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A short LSU rRNA fragment as a standard marker for integrative taxonomy in calcareous sponges (Porifera: Calcarea)

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Abstract Calcareous sponges are taxonomically difficult, and their morpho-systematic classification often conflicts with molecular phylogenies. Consequently, species descriptions that rely solely on morphological characters,and taxonomic revisions appear to provide little to no information about phylogenetic affiliations and integrative approaches, combining DNA and morphological data, are applied more frequently. However, a standardized database that combines DNA sequence and morphological specimen information is still missing for calcareous sponges. The mitochondrial cytochrome oxidase subunit 1 gene (COI) is the marker of choice for rapid species identification in many other animal taxa, including demosponges, for which COI sequences and morphological information have been compiled in the sponge barcoding database (www.spongebarcoding.org). But due to the peculiarities of calcarean mitochondrial genomes, sequencing COI in Calcarea is methodologically challenging. We here propose the use of one more commonly used DNA marker, the C-region of the 28S gene (LSU), as standard barcoding marker for Calcarea, after also considering the internal transcribed spacer (ITS) region for such proposes. Especially in the subclass Calcaronea, we observed severe problems of high intra- and intergenomic variation that impedes pan-calcarean ITS alignments. In contrast, the C-region of LSU provides a short but phylogenetically informative DNA sequence, alignable across both subclasses with the help of a newly developed secondary structure and which also can be used to address exemplary taxonomic questions. With our work, we start to close the gap of Calcarea in the sponge barcoding project (www.spongebarcoding.org) and provide a resource for biodiversity studies and potentially for DNA-guided species identification.

Keywords Integrative taxonomy · Calcareous sponges · DNA-barcode · LSU rRNA · Sponge barcoding database

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Introduction

DNA and/or amino acid sequence analyses have contributed much in the last decades to address questions concerning the systematics of sponges. For instance, the monophyly of sponges was corroborated and novel class-level relationships were inferred by phylogenomic analyses (Philippe et al. 2009). Also below class level, the classification and our understanding of sponge evolution were highly influenced by DNA sequence analyses leading to the recognition or verification of new taxa (e.g., the class Homoscleromorpha, Gavaze et al. 2012) or severe taxonomic revisions (reviewed by Wörheide et al. 2012). Especially in the class Demospongiae, the most diverse within Porifera, considerable effort was invested to establish a DNA-barcode marker to make DNA-based species identification possible in the future. For this purpose, the Sponge Barcoding Project (SBP, http://www.spongebarcoding.org, Wörheide et al. 2007) in tandem with the sponge genetree server

(SGTS, http://www.spongegenetrees.org, Erpenbeck et al. 2008), compiles and provides a database of demosponge sequences of the mitochondrial cytochrome oxidase I gene (COI) together with a morphological documentation of the corresponding voucher specimens (SBP) and phylogenetic trees (SGTS), thereby providing a valuable resource to aid in the correct identification of sponge specimens.

For calcareous sponges, such a database is as yet lacking. This is most unfortunate, because in this sponge class molecular studies are in a strong conflict with the current classification system. Although the subclass division into Calcaronea and Calcinea has been confirmed, many orders, families and even genera are not recovered as monophyletic entities (Dohrmann et al. 2006; Voigt et al. 2008). In consequence, many of the morphological diagnostic characters—most importantly the spicule types, spicule arrangement and the organisation of the aquiferous system—are either plesiomorphies or the result of convergent evolution (Manuel et al. 2003; Voigt et al. 2012b). Additionally, for many molecular clades no clear morphological synapomorphies have been identified yet (Voigt et al. 2012b), although for some others, new synapomorphies have been proposed and led to taxonomic revisions (e.g. Rossi et al. 2011; Klautau et al. 2013). Therefore, the current taxonomic system of calcareous sponges is unsatisfactory and problematic to be applied. The non-monophyly of many taxa below subclass level (Dohrmann et al. 2006; Voigt et al. 2008) makes a reasonable and taxonomically informed taxon sampling for the revision of groups difficult or even impossible. The reasons for the strong conflicts between classically recognized taxa and molecular clades in Calcarea remain to be resolved. However, first integrative approaches suggest that certain diagnostic morphological characters can be identified if molecular phylogenies serve as back bones to test hypotheses of their synapomorphy (e.g. Rossi et al. 2011); such an approach was already used to erect new genera (Klautau et al. 2013). Unfortunately, with uncertainty about recognizable morphological synapomorphies and a high number of potential homoplasies, phylogenies based on morphological characters remain largely unresolved (Manuel et al. 2003).

Therefore, if DNA studies are neglected in the description of a new morphospecies, only very limited information about its phylogenetic affiliation and evolution can be inferred, because the traditional morphology-based classification system is not reflecting the evolutionary history of the taxa. For example, for a new species of the apparently paraphyletic genus *Sycon* (in molecular phylogenies; e.g., Voigt et al. 2012a), almost nothing will be known about its phylogenetic affiliation other than its assignment to subclass Calcaronea. To overcome these problems, integrative approaches are needed utilizing DNA sequence along with morphological analyses. At the higher taxonomic level (i.e., above species) only few DNA markers have been applied to date in calcareous sponges, mainly from the small subunit (SSU or 18S) and large subunit (LSU or 28S) ribosomal RNA gene ribosomal RNA (rRNA) (Manuel et al. 2003, 2004; Dohrmann et al. 2006; Voigt et al. 2012b) or the internal transcribed spacer regions (ITS, including ITS1, 5.8S, ITS2) (e.g. Wörheide et al. 2002; Bentlage and Wörheide 2007; Klautau et al. 2013; Azevedo et al. 2015).

However, for species-level identifications or determination of phylogenetic affiliation a suitable DNA sequence marker is desirable, which must be applicable to as many taxa as possible. In other animal taxa including demosponges, the partial COI gene of the mitochondrial DNA (mtDNA) was established as a standard barcoding marker (Hebert et al. 2003). But in calcareous sponges, COI is not useful for such purposes due to the peculiar features of the calcarean mitochondrial genome, e.g. the presence of several linear mitochondrial chromosomes, a modified genetic code (Lavrov et al. 2013) and extraordinary high level of variation (Voigt et al. 2012a; Lavrov et al. 2013). For these reasons, early studies failed to find such a universally applicable mtDNA marker for species-level studies in calcareous sponges (e.g. Wörheide et al. 2000), which would aid identification to genus or species level, especially in large biodiversity surveys.

A useful DNA marker for integrative approaches should fulfil several criteria:

i) It should easily be amplified and sequenced (no amplification of infaunal/bacterial contaminant organisms, no intra-genomic variation, which may require cloning).
ii) It should be short. This is important, because DNA quality especially of specimens from older collections can be low. The shorter the amplified fragment, the higher the chances of successful PCR from such specimens.
iii) It must carry an appropriate phylogenetic signal and be alignable to outgroup taxa. For calcareous sponges, this in many cases will require the inclusion of a sequence from the other subclass (either Calcinea or Calcaronea), because the subclass division is at least unequivocally supported by morphological and molecular data (Manuel et al. 2003, Dohrmann et al. 2006; Voigt et al. 2012b).

We here evaluate the use of the ITS region of the rDNA (especially for subclass Calcaronea for which only few sequences are yet available) and a short variable LSU fragment, the C-region (following the annotation of helices of Ben Ali et al. 1999) as a standard DNA marker for intra-genus level studies in calcareous sponges. We further propose a secondary structure model for the LSU region to serve as a guide for DNA alignment.

Material and methods

Species identification

Three specimens of the genus *Leucettusa* that were not previously determined to species level were inspected and

compared to original descriptions and additional published observations (Haeckel 1872; Poléjaeff 1883; Preiwisch 1904; Dendy 1924; Brøndsted 1927; Burton 1963). We identified two samples (QM G323253, QM G323283) as *Leucettusa imperfecta* (Poléjaeff, 1883). QM G323232 was identified as *Leucettusa haeckeliana* Poléjaeff, 1883. However, the specimen was only a fragment of the lower part of the sponge, and we did not observe the rare subcortical tetractines. Another specimen (QM G313824) was classified as *Ascaltis* in a previous work (Voigt et al. 2012b). It shows the typical spined apical actines of the more recently proposed genus *Borojevia* (Klautau et al. 2013) but also shares some affinities with *Ascaltis*, namely a pseudoatrium and a thin layer of tangential spicules, as described before (Voigt et al. 2012b). In this work, we refer to the specimen as *Borojevia (Ascaltis)*. Likewise, we give the previous genus for other species previously belonging to *Clathrina* (sensu lato) or *Guancha*, and which were transferred to the genera *Ascandra, Brattegardia, Borojevia* or *Ernstia* (Klautau et al. 2013). Two specimens of *Grantessa* (SNSB-BSPG.GW974, SNSB-BSPG.GW979) show affinities to *Grantessa intusarticulata* (Carter 1886), by growth form and spicule size, but differ by possession of tetractines in the choanosomal skeleton of the radial tubes. This is also found in the recently described species *Grantessa tenhoveni* Van Soest & De Voogd, 2015; however, this species differ from our specimens by an anastomosing growth form and the sizes of spicules (Van Soest and De Voogd 2015). For now, we decided to refer to these specimens as *Grantessa* aff. *intusatriculata*. Description of these and other specimens have been submitted to the sponge barcoding website (www.spongebarcoding.org) together with the partial LSU sequence and electrophoretograms. Photos of the specimens and of sections can be found in Online Resource 1 (Calcinea) and Online Resource 2 (Calcaronea). Two specimens previously named *Leucaltis clathria* (QM G316022) and *Pericharax heteroraphis* (QM G316295) from NE Australia have recently been recognized as *Leucaltis nodusgordii* (Poléjaeff, 1883) and *Pericharax orientalis* Van Soest & De Voogd, 2015, respectively (Van Soest and De Voogd 2015).

Internal transcribed spacers (ITS) of the rRNA genes

The ITS region (ITS1, 5.8S and ITS2) was amplified for 8 specimens of Calcaronea (Online Resource 3) with primers situated in the SSU and LSU region (fwd: 5′-GTCCCTGCC CTTTGTACACA-3′; rv: 5′-CCTGGTTAGTTTCTTT TCCTCCGC) (Wörheide 1998) using previously reported PCR conditions (Wörheide et al. 2002). Sequencing reactions were carried out as described before (Voigt et al. 2012b). Direct sequencing was impossible for all eight specimens due to intra-genomic indels, leading to subsequent continuous double peaks in the reads. PCR fragments were therefore cloned using the TOPO TA cloning kit for sequencing (Invitrogen), and two to three clones per specimen were sequenced using the PCR fwd primer and a newly designed internal 5.8S reverse primer (rev: 5′-TGAGACAGAC ATGCTCCTGG-3′). Clone sequences were submitted to NCBI GenBank (accession numbers KT223587-KT223608, see also Online Resource 3).

Sequences were initially aligned with MUSCLE (Edgar 2004) and manually modified in Seaview 4 (Gouy et al. 2010) (Online Resource 4). Visualization of the alignment was generated in Geneious R6 (http:www.geneious.com, Kearse et al. 2012). Due to the high intra-genomic and intra-specific variation and apparent need to clone many samples for sequencing, we desisted from extending the taxon range, the sequencing for additional specimens, because it would be impossible to obtain a usable pan-calcarean alignment for this marker. We therefore decided to restrict the phylogenetic analysis to the LSU fragment. To compare the lengths of the new calcaronean ITS 1 sequences to the ones available (almost exclusively from subclass Calcinea), 127 sequences of calcinean ITS sequences were downloaded from GenBank. From these and our sequences, we determined ITS1 sequence length (measured for the end of SSU helix 50 to the start of 5. 8S rRNA helix B1). Lengths and sequence accessions are provided in Online Resource 5.

Large subunit of the rRNA genes (28S rDNA)

LSU rRNA gene sequences from three previous studies (Rossi et al. 2011; Voigt et al. 2012b; Imešek et al. 2013) were downloaded from GenBank (Online Resource 3). For our analyses, we extracted the ‘C-region’ of the LSU gene, which comprised between 387–418 bp. This section of the LSU is included in a slightly larger fragment (459–490 bp) that can easily be amplified from calcareous sponge samples using standard LSU primers (fwd: 5′-GAAAAGAACTTTG RARAGAGAGT-3′,rv: 5′-TCCGTGTTTCAAGACGGG-3′; Chombard et al. 1998). Inspection of the nearly complete LSU sequences region (Voigt et al. 2012b) suggested that the universal LSU forward primer can be modified to 5′-GAAAAGCACTTTGAAAAGAGA-3′ to fit calcareous sponge LSU, although application of the standard primers usually is not problematic (data not shown). In our experience, the amplification of this part of the LSU is often possible with degraded DNA extractions from museum samples and rarely yielded contaminant sequences (data not shown). However, the very aberrant sequence for *Ascandra falcata* retrieved from GenBank (Accession number HQ589006) was analysed by BLAST (Altschul et al. 1990) and shows 96 % sequence identity with nemerteans of the genus *Tetrastemma*. It therefore likely originated from contaminant DNA, and was excluded from all further analyses. Because LSU sequences from Voigt et al. (2012b) were covering a considerable larger

LSU fragment, the reverse sequencing of the C-region was performed with one of two primers that lie further upstream from the recommended barcoding primer: Helix D6-D11: 5′-ACCTTGGAGACCTGATGCG-3′ (Nichols 2005), and helix D15: 5′-CATCGCCAGTTCTGCTTAC-3′ (Voigt et al. 2012b). For amplification of a larger LSU fragment (B21–D15), the forward primer was successfully combined with the latter reverse primer in the previous study (Voigt et al. 2012b).

Secondary structure and alignment

Sequences were initially aligned manually in Seaview 4 (Gouy et al. 2010) to a previous alignment including secondary structure information for all but the C-region (Voigt et al. 2012b). This region of the LSU is highly variable among eukaryotes (Ben Ali et al. 1999; Wuyts et al. 2001) and the most variable part of calcarean LSU. Due to the high variability, secondary structure of this region is taxon specific and no universal structural model is available (Schnare et al. 1996). In order to improve the alignment and to use RNA-specific substitution models of nucleotide evolution in our phylogenetic analyses, a secondary structure model for calcareous sponges was developed as follows. From the initial alignment we extracted an alignment containing only complete sequences over the whole partition. The RNAlifold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi) was used to make an initial secondary structure prediction as bracket-dot annotation, which was added to the original alignment. Using this structural information, the alignment was refined and resubmitted to the RNAlifold server for a refined prediction. A second approach was followed by using RNAsalsa (Stocsits et al. 2009), a software dedicated to align a number of sequences using a constraint structure and minimum-free-energy predictions. Subjectively, this automated approach did not improve the alignment, but instead in some cases led to the alignment of loop positions to stem positions of sequences with extended helices. Therefore, this alignment was neglected, but the minimum-free-energy predictions for each sequence were added to our manual alignment (Online Resource 6). The minimum-free-energy predictions and the RNalifold-consensus structure were largely congruent, but in some cases we modified RNalifold model to include additional pairs that were found for a majority of minimum-free energy predictions but were missing in the RNalifold consensus structure (e.g. Helix C5). Previously published custom PERL scripts (Voigt et al. 2012b) were used to generate secondary structure annotations for all sequences and ct-files with structure information that can be used for visualization with RNAviz (De Rijk et al. 2003). These scripts are available at http://www.palaeontologie.geo.lmu.de/molpal/RRNA/index.htm.

Phylogenetic analysis

We selected 397 sites from the alignment for our phylogenetic study, manually excluding sites with single-sequence insertions or where homology of sites was uncertain. The alignment with selected sites is provided (Online Resource 6). Maximum likelihood (ML) phylogenetic analysis (including a 200 replicate bootstrap analysis) was performed with PhyML (Guindon et al. 2010) as implemented in SeaView, using a GTR+G+I model that was the best fitting standard DNA substitution model according to AIC in Jmodeltest (Posada 2008). RNA-specific models of nucleotide evolution for paired site in RNA have been shown to be beneficial for phylogenetic reconstruction (Gibson et al. 2005), but not included in all phylogenetic software packages. Until recently, a comparison of different RNA-specific models was problematic, and a priori model testing as for standard DNA substitution models was not available. The software package PHASE 3 now provides such possibilities (Allen and Whelan 2014). For model selection, likelihoods of a provided phylogeny (in this case we used the ML tree) under 7-state or 16-state models for looped sites and GTR (=REV) or HKY for standard sites (and all combinations of these) are calculated and adjusted to allow comparisons of 7- and 16-state models, and analysed by AIC (Allen and Whelan 2014). This model testing suggested the R16A+G model for stem (paired) sites in combination with GTR (=REV)+G for looped (unpaired) sites. Using these model settings, we started four independent Bayesian analyses in PHASE 3 (Gibson et al. 2005; Allen and Whelan 2014) using different random seeds. Each run consisted of 1 million burn-in generations and 10 million generations, from which every 200st was sampled. The topologies and model parameters were summarized using the *mcmcsummarize* command in PHASE 3. Parameter sampling was monitored using Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/), after generating a readable input file using a Perl script (Voigt et al. 2012b). Pairwise *p* distances were calculated in PAUP* 4.0b10 (Swofford 2003). Phylogenetic trees are available on the sponge genetree server (www.spongegenetrees.org).

Results

ITS1 region

Sequencing of the ITS1 region of eight calcaronean specimens revealed a high level of intra-specific and intra-genomic variation. Outside of the conserved SSU and 5.8S rDNA regions, the sequences between species were not unambiguously alignable due to the high variability and length differences (Fig. 1). With exception of *Synute pulchella*, molecular cloning revealed intra-genomic length variation (indels) in ITS1

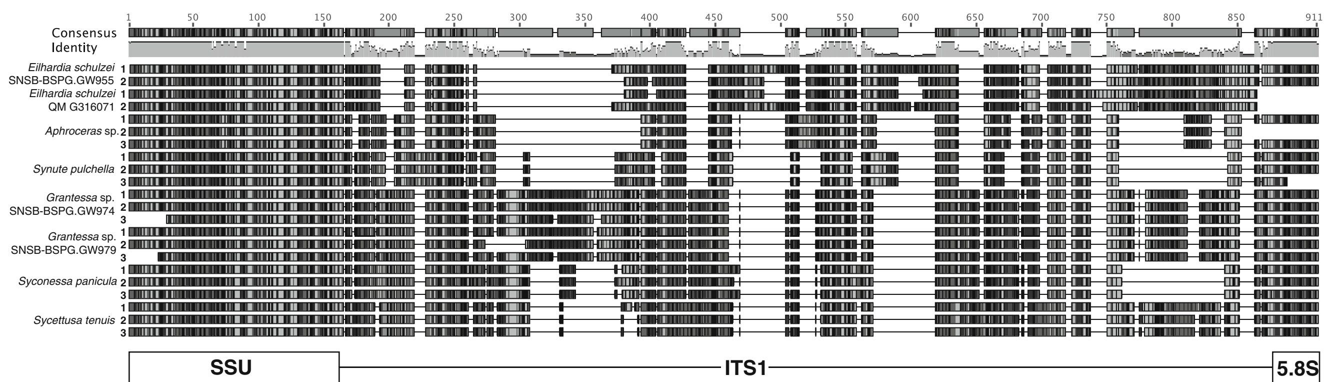


Fig. 1 Overview of the alignment and consensus of ITS sequences. For each specimen, 2–3 clones were sequenced

(Table 1), which inhibited direct sequencing. Indel motifs sometimes included short, 3 base pair repeats, which occurred in different numbers in clones (in *Eilhardia* and *Grantessa*). In all specimens but the two conspecific *Eilhardia* specimens, molecular clone sequences were recovered monophyletic in Neighbor Joining phylogenies (data not shown). In *Eilhardia*, molecular clones of individuals of both specimens formed two clades, suggesting that the variability is a shared polymorphism within the species. ITS1 of Calcaronea also seem to be substantially larger (363–543 bp, Table 1) than in Calcinea (mean 291.74 bp, from 240–396 bp), in some cases exceeding the length of the C-region of LSU.

Secondary structure model for C-region of calcarean LSU

Our predicted secondary structure model of the C-region consists of eight helixes named C1–C8 for both subclasses (Fig. 2). Structures of subclass 90 % consensus sequences with highlighted variable positions are shown in Fig. 2B. On the 90 % consensus level, sequence and structure of Helix C1 and C3 are conserved in Calcarea. Helix C5 is conserved in Calcaronea and shows two variable sites (one stem, one loop position) within Calcinea. Helix C6 is conserved on subclass level, but has subclass-specific substitutions. Helix C7 shows a subclass-specific structural difference in the helix length, consisting of three pairs in Calcinea and four in Calcaronea (with two species, *Plectroninia neocaledoniense* and *Eilhardia schulzei* with helices elongated to eight or six pairs, respectively). In both subclasses, most variation occurs in helices C2, C4 and C8. In Helix C2 and C8, the terminal (loop-site) parts of the helix are more conserved compared to many other parts of the helix.

Sequence distances and phylogenetic analyses of the LSU C-region

Uncorrected p distances of selected sites are higher within Calcinea (average 15.3 %, s=4.3 %, maximum: 25.5 %) than within Calcaronea (average 10.6 %, s=4.0 %, maximum: 22.8 %). Between subclasses, the average p distance is 29.4 % (s=1.6 %, maximum 34.6 %).

Four independent runs in PHASE 3 resulted in almost identical tree topologies (Fig. 3), the only difference being the position of *Leucaltis nodusgordii* (not shown). Because in

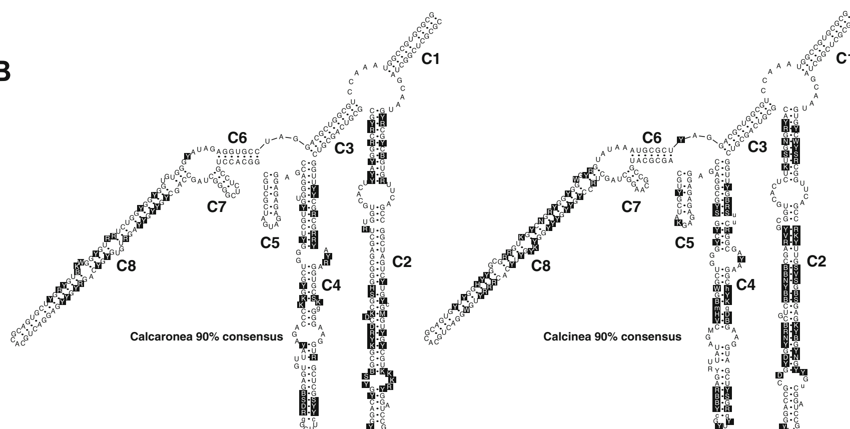
Table 1 Intra-genomic substitutions, length variation and indel motifs in ITS1 sequences

Specimen	# clones	Length ITS1	# substitutions	Indel motifs in clones
Eilhardia schulzei (SNSB-BSPG.GW955)	2	479–521	8	3x[AGC], ACT, GAATGCATCATGTTGGCG (+2 substitutions at indel begin/end), 4x[GCT]GGT, T
Eilhardia schulzei (QM G316071)	2	485–521[a]	9	3x[AGC], ACTGCT, GAATGCATCATGTTGGCG (+2 substitutions at indel begin/end),3x[GCT]GGT, 3x[GAA],T
Aphroceras sp.	3	365–366	2	T
Synute pulchella	3	363	2	none
Grantessa aff. *intusarticulata* (SNSB-BSPG.GW974)	3	534–543	2	GTT, 2x[GCA], T
Grantessa aff. *intusarticulata* (SNSB-BSPG.GW979)	3	511–540	8	AGCGTCGCCGTGTTAACAAAAACAGCGG, GTT
Syconessa panicula	3	436–437	6	CG, CGTC
Sycettusa tenuis	3	478–495	5	GTTAACACGC, CA, GA,TCA

[a] Sequences were not complete at conserved 3′ end, lengths given suggesting sequence conservation in this region

A

B



◀ **Fig. 2** Secondary structure model of LSU in Calcarea. **a** Complete LSU secondary structure of *Leucetta microraphis*. Positions of PCR and/or sequencing primers mentioned in the text are highlighted. **b** Structure of the C-region on 90 % consensus level of the two subclasses. Insertions above the consensus level are given in small letters; variable sites are provided as ambiguity codes and are highlighted by dark boxes

Calcaronea the taxon sampling remained almost identical to that of Voigt et al. (2012b), the topology will not be described here in detail. Compared to that previous analysis of 4939 bp (LSU and SSU), our phylogeny is based upon a smaller dataset of 397 bp but still revealed a similar topology, being in disagreement in six nodes (Fig. 3), of which all but one (position of Baerida) find no significant support by either, or one of the Bayesian and the ML phylogenies.

In Calcinea, the combination of datasets provided a considerable larger taxon set (48 sequences) than previous studies. The position of the root of Calcinea remains unsupported, rendering phylogenetic relations of the first branching species (*Soleneiscus* spp., *Ascandra* sp. JQ272293) unresolved. A highly supported clade contains the two remaining *Ascandra* species (*Ascandra (Clathrina) contorta, Ascandra (Clathrina) corallicola*) as paraphyletic by including *Levinella prolifera*. This clade is (with low support) the sister clade to the remaining Calcinea, whose monophyly finds substantial support in the Bayesian analyses only. Subsequently, a highly supported clade containing the type species for the new genus *Ernstia* (Klautau et al. 2013), *Ernstia (Clathrina) tetractina*, forms a highly supported clade with two undetermined '*Clathrina*' specimens, which may represent other species of this new genus.

The next bifurcation (with high support) separates two large sister clades. The first contains *E. (Clathrina) adusta*, which forms together with yet another undetermined specimen of '*Clathrina*' a highly supported sister group to a clade containing all samples in this dataset belonging to *Clathrina* sensu Klautau et al. (2013). In this *Clathrina* clade, *Clathrina aurea, Clathrina clathrus* and *Clathrina luteoculcitella* are closely related as previously reported (Klautau et al. 2013). As shown before (Imešek et al. 2013), *Clathrina blanca* (PMR-14307) from the Mediterranean is recovered to be closest related to *Clathrina ramosa*, which finds high support. Other relationships in the *Clathrina* clade find in many cases only moderate or no support, and for this reason will not be discussed in detail.

The remaining Calcinea fall in one clade, and only here species with a solenoid or leuconoid aquiferous system occur. The branching order of an undetermined *Leucascus* species, *Ascaltis (Clathrina) reticulum* and *Murrayona phanolepis* has low support, and /or differ between Bayesian an ML phylogeny. Murrayonida, represented by *Murrayona* and *Lelapiella*, is not monophyletic, as has been shown before (Dohrmann et al. 2006; Voigt et al. 2012b).

The new genus *Borojevia*, represented by four specimens, finds high support. It includes a specimen (QM G313824), which was previously assigned to *Ascaltis*, but shows also characteristics of *Borojevia*, including the typical spines on the apical actines of tetractines.

The so for only recognized species of *Brattegardia, B. (Clathrina) nanseni*, forms a highly supported clade with an yet undetermined specimen, previously assigned to *Guancha* (UFRJPor 6336) (Rossi et al. 2011). The position of *Leucaltis nodusgordii* is not highly supported, and it falls either as sister to the clade of the clade containing *Brattegardia*, or to a clade of *Leucettidae*+ *Leucettusa* in the different PHASE analyses. The latter clade reflects the relationships as described before (Voigt et al. 2012b), with *Leucettusa* as a sistergroup to the Leucettidae. The two specimens of *Leucettusa imperfecta* have identical sequences, while *Leucettusa haeckeliana* is genetically distinct from these. Within Leucettidae, *Leucetta* is paraphyletic, because of the position of *Pericharax orientalis* (as found before, Voigt et al. 2012b).

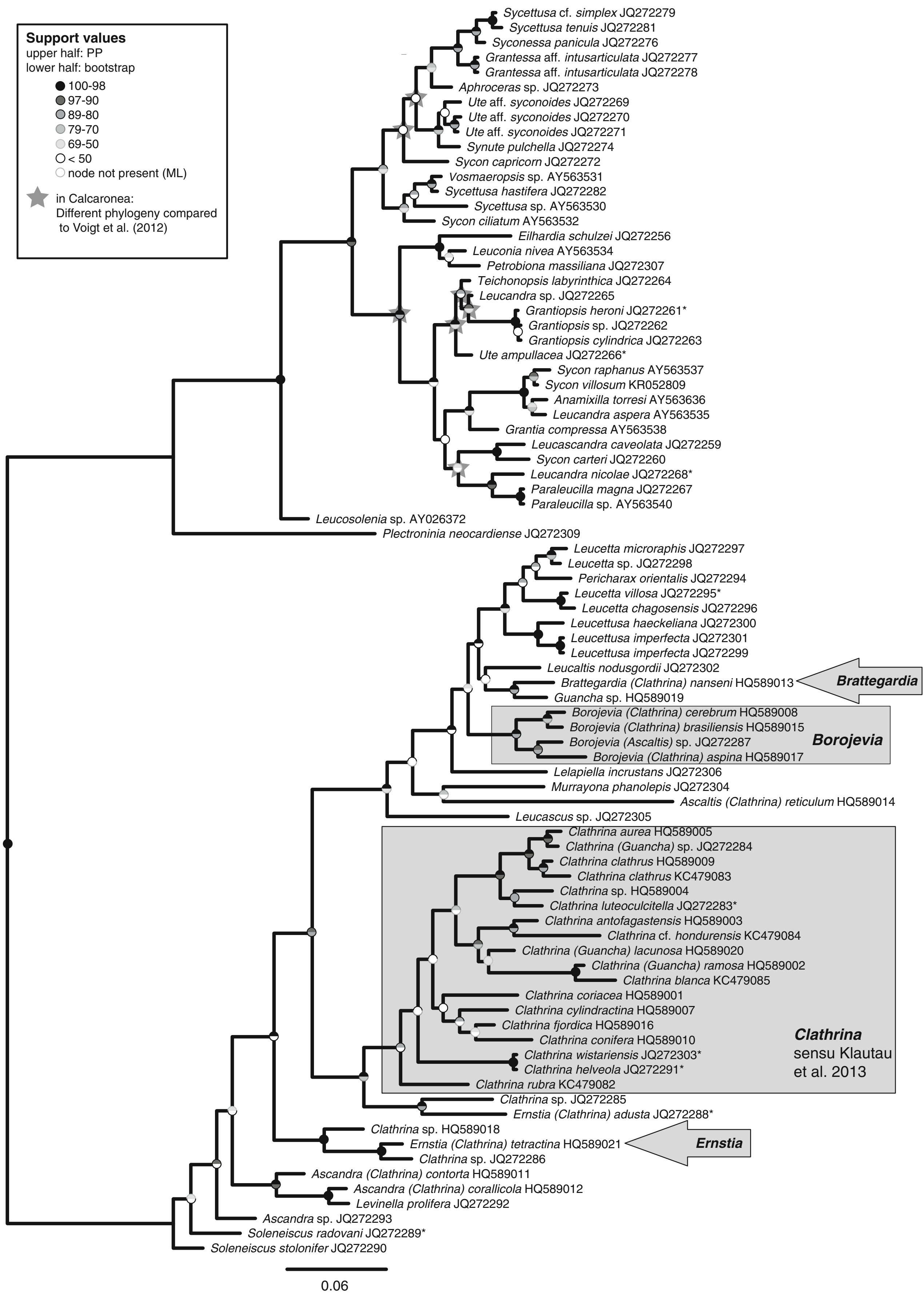
Discussion

ITS region

The ITS region has been used in a number of studies of calcareous sponges, and seems most helpful when closely related specimens are studied (Wörheide et al. 2002; Bentlage and Wörheide 2007). When more distant species are compared, the alignment is problematic, as demonstrated for ITS1 of our eight specimens of Calcaronea (Fig. 1), and outgroup taxa often cannot be aligned and included in the analysis (Klautau et al. 2013). Previous studies already reported intra-genomic variation of the ITS region (predominantly of Calcinea, Wörheide et al. 2004), which mostly were restricted to single substitutions. In the Calcaronea tested here, intra-genomic indels (Table 1) prohibited direct sequencing, which requires additional laboratory efforts (molecular cloning) to obtain ITS sequences. Intra-genomic length variation also occurs in LSU of calcareous sponges, but less frequently. Of the Calcaronea tested for ITS, only LSU of *Eilhardia* required molecular cloning (Dohrmann et al. 2006; Voigt et al. 2012b). According to our results, the ITS1 region of Calcaronea is additionally substantially larger than in Calcinea, sometimes exceeding the length of the complete LSU C-region. PCR amplification of the complete ITS-region (including complete 5.8S and ITS2) of suboptimal preserved DNA will therefore likely be more difficult compared to the smaller LSU fragment.

LSU C-region

The phylogenetic analyses of the C-region fragment already provides a surprisingly resolved phylogeny of Calcarea, which in many parts mirrors previous results with about 10


Support values
upper half: PP
lower half: bootstrap
100-98
97-90
89-80
79-70
69-50
< 50
node not present (ML)
in Calcaronea:
Different phylogeny compared to Voigt et al. (2012)
Sycettusa cf. simplex JQ272279
Sycettusa tenuis JQ272281
Syconessa panicula JQ272276
Grantessa aff. intusarticulata JQ272277
Grantessa aff. intusarticulata JQ272278
Aphroceras sp. JQ272273
Ute aff. syconoides JQ272269
Ute aff. syconoides JQ272270
Ute aff. syconoides JQ272271
Synute pulchella JQ272274
Sycon capricorn JQ272272
Vosmaeropsis sp. AY563531
Sycettusa hastifera JQ272282
Sycettusa sp. AY563530
Sycon ciliatum AY563532
Eilhardia schulzei JQ272256
Leuconia nivea AY563534
Petrobiona massiliana JQ272307
Teichonopsis labyrinthica JQ272264
Leucandra sp. JQ272265
Grantiopsis heroni JQ272261*
Grantiopsis sp. JQ272262
Grantiopsis cylindrica JQ272263
Ute ampullacea JQ272266*
Sycon raphanus AY563537
Sycon villosum KR052809
Anamixilla torresi AY563636
Leucandra aspera AY563535
Grantia compressa AY563538
Leucascandra caveolata JQ272259
Sycon carteri JQ272260
Leucandra nicolae JQ272268*
Paraleucilla magna JQ272267
Paraleucilla sp. AY563540
Leucosolenia sp. AY026372
Plectroninia neocardiense JQ272309
Leucetta microraphis JQ272297
Leucetta sp. JQ272298
Pericharax orientalis JQ272294
Leucetta villosa JQ272295*
Leucetta chagosensis JQ272296
Leucettusa haeckeliana JQ272300
Leucettusa imperfecta JQ272301
Leucettusa imperfecta JQ272299
Leucaltis nodusgordii JQ272302
Brattegardia (Clathrina) nanseni HQ589013
Brattegardia
Guancha sp. HQ589019
Borojevia (Clathrina) cerebrum HQ589008
Borojevia (Clathrina) brasiliensis HQ589015
Borojevia (Ascaltis) sp. JQ272287
Borojevia
Borojevia (Clathrina) aspina HQ589017
Lelapiella incrustans JQ272306
Murrayona phanolepis JQ272304
Ascaltis (Clathrina) reticulum HQ589014
Leucascus sp. JQ272305
Clathrina aurea HQ589005
Clathrina (Guancha) sp. JQ272284
Clathrina clathrus HQ589009
Clathrina clathrus KC479083
Clathrina sp. HQ589004
Clathrina luteoculcitella JQ272283*
Clathrina antofagastensis HQ589003
Clathrina cf. hondurensis KC479084
Clathrina (Guancha) lacunosa HQ589020
Clathrina (Guancha) ramosa HQ589002
Clathrina blanca KC479085
Clathrina coriacea HQ589001
Clathrina cylindractina HQ589007
Clathrina fjordica HQ589016
Clathrina conifera HQ589010
Clathrina wistariensis JQ272303*
Clathrina helveola JQ272291*
Clathrina rubra KC479082
Clathrina sensu Klautau et al. 2013
Clathrina sp. JQ272285
Ernstia (Clathrina) adusta JQ272288*
Clathrina sp. HQ589018
Ernstia (Clathrina) tetractina HQ589021
Ernstia
Clathrina sp. JQ272286
Ascandra (Clathrina) contorta HQ589011
Ascandra (Clathrina) corallicola HQ589012
Levinella prolifera JQ272292
Ascandra sp. JQ272293
Soleneiscus radovani JQ272289*
Soleneiscus stolonifer JQ272290
0.06

◀ **Fig. 3** Bayesian phylogeny (PHASE 3) of the LSU C-region. Support values are colour-coded; Posterior probabilities are shown as the upper half of circles, bootstrap values (ML) as the lower half

fold more positions (Voigt et al. 2012b). In contrast to Klautau et al. (2013) who used the ITS region, the C-region of the LSU allows the alignment of sequences from both subclasses, which has been further facilitated now by our secondary structure model of the region. Because the current taxonomy is in many cases in conflict with our and previous molecular phylogenies (Dohrmann et al. 2006; Voigt et al. 2012b) at the order, family and genus level, it is important to include as many species as possible when making decisions about revisions and to obtain a rooted phylogeny (Voigt et al. 2012b). Unrooted or arbitrarily rooted (e.g. midpoint-rooted) phylogenies are in principle never suitable to recognize monophyly or clades (Wilkinson et al. 2007), because depending on the actual root position clades may become paraphyletic if the root would be within them. In this respect, the proposed LSU fragment can provide further insight about root positions, but also can be helpful to choose which additional species should be included in a further analysis with additional markers. In this regard, our analysis can be used to test the suggested relationships of some recently proposed genera (Klautau et al. 2013): *Clathrina* sensu Klautau et al. (2013), *Ernstia*, *Borojevia* and *Brattegardia*, all of which have previously been assigned to either *Clathrina* sensu *lato* or *Guancha*. We can further assess their relationships within Clathrinida. The conclusions from Klautau and co-workers (Klautau et al. 2013) were drawn from unrooted (midpoint-rooted) ITS phylogenies. Their findings and suggestions will be discussed in some examples in the following with our rooted phylogeny (by including Calcaronea as an outgroup).

The relations of *Ascandra, Soleneiscus* and *Levinella*

The genera *Soleneiscus* and *Levinellidae* were not included in previous studies, which aimed to resolve relationships of Clathrinida (Klautau et al. 2013), although a closer relationship of these genera to several *'Clathrina'* species is evident (Voigt et al. 2012b). Their relationships within Clathrinida are therefore important to draw conclusions about the evolution of asconoid Calcinea and therefore cannot be neglected in such analyses. Recently, several species of *Clathrina* have been transferred to *Ascandra* (Klautau et al. 2013). Of these, *Ascandra (Clathrina) contorta* and *Ascandra (Clathrina) corallicola* are included in the analyses. Unfortunately, the sequence presented as partial LSU sequence of *Ascandra falcata*, the type species of the genus, appears to be of contaminant origin (see M&Ms), and its phylogenetic position remains uncertain. Also, support at the root of the Calcinean subtree remains low, which is not surprising considering that complete SSU and LSU showed similar results (Voigt et al. 2012b). Despite these uncertainties, we found that *Levinella prolifera* is closely related to *Ascandra (Clathrina) corallicola* and *Ascandra (Clathrina) contorta*. Additionally, affinities of an undetermined *Ascandra* specimen remain uncertain. Because also *Soleneiscus* is not recovered monophyletic, the missing type species sequence, and the low support values, the validity of the *Ascandra* sensu Klautau et al. (Klautau et al. 2013) remains to be tested. It is however clear, that such a phylogenetic test cannot ignore members of *Soleneiscus* and *Levinella*.

'Clathrina' adusta is not a member of the genus *Ernstia*

Klautau and co-workers assigned the species *'Clathrina' adusta* Wörheide and Hooper (1999) to their new genus *Ernstia* (Klautau et al. 2013) without consideration of molecular data. We find that this decision is in conflict with their genus definition, where *Ernstia* is described to have tetractines as the 'most abundant spicules or occur at least in the same proportion as the triactines.' In contrast, Wörheide and Hooper (1999) describe the skeleton of the species' skeleton as follows: 'The major part of the skeleton consists of regular triactines with more-or-less cylindrical actines, […]. A few tetractines are present, more abundant in the walls of the larger tubes.' The assignment of *C. adusta* to *Ernstia* (Klautau et al. 2013) was therefore not justified, and *C. adusta* would have better fitted in one of the newly described genera *Arthuria* or *Brattegardia*, which are among other traits diagnosed by possessing tri- and tetractines, where the former are more abundant (Klautau et al. 2013). From our phylogeny, *C. adusta* is clearly not close to the so far only recognized species of the genus *Brattegardia (Clathrina) nanseni*. Unfortunately, no certainly determined member of *Arthuria* is included in our phylogeny, and the phylogenetic affinities cannot be falsified. We nonetheless suggest transferring *'Clathrina' adusta* to the genus *Arthuria (Clathrina) adusta*, based upon the morphological data and the phylogenetic position in respect to *Brattegardia*.

A new species of *Brattegardia?*

Our phylogeny contains the sequence of a specimen (UFRJPor6336) of the no longer valid genus *Guancha*, which already previously was shown to be closely related to '*Clathrina' nanseni* (Rossi et al. 2011). Our phylogeny with more taxa confirms that close relationship, and their phylogenetic affinities to the *Leucetta-Pericharax-Leucettusa* clade, *Leucaltis nodusgordii* and *Borojevia*. The sequence variation between *Brattegardia (Clathrina) nanseni* and the UFRJPor6336 specimen (*p* distance 5.1 %) makes it likely that the latter represents a new species of this so far monotypic genus. A potential additional species of *Brattegardia* from the Norwegian-Greenland-Island Seas was mentioned before as unpublished data from Rapp and Tendal (Klautau et al.

2013), and it is possible that they referred to the same specimen.

Clathrina blanca

Imešek and co-workers (Imešek et al. 2013) found *Clathrina blanca* from the Mediterranean to be closely related to *Clathrina ramosa*, a result that we corroborate here too. In contrast, a specimen similar to *Clathrina blanca*, (*Clathrina* aff. *blanca*) from Norway was most closely related to *C. conifera* based upon an ITS-phylogeny (Klautau et al. 2013). In our phylogeny, *C. conifera* is not closely related and genetically quite distinct from a *C. blanca* from the Mediterranean (*p* distance of 10.7 %). Therefore, the Norwegian specimen despite its similarity most likely represents a different species than *C. blanca* from the Mediterranean.

LSU C-region— a DNA-barcoding marker for species identification?

DNA barcoding aims at species unambiguous determination based upon DNA sequences. For this, a DNA barcoding marker should possess a 'barcoding' gap, i.e. that the intra-specific variation can be clearly separated from the interspecific variation (Meyer and Paulay 2005). Certain sequences in our phylogeny from different species however posses identical LSU sequences: *Clathrina helveola* and *C. wistariensis* share identical sequences, as also do all specimens of the genus *Grantiopsis*, with at least two different species. *Clathrina helveola* and *Clathrina wistariensis*, which both have close type localities, are morphological distinct and differ for example by their colour (pale yellow and white, respectively) and slightly different spicule sizes (Wörheide and Hooper 1999). In *Grantiopsis*, the specimen analysed here differ for example by the relative diameter of the osculum, their aquiferous system (syconoid and sylleibid) and also sizes of spicules (e.g. size of microdiactines). In both cases, only few specimens were included in measurements of the spicules and observation of the other diagnostic characters in the species descriptions (Wörheide and Hooper 1999, 2003). From our results, it remains unclear, whether the identical sequences result from insufficient variation of the applied DNA marker in these cases or from an over interpretation of morphological intra-specific variation that may have been mistakenly interpreted as species diagnostic characters. The latter possibility would be corroborated, if studies with additional specimens demonstrate that the proposed morphological differences are indeed within a continuum of intra-specific variation. In contrast to these cases, the LSU fragment allows the separation of different phylogeographic clades in the *Leucetta chagosensis* species complex (Wörheide et al. 2002, 2008). Additionally, species delimitation is possible in other cases. For example, Burton (1963) criticised that many species of *Leucettusa*, including *L. imperfecta* and *L. haeckeliana*, were only described from one or very few specimens, and that intra-specific variation was so far neglected. He concluded that many *Leucettusa* species should be synonymized and suggested that *L. haeckeliana* and *L. imperfecta* were conspecific (Burton 1963). The phylogeny with LSU C-region data could clearly show genetic differences of the two species. Using this DNA marker it should therefore also be possible to evaluate additional, morphological more similar species, like *Leucettusa lancifer*, which shares many similarities (e.g., subcortical tetractines, v-shaped choanosomal triactines) with *Leucettusa imperfecta*. In summary, the potential for species delimitation with this marker remains to be evaluated in more detail with additional specimens for each taxon, to check if a 'barcoding gap' (Meyer and Paulay 2005) exists between most species. But at least in some very closely related species the C-region of LSU may not suffice as a species-level marker. This, however, also applies to the commonly used 'universal' DNA barcode marker—COI—in some demosponge species (Pöppe et al. 2010). In cases where morphologically different species have identical LSU sequence, additional markers like the ITS-region (Wörheide et al. 2002, 2008; Bentlage and Wörheide 2007; Rossi et al. 2011; Klautau et al. 2013; Imešek et al. 2013; Azevedo et al. 2015) or mitochondrial markers such as cytochrome oxidase subunit 3 (Voigt et al. 2012a) can be applied to refine the resolution for molecular species delimitation/determination. But even then the C-region of LSU can provide valuable information about the phylogenetic position of specimens and will be helpful to identify reliable outgroup taxa.

Conclusion

The C-region of LSU in contrast to the ITS-region allows simultaneous analyses of specimens from both subclasses, thus enabling analyses in a broader phylogenetic framework with a relatively high resolution potential. It offers a reasonable short DNA marker that can be applied to specimens with suboptimal DNA preservation, which is important for the inclusion of older collection material. Our secondary structure model of the region can substantially support and improve alignment of new sequences. In our analyses, we demonstrate the usefulness of this DNA marker for integrative taxonomic approaches, as they are required for Calcarea where severe taxonomic revisions are imminent, but a priori assumptions on morphological synapomorphies are problematic. In such cases, molecular studies have shown to allow identification of morphological traits that in return can be used as diagnostic characters (Klautau et al. 2013). By providing DNA sequence and morphological data on reference specimens via the Sponge Barcoding Project and phylogenetic trees via the

Sponge Gene Tree Server, we facilitate such integrative approaches in the future. Ideally, new species descriptions in Calcarea should included DNA data deposition of the C-region LSU and other DNA markers within the Sponge Barcoding Project database.

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