by Manuel et al. (2004): When mapped on the tree of the combined analysis with MacClade 4.06 (Maddison and Maddison, 2002), the ancestral state of

Calcarea was in fact equivocal (results not shown). A sister group relationship of Lithonida (excl. *Petrobiona*; i.e., Minchinellidae) and Baerida, as proposed by Manuel et al. (2003, Fig. 8) on the grounds of a combined morphological/18S rDNA-analysis, is not well supported in our view, because their analysis included no molecular characters of Minchinellidae, and the proposed synapomorphies (absence of an atrial cavity and no axial symmetry of the architecture of the skeleton along the body axis) can easily be interpreted as convergent losses.

The remaining Calcaronea formed a well-supported monophyletic group, with *Leucosolenia* sp. being the sister taxon of the rest of the species in the 28S rDNA and combined trees. The nested position of Baerida within 'Leucosolenida,' rendering the latter paraphyletic, is in agreement with earlier studies (Manuel et al., 2003, 2004). There was, however, some amount of uncertainty regarding the exact placement of Baerida, given that the 18S rDNA and the 28S rDNA alignments contained conflicting signal, reflected by lowered clade support in the combined analysis, so additional data is needed to resolve this issue.

There were some interesting trends concerning the other supraspecific taxa classically assigned to Leucosolenida (compare Manuel, 2006, Fig. 8): Heteropiidae and most species of *Sycon* (Sycettidae) fell into one clade, although both groups were not recovered as monophyletic. Polyphyly of *Sycon* had already been suggested by Manuel (2001) on the basis of morphological evidence, which was later confirmed with molecular data (Manuel et al., 2003, 2004). *Sycon* is a very large, cosmopolitan group and might be regarded as a kind of 'taxonomic waste bin', so this result was not surprising. Heteropiidae was found to be monophyletic by Manuel et al. (2003, 2004), which appears to be a chance result: *Sycettusa* sp. and *Vosmaeropsis* sp. were the only sampled species, and they indeed seem to be closely related, as our results confirmed. Inclusion of only two more species of Heteropiidae here led to the hypothesis of non-monophyly of Heteropiidae and *Sycettusa*. The Heteropiidae are characterized by the presence of a "subcortical layer of pseudosagittal triactines" (Borojevic et al., 2000, 2002b), which could be interpreted as an autapomorphy of this group. However, isolated pseudosagittal spicules also occur in other calcaroneans (e.g., *Sycon ensiferum* Dendy and Row, 1913), so this character might not be as strong an evidence for delimiting the Heteropiidae as was originally thought (see Borojevic et al., 2000: 234–235). The second major calcaronean clade contained all members of Grantiidae, the representatives of Jenkinidae, Amphoriscidae and Lelapiidae, as well as *S. raphanus*. Neither *Leucandra* nor Grantiidae were monophyletic, which is comprehensible, given that—like *Sycon*—both are large groups, in which a number of unspecialized, phenetically similar calcaroneans are merged. The 'family' Jenkinidae was erected by Borojevic et al. (2000) for thin-walled Calcaronea with an inarticulate choanoskeleton; in the light of our results this growth form appears to have originated several times independently instead of being due to common ancestry. A close relationship between *Aphroceras* and *Ute*, as recovered from the 18S rDNA and the combined analysis, had already been suggested by Borojevic (1966); both taxa are characterized by the presence of cortical giant longitudinal diactines (Borojevic et al., 2000, 2002b). This character also occurs in other grantiid 'genera' not included in the present study (e.g., *Sycute* Dendy and Row, 1913) and might be a synapomorphy of these taxa.

4.4. Phylogeny of Calcinea

The 18S rRNA gene apparently contains little phylogenetic information for relationships within Calcinea. Because this gene is thought to be more conserved than the 28S rRNA gene (Hillis and Dixon, 1991), this finding might indicate a more recent radiation of extant Calcinea that could only be fully resolved with the more variable 28S rRNA gene. This conclusion is supported by the fact that the branch leading to Calcinea was shorter than the branch leading to Calcaronea. Unfortunately, this hypothesis cannot be tested with palaeontological data at the moment, given the sparse fossil record of unequivocally identifiable Calcarea (see above).

A split of Calcinea into Murrayonida and Clathrinida (Borojevic et al., 1990, 2002a; Vacelet et al., 2002a), and thus the idea that the former are relicts of an ancient radiation and representatives of the latter are the product of a more recent radiation (Borojevic et al., 1990; Vacelet, 1991; see also Reitner, 1992), was rejected, because *Murrayona* and *Lelapiella* were nested at different positions within 'Clathrinida.' Inclusion of *Lelapiella* in Murrayonida in the current classification is somewhat uncertain (see Vacelet et al., 2002a), and Clathrinida are defined solely by the absence of rigid basal skeletons (see Borojevic et al., 1990, 2002a), so paraphyly of the two 'orders' of Calcinea is not particularly surprising.

Interestingly, all species of Clade_J in Fig. 3 (except *C.* aff. '*cerebrum*,' see below) possess a cortex. This clearly differentiated external layer of spicules is not present in the other species, so it might be an autapomorphy of this clade. In addition, Clade_J contains all syconoid (*Leucaltis clathria*, *Leucascus* sp.) and leuconoid (Leucettidae, *Murrayona*, *Lelapiella*) calcinean species from our dataset, whereas the other species all have an asconoid (i.e., the most simple form of) aquiferous system. The more nested position of Clade_J is therefore in good agreement with the notion that the evolution of Calcinea progressed from simple to complex forms (Borojevic et al., 1990; see also Manuel, 2006).

In all analyses, *Levinella* seemed to be somehow associated with *Soleneiscus*, albeit with weak support. The monophyly of *Soleneiscus* was recovered from the 18S rDNA and the combined analysis, but the 28S rDNA alignment contained ambiguous signal. Apart from Soleneiscidae, we were able to include more than one species from only two 'families': Leucettidae and Clathrinidae. The Leucettidae were recovered as monophyletic with high support, but internal relationships of that group were poorly resolved,

and the phylogenetic status of *Leucetta* awaits further investigation (see Wörheide et al., 2004). Clathrinidae (*Clathrina* + *Guancha*) was not recovered as a monophylum, but the majority of species did form a well-supported clade. Paraphyly of *Clathrina* with respect to *Guancha* is easily comprehensible from a morphological perspective: The latter is distinguished only by possession of a peduncle (stalk) from the former, whereas all characters that are ascribed to *Clathrina* also apply to Clathrinidae (see Borojevic et al., 1990, 2002a). The positions of *Clathrina* sp. and *Clathrina* aff. *'cerebrum'* indicate non-monophyly of Clathrinidae. The placement of the latter species implies secondary morphological simplification, because it is the only asconoid species, and the only species without a cortex, in Clade_J. The possession of spines on the apical actines of tetractines links *C.* aff. '*cerebrum*' to *C. cerebrum*. Since the 18S rDNA tree is in agreement with this, *C.* aff. '*cerebrum*' appears at the same position in both single-gene trees, and repetition of extraction, amplification and sequencing resulted in the same sequences for *C.* aff. '*cerebrum*,' we suspect that the 28S rDNA sequence of *C. cerebrum*, which was retrieved from GenBank, might have come from another *Clathrina* species.

5. Conclusion and outlook

Our study is by far the most comprehensive molecular phylogenetic analysis of Calcarea conducted to date, demonstrating that the existing 'order'- to 'genus'- level classification of calcareous sponges is probably largely artificial and does not reflect the phylogeny of the group. However, to assess the phylogenetic status of still underrepresented taxa (e.g., Amphoriscidae, Lelapiidae, Soleneiscidae), and to place pivotal taxa, such as *Paramurrayona* Vacelet, 1967, or those assigned to Sycanthidae Lendenfeld, 1891, it is crucial to further broaden taxonomic sampling in future studies. Furthermore, our results await corroboration by analyses of nuclear and/or mitochondrial protein-coding genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.04.016.

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