Molecular biodiversity of Red Sea demosponges

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Abstract

Sponges are important constituents of coral reef ecosystems, including those around the Arabian Peninsula. Despite their importance, our knowledge on demosponge diversity in this area is insufficient to recognize, for example, faunal changes caused by anthropogenic disturbances. We here report the first assessment of demosponge molecular biodiversity from Arabia, with focus on the Saudi Arabian Red Sea, based on mitochondrial and nuclear ribosomal molecular markers gathered in the framework of the Sponge Barcoding Project. We use a rapid molecular screening approach on Arabian demosponge collections and analyse results in comparison against published material in terms of biodiversity. We use a variable region of 28S rDNA, applied for first time in the assessment of demosponge molecular diversity. Our data constitutes a solid foundation for a future more comprehensive understanding of sponge biodiversity of the Red Sea and adjacent waters.

Keywords: Sponges, Porifera, Red Sea, molecular diversity, biodiversity, 28S rDNA

Introduction

As anthropogenic impacts increasingly alter coral reefs (see e.g., Hughes et al., 2003; Hoegh-Guldberg et al., 2007), understanding the more natural state of these ecosystems is becoming urgent, so we have baseline conditions against which changes in biodiversity can be compared to aid conservation efforts. Sponges (Porifera) are one of the main filter-feeding guilds on coral reefs and major players in reef food webs (Perea-Blazquez et al., 2012; de Goeij et al., 2013). Little is known about sponge biodiversity in the Red Sea in comparison to the adjacent waters of Oman, or the more distant Seychelles, India and East Africa (Van Soest and Beglinger, 2008; Berumen et al., 2013). Our current knowledge of Red Sea Porifera is based largely on the works of Keller (1889, 1891), Row (1911) and Lévi (1958, 1965, 1966), as well as on contributions by several other authors (e.g., Topsent, 1892, 1906; Burton, 1952, 1959; Kelly Borges and Vacelet, 1995; Vacelet et al., 2001; Helmy et al., 2004; Ilan et al., 2004; Helmy and Van Soest, 2005; Gugel et al., 2011). Most studies have focused on the Gulfs of Suez and Aqaba, leaving large areas of the Red Sea largely underexplored (Berumen et al., 2013). Changes of the sponge species composition in the Red Sea have been suspected (Vacelet et al., 2001), therefore a comprehensive taxonomic assessment is overdue.

In recent years several expeditions have been conducted to fill in gaps in our knowledge of marine invertebrate biodiversity of the Saudi-Arabian Red Sea and adjacent areas. Species identification is generally the most challenging part of biodiversity surveys. Sponges are especially difficult to identify, because they have relatively few taxonomically-useful characters and exhibit high ecophenotypic plasticity (see e.g., Maldonado et al., 1999; Boury-Esnault, 2006; Lopez-Legentil et al., 2010). Classical (morphological) identification of sponge species requires experience and expertise in the regional fauna, substantial preparation of samples, and is therefore challenging to carry out accurately for rapid surveys and large collections. Recently molecular approaches have been initiated that aim for rapid and unambiguous identification of sponges (Wörheide and Erpenbeck, 2007; Wörheide et al., 2008b). Genetic identification is increasingly recognized as an especially effective, rapid, and reliable technique for delineating species and identifying specimens (see e.g. on Red Sea octocorals

Haverkort-Yeh et al., 2013). High throughput extraction, PCR, and sequencing protocols facilitate the genetic study of large collections (Hajibabaei et al., 2007). Nevertheless some taxa pose technical challenges and require specialized protocols. Thus the establishment of high throughput extraction methods suitable for sponge tissue has facilitated the rapid molecular screening of sponge collections (Vargas et al., 2012).

The choice of a suitable marker is pivotal for molecular assessments of biodiversity. Classically, a region near the 5' end of the cytochrome oxidase subunit 1 (CO1) has been suggested as a "universal barcoding region" for metazoans (Hebert et al., 2003), but slow evolutionary rates in demosponges reduce species-level resolution by this marker(e.g., Shearer et al., 2002), while high evolutionary rates for Calcarea prevent the application of universal primers (Voigt et al., 2012; Lavrov et al., 2013). Despite these shortcomings, CO1 has been successfully used for species discrimination in selected sponge lineages (see for examples López-Legentil and Pawlik, 2008; Ferrario et al., 2010; Pöppe et al., 2010) and is used for the Sponge Barcoding Database (www.spongebarcoding.org) to comply with the current Barcoding of Life standards. Other markers suggested for DNA barcoding of sponges, such as a region near 3' end of CO1 (I3M11, Erpenbeck et al., 2006), were successfully applied on various sponge lineages, but are hampered by the need of nested PCR, which reduces amplification success (e.g., Erpenbeck et al., 2002; López-Legentil and Pawlik, 2008).

The nuclear large ribosomal subunit C1-D1 domain (referred to as "28S" in the following) is also used for shallow-level phylogenetic study of sponges. This marker shows considerable phylogenetic signal on lower taxonomic levels (e.g., Chombard et al., 1998; Erpenbeck et al., 2007a; Cardenas et al., 2009; Schuster et al., 2015) and has also been successfully applied for the molecular taxonomy of Calcarea (Voigt and Wörheide, 2016).

In this study we pursue a molecular survey of demosponge collections made in 2012-2013 along the length of the Saudi Arabian Red Sea, from the Gulf of Aqaba in the north to the Farasan Islands in the south. For reference, we also include specimens from Oman and Djibouti. We report on the establishment of a molecular biodiversity database of Arabian demosponges in the sponge

barcoding project (www.spongebarcoding.org) based on CO1 and 28S markers and discuss the suitability of the 28S marker for molecular identification.

Material and Methods

Demosponge samples

A total of 1,014 samples were collected during three collecting trips for demosponges covering the northern, central and southern regions of Saudi Arabia's Red Sea (see Figure 1). The northern and southern regions were sampled in the course of the Red Sea Biodiversity Survey's Phase 2 (2012, 431 samples from 34 stations at Farasan Islands, Al Qunfudah and Al Lith) and Phase 3 (2013, 377 samples from 25 stations at Al Wajh, Duba and Al Khuraybah), conducted by the King Abdulaziz University, Jeddah, Saudi Arabia, and the Senckenberg Nature Research Society, Frankfurt, Germany. Sampling in the central region (2013, 208 samples from 13 stations at Thuwal) was conducted in collaboration with the King Abdullah University of Science and Technology (KAUST). Samples were collected from depths of 1-30m either by scuba diving, dredging or hand-picking in shallow water under rocks. All samples were photographed, directly preserved in 99% ethanol and subsampled for molecular work. Morphological vouchers are stored in the Senckenberg Museum Frankfurt, Germany (RSS-1), at KAUST, or at the Florida Museum of Natural History, University of Florida (UF). Ethanol of the subsamples for molecular work was exchanged after 24h to avoid long-term storage in seawater-diluted EtOH. The subsamples for molecular work are registered in the Bavarian State Collection for Palaeontology and Geology (see Supplementary Data 1).

Extraction, PCR and sequencing

DNA was extracted using the plate-based extraction method (Vargas et al., 2010) developed for the Sponge Barcoding Project (www.spongebarcoding.org). Fragments of the mitochondrial cytochrome oxidase subunit 1 (standard barcoding fragment) were amplified using the degenerated version of universal barcoding primers dgLCO1490 (GGT CAA CAA ATC ATA AAG AYA TYG G) and dgHCO2198 (TAA ACT TCAG GGT GAC CAA ARA AYC A) (Meyer et al. 2005). For

the 28S fragment the primers 28S-C2-fwd (GAA AAG AAC TTT GRA RAG AGA GT) and 28S-D2-rev (TCC GTG TTT CAA GAC GGG) were used (Chombard et al., 1998). The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq* ® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 2 µL BSA (100µg/ml), 1 µL each primer (5µM), 7.8 µL water, 0.2µL Go*Taq*® DNA polymerase (5u/µl) (Promega Corp, Madison, WI) and 2 µL DNA template. The PCR regime comprised an initial denaturation phase of 94° C for 3 min followed by 35 cycles of 30 s denaturation at 94° C, 20 s annealing (45° C for CO1; 51° C for 28S), 60 s elongation at 72° C each and a final elongation at 72° C for 5 min. We employed a rapid PCR and sequencing screening strategy, which involved that successful PCR amplifications were sequenced with the forward primer only, followed by BLAST check of poriferan origin against NCBI Genbank (www.ncbi.nlm.nih.gov). Only sponge amplification products were sequenced with the reverse primer. In case of failed PCR or sequencing no optimization or repetition was attempted. PCR products were purified with standard ammonium acetate-ethanol precipitation (Sambrook et al., 1989) before cycle sequencing using the BigDye-Terminator Mix v3.1 (Applied Biosystems) following the manufacturer's protocol. Both strands of the template were sequenced on an ABI 3730 automated sequencer. Sequences were basecalled, trimmed and assembled in CodonCode Aligner v 3.7.1.1 (www.codoncode.com). Only sequences with CodonCode Aligner quality values higher than 50% of sequence length were considered for subsequent processing and analysis. Sequences are deposited in NCBI Genbank under accession numbers KU060308-KU060769, and with location data, specimen photos (including *in situ* photos) and additional morphological information in form of thin section photos and spicule preparations for each OTU in the Sponge Barcoding Database (SBD, www.spongebarcoding.org). See supplementary data for specimen details, accession numbers and links to the SBD.

Phylogenetic reconstructions and taxonomic comparison

The CO1 sequences from Red Sea samples were compared with material from adjacent waters such as the Arabian Sea and the Gulf of Oman. Operational taxonomic units (OTUs) were designated when at least two specimens had

identical genotypes for 28S or CO1 or both (if both fragments were available). When 28S showed intragenomic polymorphisms, the affected character positions were coded for with IUPAC ambiguity codes and this position disregarded in OTU assignment. Taxonomic assessments were attempted by phylogenetic grouping in relation to previously published sequences, and, where possible, subsequently compared with the morphological species descriptions. Taxa were named when falling in the distinct monophyletic group or if subsequently identified morphologically. To facilitate this predominantly molecular approach, sequences were incorporated in the CO1 and 28S alignments of the Sponge Genetree Server (www.spongegenetrees.org), which contains the currently most complete alignments of sponge CO1 and rDNA sequences available in public databases (Erpenbeck et al., 2008). For CO1, resulting data sets was aligned with MAFFT v7.149b (Katoh and Standley, 2013) under the FFT-NS-2 strategy. For 28S, obviously incomplete sequences for this fragment published in Genbank (probably lacking variable sites omitted for the phylogenetic reconstruction) were excluded from the data set. The resulting data set was aligned with MAFFT under the G-INS-i strategy and subsequently corrected by eye. Further comparison was performed with yet unpublished 28S and CO1 sequences originating from the South Indian Ocean, and East Indian Ocean and the Central Pacific. Phylogenetic reconstructions were performed with the avx version of RAxML 8 (8.0.26, Stamatakis, 2014) under the GTRGAMMA model and 100 rapid bootstrap replicates.

Results and Discussion

Sequencing success and OTUs

In total 253 28S and 212 CO1 sequences were successfully sequenced from 354 specimens (see Figure 2 for some OTU examples). Several hundred additional sequences with quality values below threshold were disregarded at this stage and will be added to the Sponge Barcoding Database at later stages after resequencing. Of the 354 specimens, 288 (82%) could be assigned to a total of 51 OTUs, i.e. these samples shared their genotypes with at least one other specimen.

An additional 66 specimens (13 CO1+28S, 20 CO1 only, 33 28S only) were singletons, as they had unique sequences not shared with another sample in one or both markers. For 116 specimens (33%) both fragments were sequenced, which considerably aided in the assignment of the other specimens to OTUs and the molecular taxonomic identification.

After alignment and trimming to identical 5' and 3' character positions and addition of published sequences from NCBI Genbank (which include all SBD entries), the CO1 data set consisted of 1,479 sequences and 550 characters, the 28S data set of 758 sequences and 869 characters. The maximum-likelihood trees (Supplementary data 2 and 3) display no support at deeper nodes of the trees, as expected for phylogenetic reconstructions using fast evolving markers. Unambiguous alignment of this 28S region was not possible because of the high variability of this marker, which prevents the reconstruction of a unified secondary structure on class level (see Erpenbeck et al., 2007b). As the trees are reconstructed with hyper-variable (and taxonomy-informative) character positions included, they are only suitable for the molecular biodiversity assessments.

The present approach represents a first assessment of the molecular biodiversity of Red Sea demosponges, and will form a valuable framework for subsequent biodiversity investigations. For several conspicuous Red Sea taxa, barcodes were obtained for the first time e.g., *Crella cyathophora* (OTU#42), *Hemimycale arabica* (OTU#41), *Biemna ehrenbergi* (OTU#48), *Monanchora quadrangulata* (OTU#29), *Suberea mollis* (OTU#32). For other taxa, DNA barcodes were generated here for the first time from Arabian waters e.g., *Stylissa carteri* (OTU#31), *Iotrochota baculifera, Haliclona toxia* (for the Red Sea, OTU#19), and *Pseudoceratina arabica* (OTU#54).

Specimen records including specimen photos are published as entries SBD#1268-1571 and SBD#1607-1640 in the Sponge Barcoding Database (www.spongebarcoding.org), and thin section and spicule photos will be added as well, as they become available. The database will be further expanded with molecular and morphological details of remaining specimens as they become successfully sequenced.

Taxonomic and phylogenetic implications

Twenty-five distinct sequences (21 %) newly obtained in this study can be assigned by the molecular trees to the demosponge order Haplosclerida, one of the most successful demosponge orders in terms of biodiversity (Van Soest and Hooper, 2002; Van Soest et al., 2012), but also the currently most disputed in terms composition of its families. Here, major discrepancies between morphological and molecular data challenge any plausible character re-interpretation (Raleigh et al., 2007; Redmond et al., 2007; Redmond et al., 2013) unlike in most other demosponge orders reclassified based on molecular data (see Morrow and Cárdenas, 2015). As a consequence, Haplosclerida are currently a 'neglected' major group of demosponges in terms of diversity studies (Van Soest et al., 2012). Most of the haplosclerid sequences can be assigned to *Haliclona* and *Callyspongia* species.

A further 22 distinct sequences (19%) can molecularly be assigned to the demosponge order Dictyoceratida, a morphologically particular challenging group. Dictyoceratids lack a mineral skeleton (with a few exceptions) that is typically the feature from which the most widely used morpho-taxonomic characters in sponges are derived. Members of this order possess only a character-poor spongin skeleton that has led to taxonomic misassignments as recently demonstrated through molecular phylogenies (Erpenbeck et al., 2012; Redmond et al., 2013) . Several dictyoceratid OTUs can be classified as *Hyrtios* (e.g., OTUs#24-26 and #28). The phylogenetic trees of both CO1 and 28S, recover *Hyrtios* OTU#24 close to database entries of *H. erectus* from Australia and American Samoa, respectively. *Hyrtios erectus* was originally described from the Red Sea (Keller 1889) but is widely reported through the Indian and Pacific Ocean (see e.g., Bergquist, 1965); this distribution awaits confirmation by molecular investigations that ideally would include type material (see e.g., Erpenbeck et al., 2015). The overall classification of *Hyrtios* needs revision as molecular data repeatedly recovered *Hyrtios* as nonmonophyletic (Erpenbeck et al., 2012; Redmond et al., 2013) and the currently 17 valid *Hyrtios* species might constitute a polyphyletic assemblage within the Dictyoceratida.

Limestone-excavating Clionaida species constitute another diverse fraction of the collections (e.g., OTUs#03-07). *Cliona* is one of the most species-

rich genera of the Demospongiae and molecular tools are indispensable for their identification because cryptic species are common in the genus (e.g., Barucca et al., 2007; Xavier et al., 2010). OTU#07 is a member of the *Pione vastifica* species complex, which was previously revealed by CO1 sequence data (Ferrario et al., 2010).

As noted by earlier studies, the Indo-Pacific barrel sponge *Xestospongia testudinaria* shares CO1 haplotypes with several other *Xestospongia* species, including barrel sponges from Indonesia, and even the Caribbean barrel sponge *X. muta* (e.g., Montalvo and Hill, 2011). Setiawan et al. (2015) hypothesized that the multiple shared haplotypes among these species represent shared ancestral polymorphisms. Sequence data from other markers are needed to resolve the phylogeny and species of barrel sponges.

Published sequences of several genera (e.g., *Biemna*, or *Crella*) or *Cinachyrella australiensis* form no monophyletic groups in our trees, which corroborates earlier molecular analyses (Hall et al., 2013; Redmond et al., 2013; Szitenberg et al., 2013). Molecular analyses based on type material including holotypes of the type species are indispensable to identify the nominal lineages and assist in reclassification of the others (Erpenbeck et al., 2015).

28S (C1-D1) in molecular sponge biodiversity assessments

We found that the partial 28S sequences sampled had a higher variability than the standard-CO1 DNA barcoding fragments in all demosponge subclasses analyzed here, indicating that it is well-suited to discriminate OTUs in large collections. Pairwise comparison between dictyoceratid, verongid, poecilosclerid and clionaid OTUs recovered here showed in that 28S was more divergent than CO1 in every case (see Table 1). Further, the 28S marker detected genetic differences among samples whose CO1 sequences were identical. For example, the *Spongia* samples of OTU#11, collected in Duba and Al Khorayba, differ in 28S from OTU#12, collected further south in Al Wajh, by 1 bp (0.4%), while their CO1 genotypes are identical, but sample sizes do not yet allow conclusions on a genetic separation by distance. A further comparison of Arabian sponge OTUs with unpublished Indo-Pacific specimens revealed additional cases where samples with identical CO1 sequences in Arabia and at other locations have

different 28S sequences (OTU#28, OTU#21, OTU#44, and OTU#06, representing all demosponge subclasses with the exception of Verongimorpha). OTU#06 and GW5814 constitute the only case in which specimens with identical 28S sequences had different CO1 haplotypes (differing in 2.3%, no amino acid changes). Comparisons of 28S and CO1 for Haplosclerida and among more distantly related lineages of the data set were hampered by the ambiguous alignment of the 28S fragment at these higher genetic divergences. Nuclear ribosomal DNA substitution rates are known to be significantly in Haplosclerida than in other demosponges, resulting in additional loops and elongated helices in secondary structure (Erpenbeck et al., 2004). This variability makes unambiguous alignment of even closely related haplosclerid OTUs challenging, but also illustrates the high taxonomic resolution capacity of the 28S fragment.

These results indicate that the 28S marker may be more suitable for discriminating OTUs than the standard, DNA-barcoding CO1 fragment. The presence of multiple copies of ribosomal genes in metazoan genomes facilitates amplification in case of older and more degraded material, but in turn requires sequence checks for intragenomic polymorphisms (Wörheide et al., 2004). The combination of amplification ease and resolution power makes 28S a suitable marker to consider for sponge molecular taxonomy.

Implications for distribution of Red Sea sponge taxa

The relative paucity of morphological characters used in sponge taxonomy results in the underestimation of taxonomic diversity and overestimation of the distributional range of taxa (see Palumbi et al., 1997; Klautau et al., 1999; Solé-Cava and Boury-Esnault, 1999; Bierne et al., 2003). In turn, the application of molecular tools led to the discovery of numerous cryptic species, species complexes and high degrees of endemism in sponges (e.g., Wörheide et al., 2008a; Pöppe et al., 2010; Reveillaud et al., 2010; Xavier et al., 2010; Reveillaud et al., 2011; Swierts et al., 2013).

Our analysis corroborates previous studies that show genetic differentiation to be prevalent across the Indo-Pacific in widespread sponge morphospecies. For example *Astrosclera willeyana* (OTU37) from the Red Sea are divergent in 28S from samples from the South China Sea (JQ362353, 0.7%) and Tonga (KC869525, 1.5%), supporting earlier findings of geographic

differentiation in *Astrosclera* (Wörheide et al., 2002; Wörheide, 2006). Similarly, while northern Red Sea and Omani samples of *Pseudoceratina arabica* (Keller, 1889), described from the Red Sea, are identical in 28S, they differ from Palauan samples (KC869514) by 2.5%. Conspecifity of these samples with awaits a through test.

The taxonomic status and biogeographic relationships for several other sponge taxa are insufficiently resolved. The Clionaida OTU#03 and OTU#05 differ by 6.5% in 28S. Each OTU forms a clade with different *Spheciospongia vagabunda* specimens from Indonesia published in NCBI Genbank (as "*Spirastrella" vagabunda*; AM293640: 0.6% in 28S different from OTU#03; AM293641: 1.8% different from OTU#05). This suggests the presence of sympatric species in *S. vagabunda*. The current concept of *S. vagabunda* encompasses several morphological forms and named varieties and synonyms (see Van Soest, 2015), suggesting the need for a revision.

Furthermore, *Stylissa carteri* (OTU#31) is among the most conspicuous sponge species in the Red Sea. We obtained sequences from 34 samples making it the most frequently collected and amplified species in this study. *Stylissa carteri* was originally described from the Gulf of Manaar (Dendy, 1889) and recently was the subject of a comprehensive study of population genetic structure in the Red Sea (Giles et al., 2015). Microsatellite data indicated a barrier for gene flow around the Farasan Islands separating the southern from the central and northern Red Sea *S. carteri* populations. Such a barrier could not be explained with the regional currents alone and provided the first evidence for a latitudinal environmental gradient influencing sponge population genetic structure (Giles et al., 2015). The more conservative 28S and CO1 data from *S. carteri* samples of all regions of the Saudi Arabian Red Sea coastline display no variation and are also identical to samples from the Great Barrier Reef (EU146398), and Fiji (AY561925, identified as *Axinella* sp.), implying a wide distribution of this species.

Several other OTUs (e.g., OTUs #16, #30, #48) comprise specimens of different Gulfs and Seas (Red Sea, Gulf of Oman, Gulf of Aden or the Arabian Sea). Nevertheless, sampling remains insufficient to definitively evaluate differences in molecular diversity of sponges throughout all of Arabia. There is clearly a

need for more comprehensive sampling, especially from the Gulfs of Oman and Aden, the Arabian Gulf and the Arabian Sea.

Considerations for molecular taxonomic approaches on sponges

We here explore the molecular diversity of collections of sponges from the Red Sea and some adjacent areas using a ribosomal and a mitochondrial DNA marker (a fragment of the 28S rDNA and CO1 gene, respectively). Such molecular biodiversity surveys provide a suitable framework for subsequent in-depth taxonomic studies, and are especially useful for the phenotypically character-poor and plastic demosponges.

Molecular tools are clearly most suitable to assess phylogenetic relationships in character-poor taxa like sponges at different levels, ranging from within species to phyla. However, a molecular species concept among demosponges is lacking because a distinct barcoding gap (Meyer and Paulay, 2005) and a genetic distance threshold to clearly distinguish species has not been defined and likely does not exist. For example, the genetic differences among freshwater sponges (Spongillina) or taxa of the three dictyoceratid families Spongiidae, Thorectidae and Irciniidae are extremely low (see trees in the Supplementary data 2 and 3), independent of the type of marker considered. On the other hand, some closely related species, such as the *Tethya* species, show remarkably large genetic differences despite otherwise accepted phylogenetic similarity. This suggests that barcoding gap or threshold-based species concepts will not work well in sponges.

As is the case with many other phyla, the nomenclature of sponge species is still defined on a variety of concepts based primarily on morphology that assume the consistency of traits or (in comparably few cases) geographic or ecological factors as indicators for reproductive isolation (see e.g., Setiawan et al., 2015). More work clearly is needed to unify evidence derived from molecular and morphological data to distinguish species.

Molecular characterization of type material is especially useful, as it allows a point-in-time resolution of the nomenclature in challenging species complexes. Despite the taxonomic importance of types as the only reference point for a name, type material has hardly been investigated in molecular studies

of sponges, although benefit and feasibility are demonstrated (see Erpenbeck et al., 2015). For taxonomy, providing a reference molecular sequence for new species, especially when recently collected material is used, would be most beneficial. However, this approach is most useful if more material than the single holotype is available from the same population to assess intra-species molecular diversity, emphasizing the value of specimen collections (Rocha et al., 2014). Including type material into molecular phylogenetic studies facilitates the rapid and unambiguous reclassification of taxa when para- and polyphylies are detected, and therefore advances the capacity of rapid species identification and biodiversity assessments with molecular means. Likewise, as long as no unifying species concept for sponges has been established (see e.g., Setiawan et al., 2015), rapid molecular screenings largely have to rely on the correctness of published reference sequences, which underlines the importance of carefully curated (by holotypes or otherwise morphologically verified specimens) molecular databases (Wörheide and Erpenbeck, 2007).

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Legends:

Figure 1: Collection sites (triangles) of samples used in this study (Map created with Simplemappr Shorthouse, 2010).

Figure 2: Representatives on selected OTUs of Red Sea specimens. OTU#01: GW3400, OTU#02: GW6038, OTU#05: GW3131, OTU#06: GW5951, OTU#07: GW5857, OTU08: GW5992, OTU#09: GW5798, OTU#13: GW5892, OTU#14: GW3257, OTU#16: GW3490, OTU#17: GW3123, OTU#18: GW3094, OTU#20: GW3040, OTU#24: GW3279, OTU#25: GW3251, OTU#26: GW3073, OTU#28: GW5789, OTU#29: GW5881, OTU30: GW3369, OTU#31: GW3076, OTU#32: GW3463, OTU#33: GW3365, OTU#37: GW3193, OTU#39: GW3051, OTU#40: GW6123, OTU#41: GW3517, OTU#42: GW6171, OTU#43: GW3373

Supplementary data 1:

List of species examined in this analysis, and collecting details. The specimen numbers are referring to the Bavarian Natural History Collections - Bavarian State Collection for Palaeontology and Geology (SNSB-BSPG.GW#####). Operational taxonomic units (OTUs) for the new sequences are numbered in this approach if at least two specimens with identical genotypes for the 28S *and* CO1 fragment if available. SBD numbers refer to accession numbers of the Sponge Barcoding Project (www.spongebarcoding.org).

Supplementary data 2:

Maximum likelihood reconstruction based on CO1 sequences. Specimens indicated in blue are newly sequenced for the current analysis, terminal taxa in black were previously published in NCBI Genbank with the species names as published followed by accession numbers. Deviation from the Genbank numbering scheme (XX######) indicate merged sequences of the same species (see Erpenbeck et al. 2008 for details). Blue arrows highlight different sequence types, their white numbers depict the OTU numbers. Numbers on branches indicate bootstrap support of 70% and higher. Scale bar indicates substitutions per site. Note: The sequence of *Haliclona toxia* (OTU#19) differs by 1 bp in CO1

from a published *Haliclona toxia* from Oman (JN242206). This transversion in the first codon position would be the only substitution among the almost 1,500 demosponge sequences at this position and would result in a missense mutation from the negatively charged glutamic acid to the polar uncharged glutamine. We therefore assume a sequencing error in the latter. Likewise, the sequences of *Callyspongia siphonella* (OTU#17) differ in the sequenced CO1 fragment by a silent mutation from a Red Sea conspecific published in Genbank (JX999082). As the substitution in the Genbank sample is part of a G-pentamer (a region infamous for basecalling difficulties), a double check of this sequence is suggested to confirm that *C. siphonella* only possesses a single CO1 sequence type in the Red Sea.

Supplementary data 3:

Maximum likelihood reconstruction based on 28S sequences. Specimens indicated in blue are newly sequenced for the current analysis; terminal taxa in black were previously published in NCBI Genbank with the species names as published followed by accession numbers. Deviation from the Genbank numbering scheme (XX######) indicate merged sequences of the same species (see Erpenbeck et al. 2008 for details). Blue arrows highlight different sequence types, their white numbers depict the OTU numbers. Numbers on branches indicate bootstrap support of 70% and higher. Scale bar indicates substitutions per site.