Evolution and Ecology of Antarctic Sponges

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Summary

Sponges are abundant and species-rich in Antarctic waters, and play important roles in the benthic ecosystems of the continent. The taxonomy of Antarctic sponges is, to some extent, well established, yet the phylogenetic relationships of this fauna remain unknown. Here, the first contributions to the knowledge of the evolution of Antarctic sponges are presented. A molecular phylogeny for the common Antarctic shelf glass sponge genus *Rossella* is provided. Based on nuclear and mitochondrial markers, it is shown that many of the species described for the genus, which usually are morphologically poorly defined and difficult to differentiate, likely, represent a single species (*Rossella racovitzae*) or a species complex.

The deep Southern Ocean has yielded numerous, most likely new, species of the demosponge Familiy Cladorhizidae. Cladohizidae groups sponges largely known by their carnivorous habit, which is usually accompanied by the lack, or strong modification, of the sponge aquiferous system and by a distinct stipitate body shape. Cladorhizids are also important for the broader understanding of the phylogenetic relationships of the Order Poecilosclerida. In this study, the debated phylogenetic position of the genus *Abyssocladia* is clarified using recent collections of cladorhizid sponges and a (remarkable) new species of the genus *Phelloderma* (Phellodermidae) from the Southern Ocean, and partial sequences

of the (nuclear) 28S rDNA and of the (mitochondrial) COI. The results show that *Abyssocladia* is a cladorhizid sponge and that Cladorhizidae is monophyletic, the consequences of these results for the prevalent interpretation of the evolution of poecilosclerid sponges are also discussed.

The diversity of Antarctic sponges occurring on the shelf has been compared to that of tropical and subtropical ecosystems. Less is known about the sponge communities inhabiting the deeper waters of the Southern Ocean. The lack of information on deep benthic habitats, mainly due to the fact that sampling remote ecosystem such as Antarctica or the deep sea is expensive and technically difficult, hampers the determination of the number of species that inhabit the vast area of the deep Southern Ocean. In this work, estimation methods are used to predict lower bounds for the number of deep sea sponge species occurring in the Weddell Sea, Western Antarctica, and to show that the deep sea can be as rich as the shelf and that the total number of sponge species in Antarctica could be more than previously considered.

Finally, a middle throughput DNA barcoding workflow for processing sponges was established and the performance of this analytical pipeline was analyzed based on a large collection (~8300 specimens) of sponges from Australia available for DNA barcoding. The barcoding workflow was also used to provide a comprehensive DNA-barcode database for the Ross Sea comprising ~50 species

of demosponges and covering ~80% of the sponge species characteristic for this area. The generated barcode database was used to provide evidence for a long history of *in situ* evolution in Antarctic sponges, which is congruent with previous biogeographic hypotheses suggesting an ancient origin for Antarctic sponges.

Contents

Introduction

Porifera, and poriferan systematics

Phylum Porifera comprises a group of non-bilaterian, filter-feeding, sessile metazoans roughly characterized by the presence of a single layer of flagellated cells (choanocytes) that pump water through the sponge body, and a skeleton composed of calcitic or siliceous spicules (Hooper et al., 2002). In terms of diversity, ~8,300 valid sponge species have been described from aquatic ecosystems distributed around the world, and ranging from rivers and lakes to coral reefs and abyssal plains (van Soest et al., 2011). Sponges are important ecosystem members with diverse functional roles as bioeroders, substrate stabilizers, settlement substrate for other sessile organisms, and refuge providers for a number of macro-invertebrate species (Bell, 2008). As filter-feeders, mainly specialized in ultra- and pico-plankton predation but also capable of dissolved organic matter uptake, sponges contribute to carbon cycling in shallow and deep water environments (Bell, 2008). Additional contributions to the bentho-pelagic coupling include the sponges' participation in silicon (mainly as silicon sinks; Gatti, 2002) and nitrogen (usually through bacterial-mediated nitrification/denitrification processes; Schläppy et al., 2010) cycling.

Porifera is regarded as monophlyletic and is currently subdivided into four Classes, namely Calcarea, Demospongiae, Hexactinellida, and Homoscleromor-

pha (Hooper et al., 2011; Philippe et al., 2009). Molecular phylogenetic studies have corroborated to a good extent the morphology-based classification of Hexactinellida (Dohrmann et al., 2008) and Homoscleromorpha (Gazave et al., 2010). In contrast, the lower-level systematics of Demospongiae and Calcarea remains unsettled (Dohrmann et al., 2006; Erpenbeck and Wörheide, 2007). Within these classes most nominal orders, and in some instances families and genera, have been shown to be non-monophyletic, artificial entities by molecular systematic studies (McCormack et al., 2002; Erpenbeck et al., 2006; Erpenbeck and Wörheide, 2007; Cardenas et al., 2011; Morrow et al., 2011). To some extent, the striking discrepancy observed between the morphological classification of demosponges and calcareous sponges and molecular phylogenetic hypotheses of both groups is not surprising considering the general dearth of informative characters available in sponges for systematic research, the potentially subjective interpretation of the available characters, and the strong homology assumptions necessary to build upon a reduced character set the classification of Porifera.

Molecular phylogenetics has provided sponge systematics with a powerful tool for the study of the evolutionary history of Phylum Porifera. The rapid access to informative character sets capable of resolving different depths of the tree of life has also revolutionized sponge systematics. More recently, the advent of DNA-barcoding —the use of standard DNA markers for species identification—

has allowed the integration of molecular markers in (alpha) taxonomic and ecological research (Wörheide and Erpenbeck, 2007; Wörheide et al., 2008). Thanks to these innovations, a more robust classification system for sponges is currently being developed and sponge taxonomy is evolving into a space of collaborative research involving classical morphologists and molecular systematicians.

Antarctica and Antarctic sponges

Antarctica, the southernmost continent in the planet, harbors diverse communities of benthic invertebrates often dominated by sponges (McClintock et al., 2005; Gutt, 2007). These animals can occur in high abundances and can reach remarkable biomasses accounting for up to 95% of the benthic biomass at some shelf stations (Barthel, 1992a, b). Poriferans provide structure to the sea-floor positively influencing the diversity and composition of Antarctic benthic communities and, likely, facilitating recruitment of other sessile organisms (Barthel, 1992b; Gutt and Schickan, 1998). Additionally, it has been shown that sponges serve as refuge for potentially high numbers of marine organisms including juvenile stages of many fish and diverse invertebrate taxa (Kunzmann, 1996; Barthel, 1997). Antarctica is also well known for its glass sponges, mainly of the genera *Rossella* and *Anoxycalyx*, which can reach large sizes and play key roles in the functioning of the shelf and slope benthic ecosystems. Interestingly, the diversity of Antarctic sponge associations is comparable to that found in

tropical and temperate realms. In this regard, the Antarctic shelf alone is known to harbor ~350 demosponge species, many of them endemic to the continent's waters (McClintock et al., 2005). Mainly due to the high species-level endemism, Antarctic sponges have been proposed to represent a faunistic component distinct from that of neighboring marine provinces (Sara et al., 1992). Another striking characteristic of Antarctic sponges is the alleged circumpolar and, in some cases, eurybathic distribution of many species (Sara et al., 1992; Janussen and Tendal, 2007).

Among the causes of Antarctica's faunistic distinctiveness, the continent's long geological history and relatively high isolation seem to occupy a preponderant explanatory role (Sara et al., 1992; Linse et al., 2006). Antarctica separated from Gondwana ~180 million years ago (MYA) and became progressively isolated as neighboring land masses (e.g. Australia, South America) moved away during the Mesozoic and Cenozoic (Brandt et al., 2007a). During the early Oligocene, ~30 MYA, Antarctica was already effectively isolated. By then, the opening of the Drake Passage to deep water circulation triggered the onsetting of the Antarctic Circumpolar Current (ACC), a wind driven current that flows West to East around Antarctica (Lawver and Gahagan, 2003). In parallel, the continent's continued migration to the South affected Antarctica's climate making it generally colder and further contributing to its relatively high isolation

(Lawver and Gahagan, 2003). Indeed, northward flowing cold water masses generated in Antarctica form the Antarctic Convergence when they mix with warmer subtropical waters and provide a natural, hydrographic boundary that delimits the continent (Janussen and Tendal, 2007). Additionally, the ACC flow prevents warm waters generated near the equator from reaching Antarctica's shores contributing to preserve the continent's ice sheets (Lawver and Gahagan, 2003).

It is interesting to note that most of the knowledge pertaining Antarctic benthic assemblages, sponges inclusive, comes from the study of the continent's shelf and slope communities. In steep contrast, the deep-sea is still largely under-represented in terms of collections and knowledge derived thereof (Brandt et al., 2007a; Brandt et al., 2007b; Brandt and Ebbe, 2009). For instance, the species richness of the deeper waters layers remains largely speculative. It is also remarkable that despite the relatively rich body of information concerning the taxonomy of Antarctic sponges, most of the species reported for the continent's waters have not been included in any phylogenetic study, molecular or morphological. A recent review on the status of the Census of Antarctic Marine Life (CAML) concluded that only about 2% of all sponge species reported for Antarctica had sequence information deposited in any of the international DNA sequence repositories (Grant and Linse, 2009; Grant et al., 2011). The general

lack of molecular information on Antarctic sponges, and the need for having a robust source of independent information that can be used to test many hypotheses regarding the evolution of this important group of Antarctic organisms largely motivated the development of the present work, which main objective is to further contribute to the understanding of the evolution of Antarctic sponges through the study of the molecular systematics of selected sponge groups. I also aimed to provide an estimation of the number of sponge species found in the deep waters of the Weddell Sea that can be used as a guide for future studies. Finally, a workflow for sponge barcoding was developed and used to establish a comprehensive DNA barcode database for Antarctic shelf sponges. The resulting database was used to test hypotheses concerning the causes of their diversity and to assess the potential of DNA-barcoding for the identification of Antarctic sponges.

Rossella, complex taxonomic history of a common Antarctic glass sponge

Sponges of the genus *Rossella* are, perhaps, among the most common and abundant poriferan species collected in Antarctica (Barthel, 1992a). Their ecological significance has been the focus of a number of scientific contributions, and their importance as key-stone species in Antarctic benthic communities has been established and repeatedly acknowledged by several authors (Barthel, 1992b;

Kunzmann, 1996; Barthel, 1997; Janussen et al., 2004; Gutt, 2007; Janussen and Reiswig, 2009). Despite this, the taxonomy and systematics of *Rossella* remains largely unsettled due to the ever changing number of species recognized for the genus. The World Porifera Database (WPD; van Soest et al. 2011), an authoritative online resource on the taxonomy and nomenclature of Phylum Porifera, lists ~20 valid *Rossella* species. Yet, different authors in the last century have considered this number to vary between 2 and 7, reducing most of the ~20 names listed in the WPD to synonyms of other species within the genus (Burton 1925; Koltun 1976; Barthel and Tendall 1994). The somewhat extreme synonymization rate observed in *Rossella* is, most certainly, the product of a shift away from the extremely typological taxonomic system used by nineteenth century naturalists to include in the species descriptions, for instance, notions of intraspecific variability in diagnostic features. The resulting classification schemes available for *Rossella* remain largely authoritative and in need of additional test, preferably based on independent character sets not used by previous authors to erect the species under consideration. In Chapter 1, one such a test is provided using mitochondrial and nuclear molecular markers to independently assess the infra-generic classification of the genus *Rossella*. The question on the number of *Rossella* species is addressed by noting that species, better said specimens assigned to a given species, should form monophyletic units in a phylogeny.

This application of the phylogenetic species concept allowed to formulate clear predictions about the shape of the phylogenetic tree of the genus *Rossella*, and to formally test these predictions.

On the phylogeny of carnivorous sponges —a word from the South

Carnivorous sponges are a diverse assemblage of deep-sea species currently classified, on grounds of their chelae —microscleres with a central shaft and terminal alae, exclusively present in poecilosclerid sponges—, in at least three families of Order Poecilosclerida (Vacelet, 2007). Among these families, Cladorhizidae is of special importance due to the high diversity of chelae shapes and types found in the family (Hajdu and Vacelet, 2002). Cladorhizid sponges represents a challenge for the classification of Poecilosclerida, heavily influenced by the assumption that chelae microscleres can be accurately used as predictors of the phylogenetic relationships among this sponge group (Hajdu et al., 1994; Hajdu and van Soest, 1996; van Soest, 2002). Within this chelae-based framework, Cladorhizidae share no clear synapomorphy and has been proposed to be polyphyletic (Hajdu and Vacelet, 2002). Yet, the classification of Poecilosclerida could be wrong (Vacelet, 2007). In Chapter 2, the first reconstruction of the phylogeny of the Family Cladorhizidae is presented, and the position of the genus *Abyssocladia* is used as an *experimentum crucis* for poecilosclerid classifi-

cation. *Abyssocladia* has been included in two different families, Cladorhizidae and Phellodermidae, in the suborders Mycalina and Myxillina, respectively, and the debate concerning its phylogenetic position represents a schism among sponge taxonomists regarding the relative weight chelae microscleres should have in the classification of Poecilosclerida (van Soest and Hajdu, 2002; Vacelet, 2006). We aim to provide an independent test for the different taxonomic positions of *Abyssocladia* proposed in the last 10 years using several new species of Cladorhizidae and one new species of the, so far, monotypic genus *Phelloderma* (Phellodermidae) collected in the Southern Ocean. This study represents a somewhat fortunate case-in-point in which specimens collected in Antarctic waters serve the purpose of clarifying the phylogeny of *Abyssocladia*, *Phelloderma*, Cladorhizidae, and of Poecilosclerida as a whole.

Grasping the unknown, sponge species richness prediction in the Deep Weddell Sea

The number of species inhabiting Antarctic deep waters will, most likely, remain unknown for the years to come. The abyssal plains that surrounds the continent are simply to vast to be thoroughly sampled (Brandt et al., 2007a), and the apparent high levels of patchiness shown by sponge assemblages make the entire enterprise even more difficult (Gutt and Starmans, 2003). Despite the inherent problems of deep-sea sampling, new collections mainly from expeditions to the

Weddell Sea during the last 10 years can provide a useful source of information that can be used to estimate the number of species that inhabits deep Antarctic waters (Colwell and Coddington, 1994; Gotelli and Colwell, 2001). In chapter 3, we use the most complete dataset available to date on Antarctic deep sea sponges and resort to simulation to provide lower-bounds for the number of species expected to be found in the deep Weddell Sea.

Sponge barcoding and community barcoding the Antarctic shelf sponges

The Antarctic benthos harbors rich sponge assemblages which diversity is thought to be comparable to that of tropical and subtropical ecosystems (McClintock et al., 2005). Interestingly, or better said worryingly, only a handful of Antarctic sponge species have sequence data publicly available (Grant and Linse, 2009). DNA-barcoding, the use of standard molecular markers as species identifiers (Hebert et al., 2003), can be a useful tool for the study of Antarctic benthic ecosystems allowing the rapid assessment of the diversity of a wide range of invertebrate phyla, inclusive sponges, commonly found in the Antarctic marine realm. DNA-barcodes can be used to study the phylogenetic diversity (*sensu* Lewis and Lewis, 2005) of a given ecosystem and to compare it with other ecosystems avoiding the nomeclatural noise that can be associated with groups of difficult taxonomy and systematics such as sponges. Finally, the

establishment of a barcode database for Antarctic sponges represents a first step towards thorough tests of different hypothesis, such as the circumpolar or eurybathic distribution of many species, put forward by previous authors. In Chapter 4, the results of the development of a laboratory workflow for barcoding sponges in general are summaryzed, and in Chapter 5 the first comprehensive DNA-barcoding campaign directed at Antarctic sponges is presented. The resulting dataset is used to provide insights into the diversity patterns of Antarctic sponges comparing the phylodiversity of the Antarctic shelf and other tropical and subtropical ecosystems, and to unravel patterns of molecular evolution among Antarctic sponges.

Part I

Evolution of Antarctic Sponges

Chapter 1

Nuclear and mitochondrial markers support two species of *Rossella* (Hexactinellida: Lyssacinosida, Rossellidae) in the Southern Ocean*

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Abstract

Hexactinellida (glass sponges) are abundant and important components of Antarctic benthic communities. However, the relationships and systematics in the common genus *Rossella* Carter, 1872 (Lyssacinosida: Rossellidae) are unclear and in need of revision. The species content of this genus has changed dramatically over the years depending on the criteria used by the taxonomic authority consulted. *Rossella* was formerly regarded as a putatively monophyletic group with bipolar distribution. However, molecular phylogenetic analyses have shown that *Rossella* is diphyletic, with *Rossella* sensu stricto restricted to the Southern Ocean, where it shows a circum-antarctic and subantarctic distribution. Herein, we provide a molecular phylogenetic analysis of the genus *Rossella* s.s., based on mitochondrial (16S rDNA and COI) and nuclear (28S rDNA) markers. We corroborate the monophyly of the Southern Ocean component of *Rossella* and provide evidence supporting the existence of only two *Rossella* species, namely *Rossella antarctica* Carter, 1872 and *Rossella racovitzae* Topsent, 1901, in accordance with a previous interpretation of the genus.

1.1 Introduction

Glass sponges (class Hexactinellida) are key components of Antarctic suspension feeder communities (Arnaud et al., 1998; Gutt, 2007). Antarctic hexactinellids, particularly the genus *Rossella*, can reach remarkable size, biomass and abundance (Barthel and Tendal, 1994; Janussen and Tendal, 2007; McClintock et al., 2005). At some localities, *Rossella* spp. dominate the seascape, increase the spatial heterogeneity of the seafloor (Fig. 1.1) (Gutt and Starmans, 1998; Janussen and Reiswig, 2009; Starmans et al., 1999), structure benthic communities (Barthel, 1992a,b), and locally play a major role in silicon cycling (Gatti, 2002). Large *Rossella* specimens can harbour a diverse community of invertebrates and juvenile stages of many other organisms, and serve as substratum for various taxa of other sessile invertebrates (epibionts) (Barthel, 1997; Gutt and Schickan, 1998; Kunzmann, 1996).

From a morphological perspective, *Rossella* is clearly defined by the presence of calycocomes (Fig. 1.1) among the microsclere component, the main diagnostic character of the genus (Tabachnick, 2002). Molecular phylogenetic analyses of the class Hexactinellida have shown, however, that *Rossella* as currently defined is diphyletic (Dohrmann et al., 2008, 2009). In these studies, the southern ocean (SO) *Rossella* species (hereafter *Rossella* sensu stricto) formed a clade unrelated to specimens collected off Ireland and attributed to *Rossella nodastrella*, the only North Atlantic species described for the genus (Van Soest et al., 2007).

In contrast to the relatively stable genus-level systematics, intra-generic relationships remain unclear and most *Rossella* s.s. species still require a revised and clear delineation. As a result, the number of species recognized for the genus has varied in the past, ranging from 2 to 21 species depending on the taxonomic authority (e.g. Barthel and Tendal, 1994; Koltun, 1976; see also van Soest, et al. 2011). The great variation of the number of recognized species is, to some extent, not surprising. With the sole exception of *Rossella antarctica*, all remaining species lack clear morphological apomorphies (cf. Barthel and Tendal, 1994), or their diagnostic characters are weak (e.g. external morphology, shape and size of dermal megascleres). In addition, the majority of characters used for species delimitation in the genus are continuous, making differences between species mainly "of grade" and subject to diverse interpretations. Calycocome sizes, for instance, tend to overlap between species, as does the size of other taxonomically important spicules (Barthel and Tendal, 1994). External body shape is variable even within species (Tabachnick, 2002). Finally, the lack of appropriate sam-

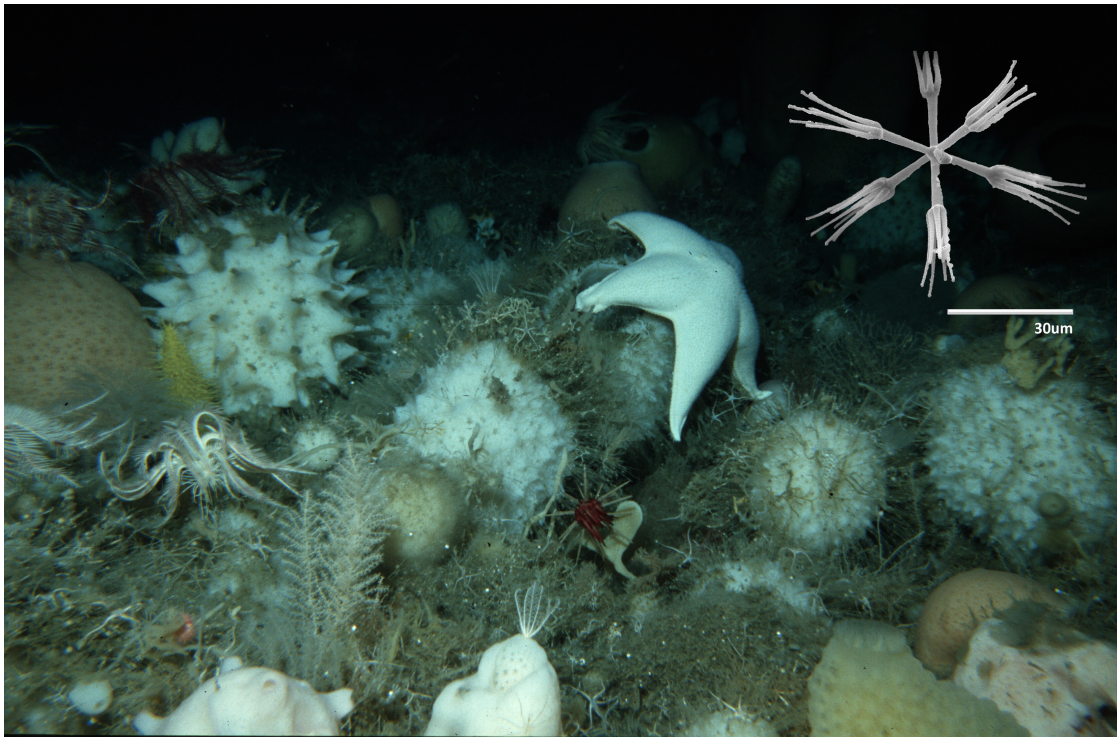

Figure 1.1: Seafloor dominated by *Rossella* spp. in the Weddell Sea, Antarctica (latitude: -70.8717, longitude: -10.5233; depth=254m); Photo: Gutt, J. and Starmans, A. (2004): Sea-bed photographs (benthos) along ROV profile PS56/127-1. doi:10.1594/PANGAEA.198695. Inset: calycocome of *Rossella antarctica*; Photo: C. Göcke.

pling has, to some extent, hampered the systematic evaluation of the variability of the main characters used for distinguishing different species.

Clarifying the systematics of *Rossella* is an important task of potential benefit to other areas of Antarctic research. *Rossella* species are structurally important in Antarctica (see above), and their distribution, as that of many other Antarctic sponges, is thought to be circum-antarctic (Janussen and Reiswig, 2009; Sara et al., 1992). The role that different *Rossella* species play in structuring Antarctic communities, as well as whether some or all species are, indeed, a circumpolar cohesive unit, strongly depends upon the clear

delineation of those species. Here, we provide a phylogenetic analysis of the genus *Rossella* based on mitochondrial (16S rDNA, COI) and nuclear (28S rDNA) markers and including 7 of the 8 species currently recognized as valid (*sensu* Barthel and Tendall, 1994). We aim to further corroborate the monophyly of *Rossella* s.s. (i.e., apart from '*Rossella nodastrella*') and to test different morphology-based taxonomic arrangements that have been proposed for the genus throughout its taxonomic history. Finally, we discuss future work necessary to reconcile the current morphology-based classification of *Rossella* s.s. with the molecular phylogeny and to further clarify the evolution of this important SO taxon.

1.2 Materials and Methods

Specimens (see Suppl. Materials for details) were collected by trawling during the German ANDEEP (2006/2007) and ANT XXIV/2-SYSTCO Expedition (2007/2008) to the Weddell Sea (West Antarctica), photographed and fixed in 96% ethanol. Sponges were determined to species level using standard procedures (e.g. Janussen et al., 2004) and pertinent literature. We adopted the *Rossella*-concept of Barthel and Tendal (1994) as an operational taxonomy for the identification of Antarctic specimens. Barthel and Tendal (1994) already considered the synonymy of the ~19 *Rossella* species listed in the World Porifera Database (van Soest, et al. 2011) reducing the number of Antarctic *Rossella* species to only 7, namely *R. antarctica*, *Rossella fibulata* Schulze & Kirkpatrick, 1910, *Rossella levis* (Kirkpatrick, 1907), *Rossella nuda* Topsent, 1901, *Rossella racovitzae*, *Rossella vanhoeffeni* Schulze & Kirkpatrick, 1910 and *Rossella villosa* Burton, 1929. Two

of the specimens analysed were labeled as *conferre* because diagnostic characteristics of more than one *Rossella* species were found to coexist in them. For instance, *R.* cf. *vanhoeffeni* showed calycocomes with S-shaped rays (characteristic of *R. vanhoeffeni*) and large middle pieces (as in *R. racovitzae*), and *R.* cf. *fibulata* had the species' characteristic heterodiactins in low abundances coexisting together with spicules typical of *R. racovitzae*. In these two cases, even though the assignment to *R. vanhoeffeni* or *R. fibulata*, respectively, appears pertinent we have preferred to act conservatively, assigning both specimens as *conferre*, in light of the observed character mixture that occur in them. Detailed descriptions of all specimens will be published elsewhere (Göcke and Janussen, pers. comm.).

DNA was extracted from small pieces of tissue with the NucleoSpin DNA tissue extraction kit (Macherey-Nagel) following the manufacturer's protocol. Three different molecular markers i.e. partial 28S rDNA (~1.2kb), partial 16S rDNA (~0.5kb), and the standard barcoding (Folmer et al. 1994) fragment of COI (~0.6kb) were amplified using 12.5 μl reaction volumes of GoTaq (Promega) supplemented with BSA. Three-step PCR protocols, including an initial denaturation step of 94°C 3min, 35 to 40 cycles of 94°C 30s, 50 – 40°C 30s, 72°C 60s, and a final extension of 5min at 72°C were used for all markers (see Table 1.1 for details on the annealing temperature for each primer). For 28S rDNA and COI we designed *Rossella*-specific primers (Table 1.1) to avoid co-amplification of non-target organisms; 16S rDNA primers were as in Dohrmann et al. 2008. PCR products were cleaned by standard ammonium acetate-ethanol precipitation or ExoSap-IT (Affymetrix) enzymatic PCR clean-up and sequenced in both directions using the same

Table 1.1: Primers and annealing temperatures used for amplification of 28S rDNA and COI.

Name	**Sequence 5′->3′**	**Annealing Temperature**	**Reverse primer name and source**
Hexa28SInt4	CTCAGCTTTTCARGGGGTC	50°C	NL4F Nichols (2005)
RossellaCOI_F1	ATATCGGYACATTATACC	40°C	dgHCO2189 Folmer et al. (1994)

primers used for PCR and the BigDye Terminator 3.1 chemistry (Applied Biosystems). Sequencing reactions were precipitated with sodium acetate-ethanol and subsequently analyzed on an ABI 3700 Genetic Analyzer at the Sequencing Service of the Department of Biology, LMU München. Trace files were assembled in CodonCode Aligner (CodonCode Corporation); hexactinellid origin of all obtained sequences was verified using NCBI BLAST (Johnson et al., 2008). Sequences are deposited at EMBL under accession numbers HE80191 to HE80223.

1.2.1 Outgroup choice and sequence alignment

New sequences were manually aligned in SeaView 4 (Gouy et al., 2010) to published alignments (Dohrmann et al., 2011). However, we restricted the taxon set to representatives of the families Leucopsacidae and Rossellidae, as well as *Clathrochone clathroclada* (Lyssacinosida incertae sedis). Leucopsacidae and *C. clathroclada* have been shown to be successive sister groups to Rossellidae (Dohrmann et al. 2011), and were therefore used as outgroups. Alignments were concatenated into a supermatrix and ambiguously

alignable regions removed. The final alignment was ~1.2 kb long and is available at OpenData LMU (http://dx.doi.org/10.5282/ubm/data.42).

1.2.2 Phylogenetic analysis

Using the concatenated alignment, we inferred both Maximum likelihood (ML) and Bayesian phylogenetic hypotheses with RAxML 7.2.8 (Stamatakis, 2006) and PHASE 2.0 (http://www.bioinf.manchester.ac.uk/resources/phase/), respectively. The GTR model of nucleotide substitution (Tavaré, 1986) was used for 16S rRNA, COI as well as for 28S rRNA single-stranded regions (loops). Among-site rate variation was modelled using a discrete approximation of a gamma distribution with 4 categories (+G; Yang, 1994, 1996). For the stem regions (paired sites) of the 28S rRNA we used the S16 and S7A models of sequence evolution (Savill et al., 2001) for the ML analysis. We searched for the ML tree using 20 independent tree-search replicates and assessed branch support with 1000 bootstrap pseudo-replicates (Felsenstein, 1985), using the "rapid bootstrap" algorithm described by Stamatakis et al. (2008). In the Bayesian analysis, two independent Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations after a burn-in of 250,000 generations, sampling every 100 generations. Model specifications for the Bayesian analysis were the same as for the ML analysis (i.e. GTR+G for 16SrDNA and COI); however we only used the S7A model for the 28S rRNA stem regions because is was difficult to achieve chain convergence using the S16 model. Branches were considered well supported if the posterior probability was >0.95 in the Bayesian analysis and the bootstrap frequency was >70 (Hillis and Bull, 1993).

1.2.3 Partition addition bootstrap and alternative lineage attachment analysis

To assess the influence of the individual partitions or combinations thereof on the ML topology inferred from the concatenated data matrix (i.e. the total evidence ML tree), we performed ML bootstrap analyses (1000 pseudoreplicates) for each individual marker and for all combinations of two markers using RAxML 7.2.8. For all these analyses, we used the same model settings as in the total evidence analysis for the corresponding partition (e.g. GTR+G for 16S rDNA and S16 for 28S rDNA stems). After each analysis, we determined the partition-specific bootstrap support (*sensu* Struck et al., 2006) for the branches present in the total evidence tree using consensus from the phyutility package (Smith and Dunn, 2008).

We also assessed alternative branching positions of different *Rossella* species using linmove from the phyutility package. In brief, linmove screens a set of phylogenetic trees and reports the frequency with which alternative placements of a branch occur in that set. The analysis facilitates the visualization of alternative branching positions of a lineage showing low bootstrap support values, which allows to determine whether poorly supported branches have only a few attachment points occurring with high frequency or branch off at several multiple positions with low frequency.

1.2.4 Testing hypotheses of relationships within *Rossella*

Different taxonomic arrangements proposed for *Rossella* s.s. can be translated into specific phylogenetic hypotheses and evaluated with available statistical tests (e.g.,

Goldman et al., 2000; Huelsenbeck, 1997; Huelsenbeck and Crandall, 1997; Whelan et al., 2001). We evaluated the status of six taxonomically unstable *Rossella* species, namely *Rossella fibulata* Schulze & Kirkpatrick, 1910, *Rossella levis* (Kirkpatrick, 1907), *Rossella nuda* Topsent, 1901, *Rossella racovitzae* and *Rossella* cf. *vanhoeffeni* Schulze & Kirkpatrick, 1910. These species have been repeatedly lumped together and segregated again. Specifically, Koltun (1976) united them (together with *Rossella villosa* Burton, 1929, not included here) in a broadly defined *R. racovitzae* (hereafter *R. racovitzae* sensu lato), leaving only two valid *Rossella* species (i.e. *R. antarctica* and *R. racovitzae* s.l.). In contrast, the commonly accepted taxonomic arrangement proposed by Barthel and Tendal (1994) considers all the above listed species valid.

We used the parametric likelihood-ratio test of monophyly (hereafter pLRTm) proposed by Huelsenbeck et al., (1996) to test whether different phylogenetic hypotheses constraining one or all of the above 'species' (see Table 1.2 and Suppl. Materials for details) to be monophyletic were not significantly worse than the unconstrained ML tree. Because the pLRTm has been shown to be too liberal when the substitution model cannot be accurately specified (Buckley, 2002; Emerson et al., 2001; Huelsenbeck et al., 1996), we also used the approximately unbiased (AU) test of Shimodaira (2002) to corroborate the results obtained using the parametric bootstrap approach. We considered a phylogenetic hypothesis to be significantly worse from the ML tree when both the AU test and the pLRTm rejected their respective null hypothesis.

We used the AU test implemented in CONSEL (Shimodaira and Hasegawa, 2001) with site-wise log-likelihood values obtained from RAxML 7.2.8, to test six different

monophyly hypotheses against the ML phylogeny. For the pLRTm, different computer programs were used to simulate data matrices under the null hypothesis (i.e. monophyly of a given species) and analyze them enforcing or not enforcing a given monophyly constraint. For simulation, we first estimated a constrained ML tree with RAxML 7.2.8 and used it as a backbone to estimate model parameters and branch lengths with optimizer from the PHASE 2.0 package. This step was necessary to produce model and tree files in the format required by PHASE 2.0's simulate program. Using simulate, we simulated 1000 alignments for each monophyly constraint (see Suppl. Materials). Each simulated alignment had the same number of nucleotides per partition as the original (empirical) data set. For all simulations, we used the GTR+G model for 16S rDNA, COI, and 28S rRNA loops, and the S16 model for 28S rDNA stems; the model parameters specific to a given monophyly hypothesis were derived from the corresponding constrained ML tree. One constrained and one unconstrained ML tree were then estimated with RAxML 7.2.8 from each alignment simulated under the null hypothesis, and the difference between the log-likelihood values of the two trees was used to build the null distribution for the corresponding monophyly test (Huelsenbeck et al., 1996; Goldman et al., 2000). In all of the above tests, specimens determined to genus-level only (i.e. as *Rossella* sp.) were not used in the constraints.

1.3 Results

We recovered a phylogenetic tree congruent with published analyses of the class Hexactinellida (Dohrmann et al., 2008, 2009, 2011). Bayesian and ML analyses recovered

generally similar trees, but the Bayesian phylogeny did not include a clade of *R. fibulata*+*R. racovitzae*, which was present in the ML phylogeny with moderate support. Both independent MCMC runs of the Bayesian analysis converged to the same consensus topology. The ML topology was not sensitive to model choice for the 28S rDNA stem sites, as analyses using S7A and S16 resulted in the same phylogeny.

Our phylogenetic analyses (Fig. 1.2) recovered a well supported clade comprising all SO *Rossella* spp. (i.e. *Rossella* s.s.) which nested deeply within the family Rossellidae. *Rossella nodastrella*, the only '*Rossella*' species described from the northern hemisphere, was not included within *Rossella* s.s., but formed a clade together with *Aulossaccus* and *Acanthascus* as previously reported (Dohrmann et al., 2008, 2009). *Rossella antarctica* specimens formed a highly supported clade in both Bayesian and ML analyses. Specimens belonging to other morphologically defined *Rossella* s.s. species formed a large clade hereafter named *Rossella racovitzae* sensu lato. Within this clade, other morphologically defined *Rossella* s.s. species were not recovered as monophyletic, but were polyphyletic in both the ML and Bayesian tree. Support values within *R. racovitzae* s.l. were generally low (<50%), with only some branches showing moderate (50–70 %) bootstrap support in the ML analysis. In contrast, the Bayesian analysis assigned high posterior probabilities (PP >0.95) to most branches within *R. racovitzae* s.l.

1.3.1 Partition addition bootstrap analysis and lineage movement

Bootstrap values assigned to the branches of the total evidence ML tree varied between different partitions or combinations thereof (Fig. 1.2). In general, bootstrap support

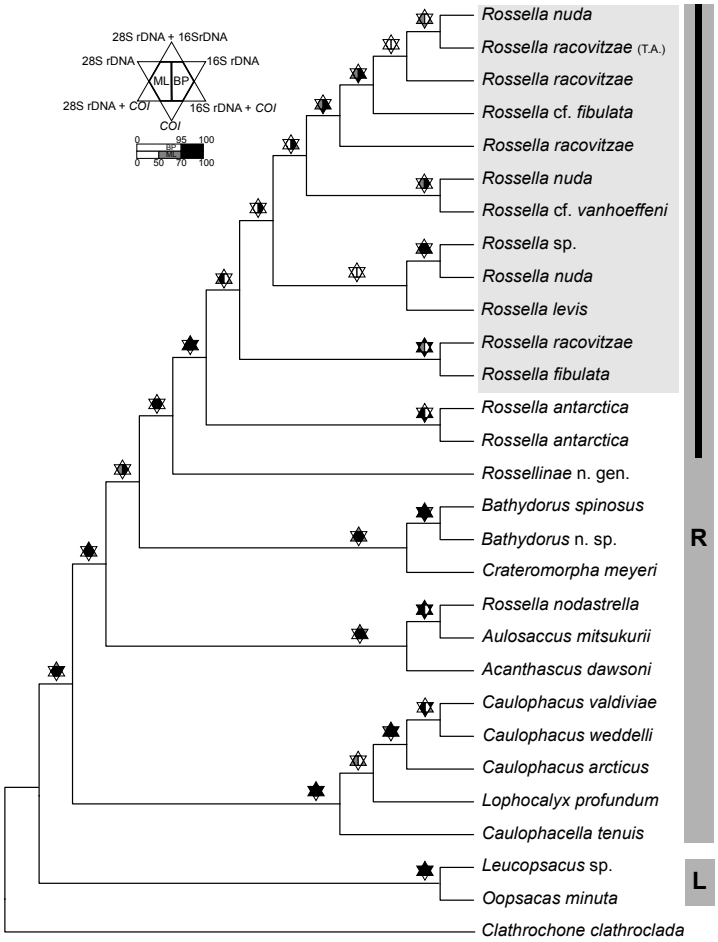

Figure 1.2: Phylogenetic relationships (cladogram) of Southern Ocean *Rossella*. The tree corresponds to the total evidence maximum likelihood topology. The vertices of the stars above the branches show the bootstrap value obtained for a given branch when using a single partition or a combination of partitions (see inset). The centers of the stars show, on the left, the bootstrap value of the maximum likelihood total evidence analysis, and on the right, the posterior probability obtained for the branch in the Bayesian analysis. Dark gray bars on the right annotate the family Rossellidae (R) and Leucopsacidae (L); Clathrochone is currently *incertae sedis* in Lyssacinosida. Within Rossellidae, *Rossella sensu stricto* is indicated with a black bar and *Rossella racovitzae sensu lato* highlighted in ligth gray. Information about other specimens included in the analysis can be found in Dohrmann et al. (2008, 2009). Both topologies, ML and Bayesian, as well as partition specific trees with branch-lengths and support values are provided in the Suppl. Materials.

increased when more data were added to the analysis. However, there was conflict between partitions in some specific cases. For instance, the *R. antarctica* clade was not supported by 16S rDNA sequences alone but received high bootstrap support from the COI partition. When the two markers were combined, bootstrap support was only moderate (50–70%) in contrast to the high support (>70%) assigned to this clade in the total evidence analysis. Within *R. racovitzae* s.l. support was low when single partitions or combinations of two partitions were used for the analysis, and was only moderate in the total evidence phylogeny. Lineage movement analysis revealed that morphospecies included in *R. racovitzae* s.l. were not monophyletic in any of the bootstrap pseudo-replicates, invariably forming clades with specimens belonging to different morphospecies.

1.3.2 Hypothesis testing

All five alternative (constrained) phylogenetic hypothesis explored in this study using the pLRTm where found to be significantly worse ($p < 0.001$) than the unconstrained ML tree (Table 1.2). The decay in the likelihood values of the constrained ML phylogenies was highly related to the number of constraints. Trees constrained to make single species monophyletic (i.e. *R. fibulata* or *R. nuda* or *R. racovitzae* s.s.) showed higher log likelihood values than trees constrained to make all species monophyletic (i.e. *R. fibulata* and *R. nuda* and *R. racovitzae* s.s.). The tree showing the lowest log likelihood value enforced the monophyly of *R. levis* + *R.* cf. *vanhoeffeni* in addition to all single species constraints. *R. levis* and *R.* cf. *vanhoeffeni* have a "great deal of overlap" in

morphological traits (cf. Barthel and Tendal, 1994), thus there is an expectation of close relationship between them.

The AU test also rejected all alternative hypotheses tested against the uncostrained ML tree, corroborating the results from the pLRTm. Moreover, the AU test results were insensitive to model selection, leading to identical conclusions when either the S16 or the S7A model was applied to 28S rDNA stems.

Table 1.2: Constrained phylogenetic hypotheses tested using the pLRTm and AU-tests. δ=2(best unconstrained log likelihood - best constrained log likelihood), where best constrained log likelihood refers to the log likelihood of the ML tree found under the constraint to be tested. δmin and δmax correspond to the minimum and maximum δ values obtained from the parametric bootstrap simulation.

Monophyly constraint	**Best log likelihood**	**pLRTm** $\delta(min\delta - max\delta)$	**AU-test**
No constraint	-6073.8647	N.A.	N.A
R. fibulata	-6094.4883	$p < 0.001$ 41.25 (-6.56–17.10)	$p = 0.003$
R. nuda	-6118.8902	$p < 0.001$ 90.05 (-5.13–18.89)	$p = 0.038$
R. racovitzae	-6089.0588	$p < 0.001$ 30.39 (-1.77–18.10)	$p = 0.001$
R. fibulata+*R. nuda* +*R.racovitzae*	-6146.8112	$p < 0.001$ 145.89 (-1.09–38.86)	$p = 0.001$
R. fibulata+*R. nuda* +*R. racovitzae*+*R. levis* +*R. vanhoeffeni*	-6153.1090	$p < 0.001$ 158.49 (-0.84–32.49)	$p = 0.001$

1.4 Discussion

The last 50 years of taxonomic history have seen *Rossella* s.s. expanding from two species, *R. antarctica* and *R. racovitzae* (*Rossella*-concept of Koltun, 1976), to eight species (*Rossella*-concept of Barthel and Tendal, 1994). All six species resurrected by Barthel and Tendal (1994) were included in a broad and 'highly polymorphic' *R. racovitzae* by Koltun (1976). Here, we have sequenced two mitochondrial markers and one nuclear marker in an attempt to clarify the systematics of *Rossella* using an independent set of characters not used by previous authors. Our results corroborated the existence of a monophyletic SO *Rossella* component unrelated to the N Atlantic *R. nodastrella* —which still requires generic reallocation—as reported previously (Dohrmann et al., 2008, 2009). The SO *Rossella* included two main clades corresponding to Koltun's *Rossella* species: a well supported *R. antarctica* clade was recovered as sister to a moderately supported group of specimens assigned to various nominal species and here referred to as *R. racovitzae* sensu lato. The taxonomy of the species included within *R. racovitzae* s.l. is not straightforward as many characters used for species delimitation inside this clade overlap or are prone to authoritative (subjective) interpretation (Table 1.3). In contrast, *R. antarctica* can be readily identified and can be clearly distinguished from all other *Rossella* s.s. species based on morphology. Congruent with the morphological evidence, the analysis of COI sequences revealed clear diagnostic characters for *R. antarctica* with respect to *R. racovitzae* s.l. (Fig. 1.3); in contrast, no diagnostic characters were found for all other *Rossella* species in the standard barcoding partition.

Table 1.3: Selected morphological characters used for the taxonomy of the SO *Rossella* after Barthel and Tendal (1994).

	Rossella						
Character	***antarctica***	***fibulata***	***levis***	***nuda***	***racovitzae***	***vanhoeffeni***	***villosa***
Max. height (cm)	30	80	30	75	20	30	30
Max. diameter (cm)	15	70	33	30	10	26	16
Conules 0=absent 1=present	1	0,1	1	0,1	1	0,1	0,1
Protruding surface spicules 0=diactine 1=pentactine	0,1	0	0	0	?(1)	0	0
Dermal spicules 0=pentactine 1=hexactine	0	0	0	0,1	0,1	0,1	?
Atrial spicules 0=pentactine 1=hexactine	1	0,1	0	1	1	1	?
Basal spicule tuft 0=absent 1=present	0,1	1	1	1	1	1	1
Calycocome diameter (μl)	70-100	164-350	130-230	>250	200-400	240-380	185-260
Calycocome primary ray length (μl)	12-15	16	8-12	15	?	14	?
Calycocome center piece size (μl)	2-4	10-25	6-12	25	?	<14	?

Figure 1.3: Maximum likelihood phylogram of Southern Ocean *Rossella* based on the total evidence data matrix. For the bootstrap values of the nodes see Figure 1. Highlighted are the two main *Rossella* s.s. clades obtained with their corresponding COI diagnostic characters: consensus sequence on top of the alignment, positions identical to the consensus represented with dots. Scale bar, expected number of substitutions per site.

The analysis of the alternative branching positions of specimens included within *R. racovitzae* s.l. revealed that specimens morphologically assigned to the same nominal species were not monophyletic in any bootstrap tree of the total evidence ML analysis. Despite the fact that the low bootstrap values inside *R. racovitzae* s.l. hindered a positive interpretation of the intra-clade structure in the total evidence ML tree, our analysis strongly supports the rejection of most *Rossella* s.s. species (*sensu* Barthel and Tendal, 1994). This interpretation is also favoured by the results of the pLRTm and AU tests of monophyly applied to phylogenetic hypotheses constrained to group different *Rossella* species.

It is important to note that the high posterior probability values assigned to the branches within *R. racovitzae* s.l. in the Bayesian phylogeny should be interpreted with caution in light of the generally low ML bootstrap support values. Bayesian posterior probabilities can be too liberal (e.g., Douady, 2003; Suzuki et al., 2002); thus, the high posterior probability values observed inside *R. racovitzae* s.l. could be an overestimation. On the other hand, both ML and Bayesian analyses assigned congruent support values to clades within *R. racovitzae* s.l., indicating that a certain degree of intra-clade structure can be detected. Whether certain morpho- or ecotypes can be associated to these clades, or whether the internal subdivision of *R. racovitzae* s.l. represents an instance of incipient speciation deserves to be further investigated using more variable markers.

In conjunction with the absence of clear apomorphic characters for most currently valid *Rossella* s.s. species, our results indicate that their inclusion into a broadly defined

R. racovitzae is justified. This entails that this 'species complex' is responsible for a bigger biomass and abundance apportionment among the Antarctic epibenthic megafauna than previously thought, making its ecological significance even larger than already acknowledged (Gutt and Starmans, 1998).

The broad morphological variation in both external and spicule morphology found in *R. racovitzae* s.l. is, to some extent, not surprising. High homoplasy levels in both macro- and micro-spiculation have been repeatedly demonstrated for several sponge taxa (Cardenas et al., 2011; Dohrmann et al., 2006; Erpenbeck et al., 2006). Whether specific morphological variations within *R. racovitzae* s.l. result from plasticity or are associated to specific genotypes needs to be further clarified using more variable markers and increased specimen sampling. According to morphological characters, *R. racovitzae* s.l. can be subdivided into several groups, which could be retained as subspecies to facilitate future studies of the causes of the observed morphological cohesiveness, and provide a finer resolution for ecological studies (see Lopes et al., 2011). The taxonomic rearrangement of *Rossella* awaits the revision of both type and recent material, and is out of the scope of the present contribution. Ongoing efforts to provide a formal revision of *Rossella* will be published elsewhere (Göcke and Janussen, pers. comm.), we hope that the results of our molecular analyses will serve as a guide for future efforts in this respect.

Finally, from a biogeographic perspective, the circum-Antarctic cohesiveness of both *R. antarctica* and *R. racovitzae* s.l. remains to be tested. In this study, the only specimen from east Antarctica (collected in Terra Adelie) included in the analysis was

deeply nested in a clade composed of specimens from the Weddell Sea. However, any conclusion about the biogeography of *Rossella* in the SO derived from our current dataset seems premature given the restricted geographic coverage of our sample.

1.5 Conclusion

We have obtained a phylogeny of Southern Ocean (SO) *Rossella* species corroborating their monophyly and showing the existence of two clades corresponding to *R. antarctica* and a broadly defined *R. racovitzae*, in accordance with Koltun's (1976) interpretation. Future taxonomic work will be required to reconcile the morphology-based classification of SO *Rossella* species with our molecular results. Furthermore, sampling and the use of highly variable molecular markers would allow the assessment of open questions, in particular the circumpolar distribution of SO Rossella and the causes of the high morphological diversity within *R. racovitzae* s.l.

Chapter 2

Chelae clash: molecular phylogeny of *Abyssocladia* (Cladorhizidae: Poecilosclerida) and *Phelloderma* (Phellodermidae: Poecilosclerida) suggests a diversification of chelae microscleres in cladorhizid sponges.*

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* This chapter will be submitted for stand-alone publication.

Abstract

The taxonomic placement of *Abyssocladia* Lévi, 1964 (Poecilosclerida) is controversial, having been assigned at various times to 3 different families (Mycalidae and Cladorhizidae, and Phellodermidae) in 2 different suborders (Mycalina and Myxillina, respectively), since its inception in 1964. It shares the lack of an aquiferous system and general body-plan with the carnivorous sponge family Cladorhizidae (Mycalina), yet also has chelae microscleres almost identical to those in *Phelloderma* Ridley and Dendy, 1886 (Phellodermidae, Myxillina). The ongoing debate on the position of *Abyssocladia* ultimately reduces to a discussion on the use of chelae microscleres as evolutionary proxies in Poecilosclerida. Here, we infer the phylogenetic relationships of the genera *Phelloderma* and *Abyssocladia* using two independent molecular markers (28S rDNA and COI), showing that *Abyssocladia* is not closely related to *Phelloderma* and belongs in Cladorhizidae. We suggest that despite their complexity, chelae can evolve independently in different poecilosclerid lineages and as such might be potentially misleading as indicator of the phylogenetic history of the group.

2.1 Introduction

Within the species-rich demosponge Order Poecilosclerida Topsent, 1928, members of the Family Cladorhizidae Dendy, 1922 are well-known for their carnivorous feeding (Vacelet and Boury-Esnault, 1995). Carnivorous sponges passively prey on small crustaceans and other micro-invertebrates, and have a body-plan that is atypical for sponges because it lacks an aquiferous system, or if present, have one which is highly modified and not used for filter-feeding. This remarkable trait, in association with its apparent high species richness (~116 spp in 7 genera in Cladorhizidae; including *Cercicladia australis* Rios, Kelly and Vacelet, in press), has made cladorhizid sponges the subject of special attention among sponge biologists (Riesgo et al., 2007; Vacelet and Duport, 2004), especially with regard to their taxonomy (Ise and Vacelet, 2010; Lehnert et al., 2006; Lehnert et al., 2005; Vacelet, 2008; Vacelet et al., 2009).

From a systematic perspective, Cladorhizidae is also a problematic taxon within Poecilosclerida as it lacks any "strong" synapomorphy. Its high diversity of chelae —microscleres with alae at each end of a central shaft (Fig. 2.1 Boury-Esnault and Rützler, 1997)— is at odds with the current classification of poecilosclerid sponges. This classification is largely influenced by the assumption that chelae are homologous in that group and can be used to reconstruct phylogenetic relationships due to their morphological complexity and selective neutrality (Hajdu et al., 1994; Hajdu and van Soest, 1996; Vacelet, 2007; van Soest, 2002). A number of likely evolutionary innovations that have been used to diagnose cladorhizids and could potentially represent "strong" synapomorphies (e.g. carnivory, the absence or major modification of the aquiferous system, a stipitate symmetrical body-shape, a special arrangement of megascleres and/or microscleres, and the presence of sigmancistra among this last spicule type), have been questioned because taxa in other poecilosclerid families (e.g. *Esperiopsis koltuni* Ereskovsky and Willenz, 2007 in Esperiopsidae Hentschel, 1923; species of *Eucheliplu*ma Topsent, 1909 in Guitarridae Dendy, 1924) also exhibit these characters rendering them either homoplasious or symplesiomorphic (Ereskovsky and Willenz, 2007; Hajdu and Vacelet, 2002). This interpretation, however, relies principally on the assumed "correctness" of the currently accepted taxonomic classification of the order Poecilosclerida, which has not been supported by independent evidence, e.g., in any molecular phylogeny published to date (Erpenbeck and Wörheide, 2007).

Vacelet (2007) first recognised the important position of cladorhizid sponges within poecilosclerid systematics; he suggested that the diversity of microscleres in carnivorous

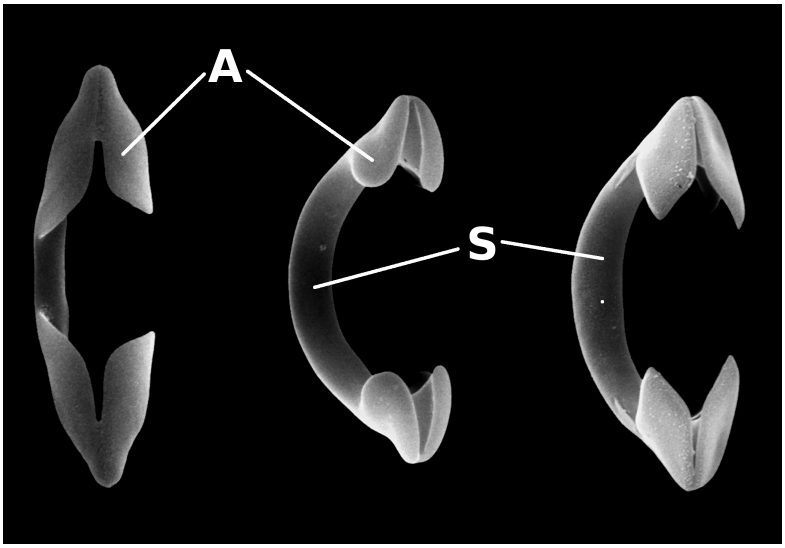

Figure 2.1: Chelae microscleres present in representatives of the order Poecilosclerida. From left to right: palmate chelae of *Clathria australiensis*, arcuate chelae of *Hamigera dendyi* and anchorate chelae of *Crella incrustans*. S=shaft, A=alae. Images not at scale. Electron microscope photos: J. N. A. Hooper.

sponges could have resulted from its convergent evolution. Cladorhizidae is the only poecilosclerid family that possesses all basic forms of chelae (palmate, anchorate, arcuate; Fig. 2.1). The diversity of chelae within the groups means that Cladorhizidae can fit the definition of several poecilosclerid families in the sub-orders Mycalina Hajdu, van Soest & Hooper, 1994 and Myxillina Hajdu, van Soest & Hooper, 1994 suggesting that the subordinal classification of Poecilosclerida requires closer scrutiny (as suggested by Erpenbeck and Wörheide, 2007).

Abyssocladia provides an exemplary case-in-point to test the "puzzling" problem cladorhizid microscleres represent within the current poecilosclerid classification. The position of *Abyssocladia* has been controversial, van Soest and Hajdu (2002) included *Abyssocladia* within the genus *Phelloderma* Ridley & Dendy, 1886 based on the "similar

and peculiar shape of their isochelae", changing thereby its sub-ordinal assignment from the Mycalina to the Myxillina. *Abyssocladia* was, however, relocated in Cladorhizidae by Vacelet (2006) who considered the shape of a single spicule insufficient to justify the (sub-ordinal) transfer. From an evolutionary perspective, testing the phylogenetic position of *Abyssocladia*, whether in Cladorhizidae (Mycalina) (with which it shares the lack of an aquiferous system, a stipitate symmetric body and a carnivorous habit), or in Phellodermidae (Myxillina) (as indicated by the shape of its arcuate chelae), is not a trivial matter. There are important implications for the validity of established or traditional morphological characters (e.g. chelae microscleres) long used for the classification of poecilosclerid sponges. If chelae are not good indicators of the evolutionary history of the order, any interpretations of its evolution based on this character will be inaccurate.

Here, we present a phylogenetic analysis of the genera *Abyssocladia* and *Phelloderma* and clarify their relative position within the order Poecilosclerida. We sequenced two independent molecular markers (i.e. 28S rDNA and COI) for all non-monotypic cladorhizid genera and a new species of *Phelloderma* collected in the Southern Ocean, and assess alternative hypotheses on the position of *Abyssocladia* advanced by researches using morphological observations. We provide initial insights into the evolution of chelae within Cladorhizidae and discuss implications for the systematics of Poecilosclerida.

2.2 Materials and Methods

We obtained genomic DNA from new species of the genus *Phelloderma* and of the family Cladorhizidae collected in Antarctica during the German ANT XXIV/2-SYSTCO

Expedition (2007/2008) and the New Zealand's BioRoss (2004) expedition, and also from one specimen of *Asbestopluma hypogea* Vacelet & Boury-Esnault, 1996 collected by Jean Vacelet off Marseille (Table 2.1), using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. After extraction, the D13-E13 domains of the 28S rDNA and the standard barcoding (Folmer) fragment of the mitochondrial cytochrome oxidase 1 gene regions were amplified using primers NL4F and NL4R (Nichols, 2005) and dgLCO1490 and dgHCO2198 (Meyer et al., 2005) respectively. PCR products were either sequenced directly after ExoSAP-IT (Affimetrix) clean-up or cloned using the TOPO TA Cloning kit (Invitrogen). Positive colonies (at least 8) were picked and boiled in 10μl HPLC grade water for 5 minutes to release the DNA; DNA was then amplified using T3/T7 primers. PCR products of the expected size were excised from a 1.5% agarose gel and sequenced in both directions using the T3/T7 primers and the BigDye Terminator version 3.1 chemistry (Applied Biosystems). Sequencing reactions were precipitated using standard ethanol-sodium acetate precipitation and analyzed on an ABI 3700 Genetic Analyzer at the Genomic Sequencing Unit of the Department of Biology, LMU München. The resulting chromatograms were visualized and assembled in CodonCode Aligner (Codon Code Corporation). Poriferan origin of all sequences was determined using NCBI BLAST (Johnson et al., 2008). Sequences are deposited at the EMBL (Table 2.1).

Table 2.1: New poecilosclerid specimens sampled for this study. Institution: NIWA, National Institute of Water and Atmospheric Research, Wellington, New Zealand; SMF, Senckenberg Museum Frankfurt, Frankfurt am Main, Germany; QM, Queensland Museum, Brisbane, Australia.

				Location		Accession number	
Suborder	Family	ID	Institutions/Voucher-No.	Latitude	Longitude	28S rDNA	COI
Mycalina	Cladorhizidae	*Abyssocladia* sp.	SMF/102-9v143	—	—	HE611627	HE611581
		Asbestopluma hypogea	—	—	—	HE611628	HE611582
		Asbestopluma obae	NIWA/28893	-71.7675	171.1092	—	HE611583
		Cladorhiza cf. *gelida*.	SMF/11753	79.1333	4.4833	HE611629	HE611584
		Cladorhiza sp.	SMF/11751	-62.0145	-2.9833	HE611630	HE611585
		Chondrocladia sp.	SMF/11752	-70.0669	-3.3267	HE611631	HE611586
	Desmacellidae	*Neofibularia hartmani*	QM/G306606	-22.2011	155.2353	—	HE611587
		Neofibularia irata	QM/G307266	-23.4669	151.9342	HE611632	HE611588
	Mycalidae	*Mycale mirabilis*	QM/G300561	-25.6167	113.3833	—	HE611589
			QM/G306269	-25.3336	153.0181	HE611633	HE611590
			QM/G307148	-23.4514	151.9019	—	HE611591
			QM/G305553	-21.7847	152.4511	—	HE611592
Microcionina	Microcionidae	*Clathria abietina*	QM/G300508	-11.4517	136.4686	—	HE611593
			QM/G306048	-19.4672	117.5675	HE611634	HE611594
			QM/G306154	-19.9333	118.2167	—	HE611595
		Clathria cervicornis	QM/G304846	40.3050	151.5000	—	HE611596
		Clathria cancellaria	QM/G311840	-28.4869	113.5639	—	HE611597
		Clathria reinwardti	QM/G306423	7.2842	134.5014	—	HE611598
		Clathria kylista	QM/G307278	-23.4669	151.9342	—	HE611599
			QM/G314045	-23.8939	252.4111	—	HE611600
			QM/G305498	-21.7847	152.4503	—	HE611601
		Clathria conectens	QM/G305135	-14.8192	145.5192	—	HE611602
			QM/G304980	-22.3347	152.7178	—	HE611603
		Clathria rugosa	QM/G300696	-22.3000	166.4167	HE611635	HE611604

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						Comes from the last page...
	Acarnidae	*Paracornulum dubium*	QM/G300541	-12.4000	130.8167	—	HE611605
		Paracornulum sp. 819	QM/G300607	-20.5667	149.0834	—	HE611606
	Rhabderemiidae	*Rhabderemia sorokinae*	QM/G3112904	-10.2183	148.1025	—	HE611607
Myxillina	Coelosphaeridae	*Lyssodendoryx* sp. 489	QM/G300820	-10.4167	141.7678	—	HE611608
	Crambeidae	*Monanchora* sp. 994	QM/G300289	-11.8000	149.2667	—	HE611609
		Monanchora sp. 0605	QM/G300289	-19.6853	118.1014	HE611636	HE611610
		Monanchora clathrata	QM/G313145	1.2189	103.8347	HE611637	HE611611
			QM/G301219	1.1667	103.7500	HE611638	HE611612
			QM/G304489	4.6667	1119.0167	HE611639	HE611613
			QM/G305838	9.6850	118.7347	HE611640	—
			QM/G301225	40.4269	116.7833	HE611641	—
	Crellidae	*Crella* sp. 4778	QM/G304373	-14.5836	145.3356	—	HE611614
		Crella incrustans	QM/G304056	-35.0834	137.7347	—	HE611615
		Crella spinulata	QM/G300194	-28.3000	113.8000	HE611642	—
	Hymedesmiidae	*Phorbas fictitioides*	QM/G305473	-21.9686	152.4675	—	HE611616
			QM/G307601	-23.4347	151.8850	HE611643	HE611617
			QM/G304247	-14.4675	145.4358	HE611644	HE611618
			QM/G305520	-21.7847	152.4503	—	HE611619
		Phorbas sp. 1539	QM/G304718	-10.2675	124.0342	HE611645	HE611620
	Iotrochotidae	*Iotrochota baculifera*	QM/G306386	7.2514	134.3181	—	HE611621
			QM/G313319	-15.3619	137.5550	—	HE611622
		Iotrochota coccinea	QM/G304254	-14.7006	145.4347	—	HE611623
			QM/G3044256	-14.7006	145.4347	—	HE611624
		Iotrochota acerata	QM/G300550	-28.7839	113.9681	—	HE611625
	Phellodermidae	*Phellloderma* sp.	SMF/11726	-64.48	2.8753	HE611646	HE611626

2.2.1 Phylogenetic analyses

New sequences were manually aligned to existing demosponge (structurally annotated) 28S rDNA and (Folmer) COI (for details see `http://www.spongegenetrees.org/` Erpenbeck et al., 2007; Erpenbeck et al., 2004; Erpenbeck et al., 2008) data matrices. In addition, both datasets were supplemented with poecilosclerid sequences generated for the Sponge Barcoding Project (Table 2.1; `http://www.spongebarcoding.org` Wörheide and Erpenbeck, 2007). Phylogenetic analyses were performed on each data matrix separately. For COI, the computer programs RAxML 7.2.8 (Stamatakis, 2006) and MrBayes 3.1.2 (Ronquist, 2003) were used to infer a bootstrapped (1000 fast bootstrap pseudoreplicates Stamatakis et al., 2008) Maximum Likelihood (ML) and a Bayesian phylogenetic tree respectively. The GTR model of sequence evolution (Tavaré, 1986) was used in both analyses, and among-site rate variation was modeled using a discrete Gamma with 4 rate categories (Yang, 1994). The GTR model is the only DNA model available in RAxML, we used this same model for the Bayesian analysis to facilitate comparisons between ML and Bayesian phylogenies and because over-parameterization, normally, does not negatively affect Bayesian phylogenetic inference (Huelsenbeck and Rannala, 2004). For the Bayesian analysis, two independent runs with one cold and 5 heated Metropolis Coupled Monte-Carlo Markov Chain (MCMCMC) chains each were set to sample trees every 500 generations for a total of 10,000,000 generations using the default prior and temperature settings available in MrBayes 3.1.2. After completion, 25% of the samples were discarded as burn-in and a 50% majority-rule consensus tree was calculated. Chain convergence was assessed using AWTY (Wilgenbusch et

al. 2004). With the 28S rDNA dataset, we inferred a bootstrapped (1000 fast pseudo-replicates) ML and a Bayesian phylogeny with the programs RAxML 7.2.8 and PHASE 2.0 (`http://www.bioinf.manchester.ac.uk/resources/phase/`), respectively. For the analysis of this dataset, we used the second most general structural model available (i.e. S7A see Savill et al., 2001) in PHASE for helices (paired-sites) and the GTR+GAMMA model for the unpaired sites of the RNA molecule. Structural annotation of the 28S rDNA dataset was done following (Erpenbeck et al., 2007; Erpenbeck et al., 2004). For the Bayesian analysis, two independent PHASE 2.0 Monte-Carlo Markov Chain (MCMC) runs were set to sample every 500 generations for a total of 10,000,000 generations; prior to each run, 500,000 generations were discarded as burn-in. In order to evaluate the probability of alternative topological arrangements for *Abyssocladia* and *Phelloderma* as well as other groupings proposed in the taxonomic literature, trees sampled during the COI Bayesian analyses were imported into PAUP*4.0 (Swofford, 2003) and filtered using constraints corresponding to the different morphology-based hypotheses. In a Bayesian context, the posterior probability of a bipartition is the frequency with which the bipartition occurs in the trees sampled from the posterior distribution during MCMC (Lewis, 2001).

2.3 Results

2.3.1 COI phylogeny

The inferred COI Bayesian phylogeny (Suppl. Materials) supported the monophyly of chelae-bearing poecilosclerids, and, from this order, the exclusion of several genera that

do not bear chelae, such as *Rhabderemia* Topsent, 1890 (Rhabderemiidae, Microcionina) and the desmacellid genera *Biemna* Gray, 1867 and *Neofibularia* Hechtel, 1965 (Mycalina). In contrast, the ML phylogeny (Suppl. Materials) inferred a polyphyletic chelae-bearing Poecilosclerida with representatives of the genera *Crambe* Vosmaer, 1880 and *Monanchora* Carter, 1883 forming a poorly supported clade with sequences attributed to species of *Niphates* Duchassaing & Michelotti, 1864 (Haplosclerida) and *Scopalina* Schmidt, 1862 (Halichondrida).

In general, both COI phylogenies (Fig. 2.2) showed at least some level of disagreement with the current classification of poecilosclerid sponges (*sensu* Hooper and van Soest, 2002), with most suborders —Suborder Latrunculina Kelly & Samaai, 2002 was not included in the present analysis— not recovered as monophyletic in either ML or Bayesian trees. Suborder Myxillina was polyphyletic in all trees sampled in the Bayesian MCMCMC, comprising four independent clades. Relationships among chelae-bearing Microcionina Hajdu, van Soest & Hooper, 1994 were unresolved in the Bayesian consensus tree but the monophyly of these taxa received low posterior probability ($p = 0.2061$). Within Mycalina, Mycalidae Lundbeck, 1905 and Cladorhizidae were not related to members of Podospongiidae de Laubenfels, 1936, which was polyphyletic. *Abyssocladia* was not closely related to *Phelloderma* (Myxillina) but was included within Cladorhizidae, which formed the sister clade of Mycalidae (with high support in both the ML and Bayesian trees). A topology consistent with the monophyly of *Abyssocladia* + *Phelloderma* was not found among the trees ($N = 15001$) sampled during the arbitrarily chosen chain one of the MCMCMC of the Bayesian analysis.

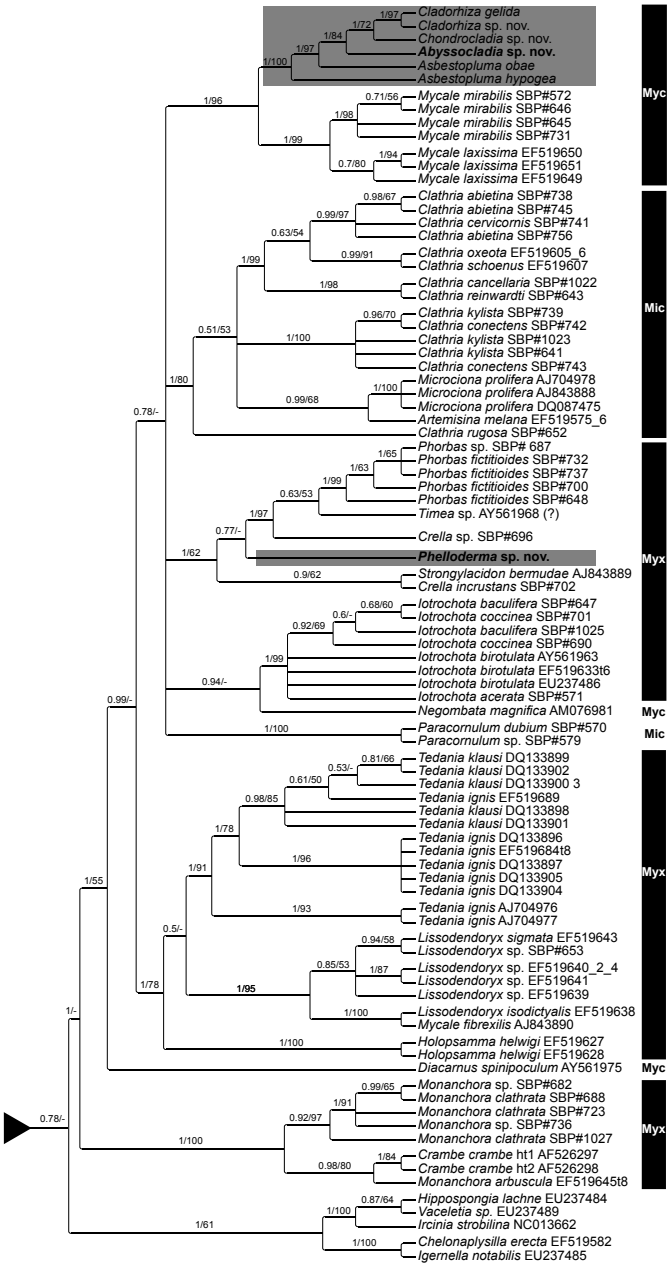

Figure 2.2: Phylogenetic hypothesis of chelae-bearing poecilosclerid sponges based on partial COI sequences. The topology is based on the results of the Bayesian analysis. Support values (posterior probability/bootstrap proportion) above branches. Vertical bars indicate sub-ordinal membership: Myc=Mycalina, Mic=Microcionina, Myx=Myxillina. Highlighted in light gray genera belonging to the families Cladorhizidae and Phellodermidae. The genera *Abyssocladia* and *Phelloderma* are in bold face. SBP# refer to the Sponge Barcoding Project reference numbers, accession numbers follow the Sponge Gene Tree Server formats. The complete ML and Bayesian phylogenetic trees with support values and branch-lengths are provided as Supplementary Materials.

2.3.2 28S rDNA analysis

Despite the lesser number of Poecilosclerida samples which were available for the 28 rDNA analysis, the results from this analysis corroborated those from the COI analysis (Fig. 2.3). Poecilosclerid genera lacking chelae (i.e. *Rhabderemia* and *Neofibularia* in the present dataset) were not recovered as being related to chelae-bearing genera in either the ML or Bayesian phylogenies (Suppl. Materials). Chelae-bearing taxa belonging to different suborders formed clades that contradicted the currently hypothesized subordinal classification of Poecilosclerida. For example, *Coelocarteria* Burton, 1934 (Mycalina) was not related to Cladorhizidae+*Mycale*, but rather to representatives of *Clathria* (Microcionina). Regarding the phylogeny of *Abyssocladia* and *Phelloderma*, these two genera were not indicated as closely related in the 28S rDNA ML or Bayesian phylogenetic trees. In accordance with the COI results, *Abyssocladia* was included within the family Cladorhizidae which, together with *Mycale*, formed a highly supported clade. *Phelloderma*, in contrast, was related to representatives of *Lissodendoryx*, *Crella*, and *Phorbas* Duchassaing & Michelotti, 1864 (Myxillina); all these genera formed a highly supported monophylum.

2.4 Discussion

The current classification of poecilosclerid sponges is based principally on the assumption that chelae microscleres reflect the phylogenetic history (*cf.* van Soest, 2002). Nevertheless, chelae morphology can be homoplasic and their presence/absence has been demonstrate to be environmentally plastic in at least one poecilosclerid genus (see

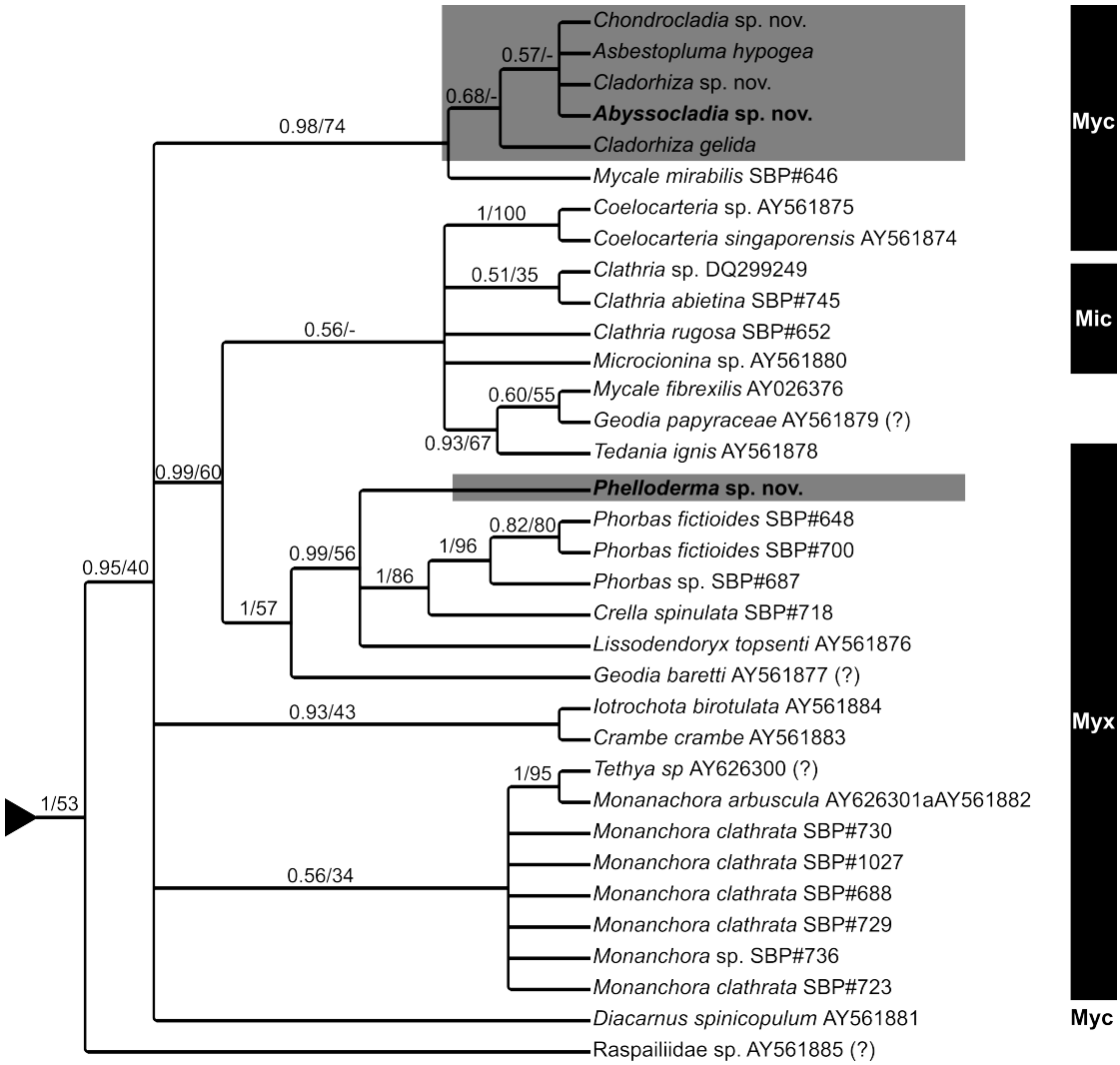

Figure 2.3: Phylogenetic hypothesis of chelae-bearing poecilosclerid sponges inferred from structurally annotated partial 28S rDNA sequences. The topology is based on the results of the Bayesian analysis. Support values (posterior probability/bootstrap proportion) above branches. Vertical bars indicate sub-ordinal membership: Myc=Mycalina, Mic=Microcionina, Myx=Myxillina. Highlighted in light gray genera belonging to the families Cladorhizidae and Phellodermidae. The genera *Abyssocladia* and *Phelloderma* are in bold face. SBP# refer to the Sponge Barcoding Project reference numbers, accession numbers follow the Sponge Gene Tree Server formats. Genbank sequences of doubtful taxonomic affiliation are annotated with a question mark (?). The complete ML and Bayesian phylogenetic trees with support values and branch-lengths are provided as Supplementary Materials.

Maldonado et al., 1999). The alternative taxonomic positions proposed for *Abyssocladia* (see Introduction and Vacelet, 2006; van Soest & Hajdu, 2002), represent an almost perfect case-study on the use, the mis-use, and the value of chelae for systematics of Poecilosclerida. Based on the morphology of chelae, *Abyssocladia* belongs in Phellodermidae. However, the lack of an aquiferous system, the overall body-shape, and the spicule complement and skeletal organization suggest affinities of the genus to Cladorhizidae.

Here we have shown that *Phelloderma* and *Abyssocladia* are not closely related, and that *Abyssocladia* belongs in Cladorhizidae. This family was recovered as monophyletic, with high bootstrap support and posterior probability in our analysis of COI. In the 28S rRNA ML and Bayesian phylogenies Cladorhizidae received low bootstrap support and posterior probabilities. It is worth noting, however, that the uncertainty regarding the monophyly of Cladorhizidae in these analyses was caused by the unstable position of *Mycale* in the Bayesian phylogeny and its inclusion within Cladorhizidae in the ML analysis, and was not caused by the exclusion of any cladorhizid genera from the family. With respect to the relationships between *Abyssocladia* and *Phelloderma*, a topology compatible with an *Abyssocladia* + *Phelloderma* clade was not sampled during the Bayesian MCMCMC analysis of COI or 28S rRNA sequences, indicating that the posterior probability of a tree including this clade is negligible in comparison with that of the optimal and near-optimal trees —in other words, the hypothesized clade is artificial. This should not be surprising since these genera differ greatly in their body-shape; *Abyssocladia* is stalked with a spherical or disc-shaped body (Vacelet, 2006)

while *Phelloderma* is subglobular with a cork-like cortex, papillae, and a general skeletal organization resembling Family Suberitidae (Ridley and Dendy, 1886; van Soest and Hajdu, 2002). Regarding the relationships of Cladorhizidae, this family was shown as sister to Mycalidae as noted above, however this sister group relationship should be treated with caution pending a more complete taxonomic sampling within 'Mycalina'.

From an evolutionary perspective, our results indicate that a diverse complement of chelae have been independently acquired in the cladorhizid lineage (Fig. 2.4). Historically, chelae have been interpreted as being selectively neutral (see Hajdu et al., 1994). This alleged neutrality has led to their use as a taxonomic indicator for the classification of the Poecilosclerida. However, due to their functional role in the capture of prey (Vacelet and Duport, 2004), it is likely that cladorhizid chelae are acted upon by strong selective pressures. Thus, it is probable that the carnivorous habit of cladorhizid sponges has led to the diversity of chelae forms observed among modern representatives of this family (see Introduction and Price et al., 2010 for an example). More speculatively, innovations in chelae morphology within cladorhizid genera might have had an impact on the speciation rates within the family, as judged by its relatively high species richness (see Introduction) and species-specific chelae morphologies. At present, testing this hypothesis is not possible due to the sparse intra-genus sampling available. A more thorough sampling of the family and of other carnivorous sponges currently classified in different poecilosclerid families (Esperiopsidae and Guitarridae) will be required to establish a correlation (if any) between carnivory, chelae diversity and speciation rates in Cladorhizidae, and carnivorous sponges in general.

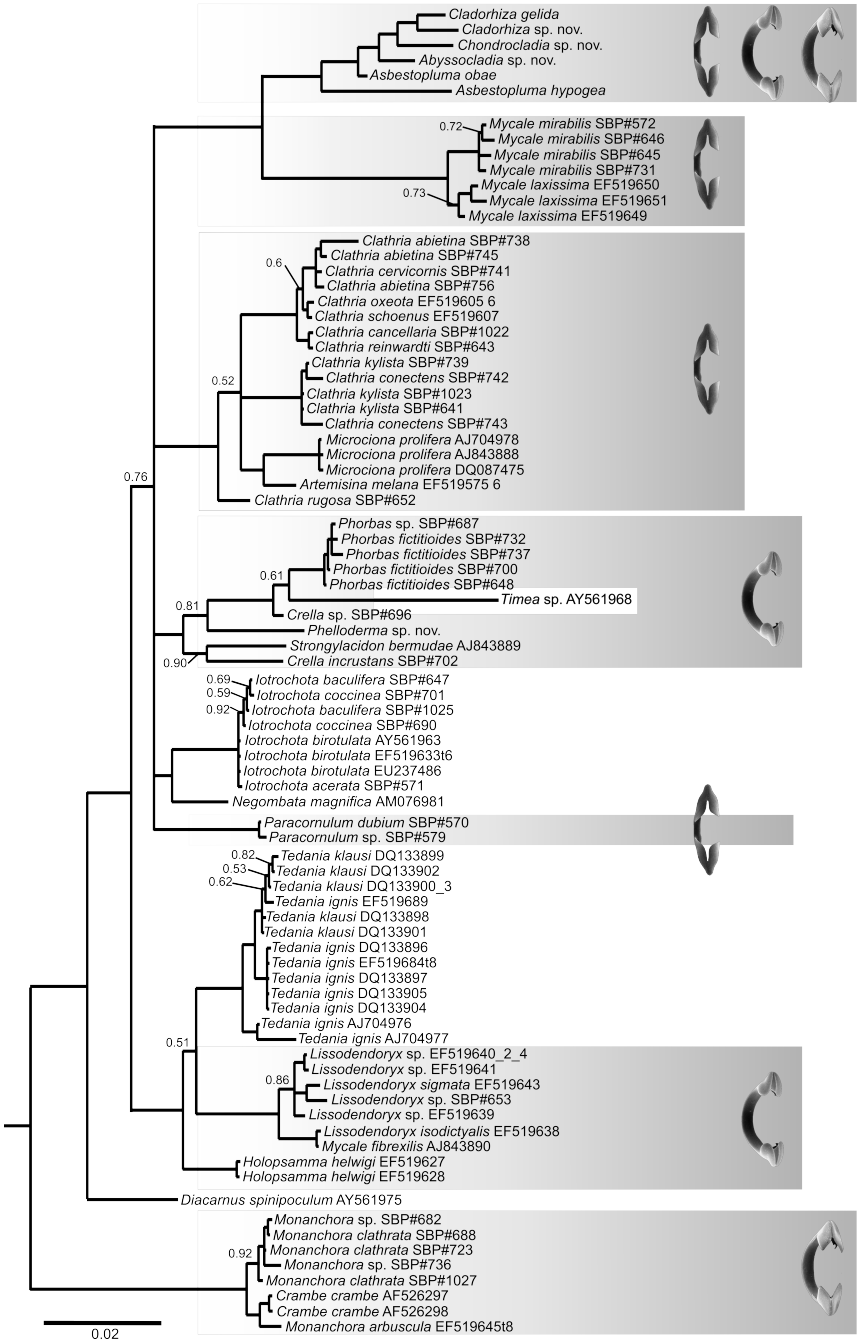

Figure 2.4: Distribution of basic chelae types in different poecilosclerid clades found in the Bayesian COI gene tree (50% majority rule). Chelae type, from left to right: Palmate, Arcuate and Anchorate. The specific chelae form in a given clade can vary within the general morphotype. The chelae forms illustrated are only for schematic purposes. Chelae are not at scale. Support values are shown for branches with posterior probabilities less than 0.95. SBP# refer to the Sponge Barcoding Project reference numbers, accession numbers follow the Sponge Gene Tree Server formats

Our findings have broader implications on the systematics of Poecilosclerida beyond those of only *Abyssocladia* and *Phelloderma*. The use of chelae microscleres in the classification of Poecilosclerida must be made with caution, and the relative importance of this character must be weighted against other sources of evidence. Also, hypotheses about the evolution of chelae microscleres within Poecilosclerida deserve to be reevaluated. We found genera bearing anchorate chelae (e.g. *Monanchora* and *Crambe*) branching off as a sister to all other chelae-bearing poecilosclerids indicating that palmate chelae are derived with respect to anchorate forms. This is in contradiction with the prevailing interpretation of the polarity of chelae transformation series (Fig. 2.4). Future studies on the molecular phylogeny of poecilosclerid sponges are still necessary to further clarify the evolutionary history of chelae microscleres.

2.5 Conclusion

We have presented a phylogeny of the genera *Abyssocladia* and *Phelloderma* using independent molecular datasets, clarified their relative position within the order Poecilosclerida and evaluated hypotheses concerning their morphological characters. Our molecular data support the proposal of Vacelet (2006) that *Abyssocladia* is a cladorhizid, and that this family is monophyletic and related to *Mycale* (suborder Mycalina). Conversely, *Phelloderma* formed a clade with sponges now classified in the suborder Myxillina, and as such, *Phelloderma* and *Abyssocladia* are only distantly related, contrary to previous morphological hypotheses based on chelae morphologies (van Soest and Hajdu, 2002). This result implies the independent acquisition of a diverse chelae com-

plement in Cladorhizidae, likely resulting from an evolutionary diversification related to the carnivorous habit of cladorhizid sponges. These findings have significant implications for the systematics of the Poecilosclerida, as foreseen by Vacelet (2006), and in particular the alleged taxonomic importance of chelae morphotypes for higher taxonomy. The present analyses have provided only a small contribution to a re-evaluation of Poecilosclerida using two independent molecular markers, but highlights the significance and growing potential of molecular-based approaches to reciprocally illuminate morphological evidence and to better resolve the evolutionary history of Poecilosclerida —the most diverse order of Demospongiae Porifera.

Part II

Ecology of Antarctic Sponges

Chapter 3
Counting in the abyss: sponge species richness estimation in deep Antarctic waters*

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* This chapter will submitted for stand-alone publication.

Abstract

Sponges are key components of Antarctic benthic communities. Several authors have pointed out their role providing habitat and refuge to many marine organisms, and sponge species richness in the Antarctic shelf matches that of tropical ecosystems. However, in contrast to the shelf, deep-sea Antarctic communities have been less studied and only sparsely sampled. Expert-based estimations on the number of sponge species inhabiting the deep (>600m) Antarctic waters have yielded mixed results. Herein, we present the first semi-quantitative estimation of the sponge species richness inhabiting deep Antarctic (Weddell Sea) waters. We use non-parametric extrapolation methods and a large number of deep-sea samples collected during the German AnDeepI-III and Systco expeditions. We show that the sponge fauna inhabiting the deep Antarctic waters is rich, matching the self in sponge species depending on the degree of patchiness used for the extrapolation.

3.1 Introduction

Sponges (Porifera) are ecologically important components of Antarctic benthic communities. On the Antarctic shelf, sponges can locally dominate the seafloor contributing with up to 96% of the benthic biomass at some stations (Barthel, 1992a). Big glass sponges of the genera *Rossella* and *Anoxycalyx* have been shown to play important roles in the bentho-pelagic coupling (Gatti, 2002), and to provide refuge and habitat to numerous marine invertebrates (Barthel, 1997; Kunzmann, 1996; Gutt and Schickan, 1998). In terms of species richness the Antarctic shelf is comparable to tropical ecosystems (McClintock et al., 2005), and the level of endemism among Antarctic sponges is high: ~50% of the species described from Antarctica are restricted to the continent's waters (Barthel, 1992a; Sara et al., 1992). In contrast to the shelf, little is known about the diversity of Antarctic deeper (>600m) sponge assemblages which have been only sparsely sampled (Brandt and Ebbe, 2009; Brandt et al., 2007b; Janussen et al., 2004; Janussen

and Tendal, 2007). Expert-based estimations on the number of siliceous sponge species inhabiting deep Antarctic waters have yielded contrasting results (~30 spp. McClintock et al., 2005; ~150spp. Brandt et al., 2007a), leaving the total number of sponge species undetermined.

The determination of the species richness of a given area constitutes the first step towards an in-depth understanding of any biotic community. Yet, species richness is difficult to measure because the number of species recorded for a given community is expected to increase as the number of individuals sampled from that community increases (Gotelli and Colwell, 2001). In theory, if enough samples are taken from a community, the number of species accumulated over all samples (i.e. the species accumulation curve or collector's curve) is expected to reach an asymptote (Colwell and Coddington, 1994). However, reaching an asymptotic species accumulation curve is often not possible, especially in diverse communities. In addition, extensive sampling may be difficult in isolated areas, or the costs of taking even a small number of samples from some communities (like the deep sea) can be prohibitive. In these cases, the species richness of the community can only be approximated using a number of methods that allow for the estimation of species richness from samples through extrapolation. These methods provide lower bound species richness values for communities where the current species accumulation curve shows no clear asymptote (Chao and Shen, 2004; Colwell and Coddington, 1994).

Here, we use non-parametric extrapolation methods to estimate the number of siliceous sponge species inhabiting deep Antarctic (>600m, Weddell Sea) waters. We use

a large number of semi-quantitative samples collected during the German ANDEEPI-III (2006/2007) and ANTXXIV/2-SYSTCO (2007/2008) expeditions to the Weddell Sea, West Antarctica, and covering a depth range between -480 and -5330 m. We aim to independently corroborate previous expert-based predictions and offer base-line estimates that can help guiding future research and sampling of Antarctic deep-sea communities in light of the threats posed by climate change and human activities to these precious ecosystems.

3.2 Materials and Methods

3.2.1 Sampling and species determination

Samples were obtained during the German ANDEEPI-III (2006/2007) and ANTXXIV/2-SYSTCO (2007/2008) expeditions to the Weddell Sea (West Antarctica) by Agassiz trawling or an epibenthic sledge, and in a few cases also box corer and a multicorer. Specimens from sponge-containing stations (N=54) were preliminary sorted and identified on board, and fixed in Ethanol 95% for final taxonomic identification at Senckenberg Museum, Frankfurt. The number of individuals collected by species at each station was also documented on board. All sponges and their preparations are deposited at the Senckenberg Research Institution and Nature Museum, Frankfurt.

3.2.2 Richness estimation, the role of patchiness and sufficient sampling the Antarctic deep

After taxonomic determination, a species per station matrix was constructed and analyzed using the program EstimateS (Version 8.2, R. K. Colwell, `http://purl.oclc.`

org/estimates). Species richness was estimated using the Chao1, Chao2, ACE, ICE, First Order Jackknife (Jack1), Second Order Jackknife (Jack2) and Bootstrap (Boot) non-parametric estimators (for details on the estimators see Chazdon et al., 1998; Colwell and Coddington, 1994). We analyzed three datasets corresponding to the classes Demospongiae and Hexactinellida together (i.e. Siliceous sponges), and to each class separately. For each dataset we assessed the role of patchiness in the predicted species richness values using the "shuffling" option available in EstimateS (Chazdon et al., 1998). We used patchiness values (A) of 0, 0.25, 0.5, 0.75. For all simulations we ran 500 permutations; running 1000 permutations on selected cases yielded similar results (not shown). We consistently obtained CV values >0.50 for the abundance and incidence distributions, therefore we used the classic formulas for both estimators as suggested by the EstimateS manual. Finally, we estimated the number of samples necessary to detect 100% and 75% of the species predicted by the Chao2 estimator using the method described in Chao et al. (2009).

3.3 Results

3.3.1 Demospongiae

Demosponge predicted asymptotic species richness values using the un-shuffled dataset ranged between 94 and 158 species depending on the estimator used (Fig. 3.1). Figure 3.2 shows the predicted asymptotic species richness values under different patchiness settings. After shuffling, when no patchiness was assumed, the number of predicted species by each estimator dropped with respect to the un-shuffled predicted richness

and varied between 91 (Boot) and 144 (ICE). At intermediate patchiness levels (A=0.50) the number of species predicted by each estimator approached the un-shuffled predicted richness levels, and at high patchiness (A=0.75) the number of species predicted almost duplicated the values predicted under no patchiness conditions. In general, the ICE was the most liberal estimator in all simulations except when patchiness was set at A=0.25, under this condition the first order jacknife predicted more species (135) than all other estimators. The bootstrap estimator (Boot) was the most conservative estimator under all conditions used for simulation. Abundance-based estimators yielded lower richness values and, asymptotically, were less sensitive to variation in patchiness than occurrence-based estimators; Chao1 and ACE were (asymptotically) insensitive to variations in patchiness (Fig. 3.2). A complete inventory of deep-sea demosponges would require 382 new samples; 30 new samples would yield 75% of the predicted species.

3.3.2 Hexactinellida

The predicted asymptotic species richness of glass-sponges (Hexactinellida) ranged between 32 and 47 species (Fig. 3.1). Assuming no patchiness, asymptotic richness values ranged between 30 and 40 species and when a high patchiness level (A=0.75) was used, the simulation resulted in 33 to 136 species predicted by the estimators (Fig. 3.2). For hexactinellids, the second order jackknife gave the most liberal estimation for the un-shuffled data matrix and for all patchiness values except A=0.50 when it ranked second in the asymptotic number of species predicted. The behavior of the estimators was variable, the asymptotic number of species predicted by most estimators was

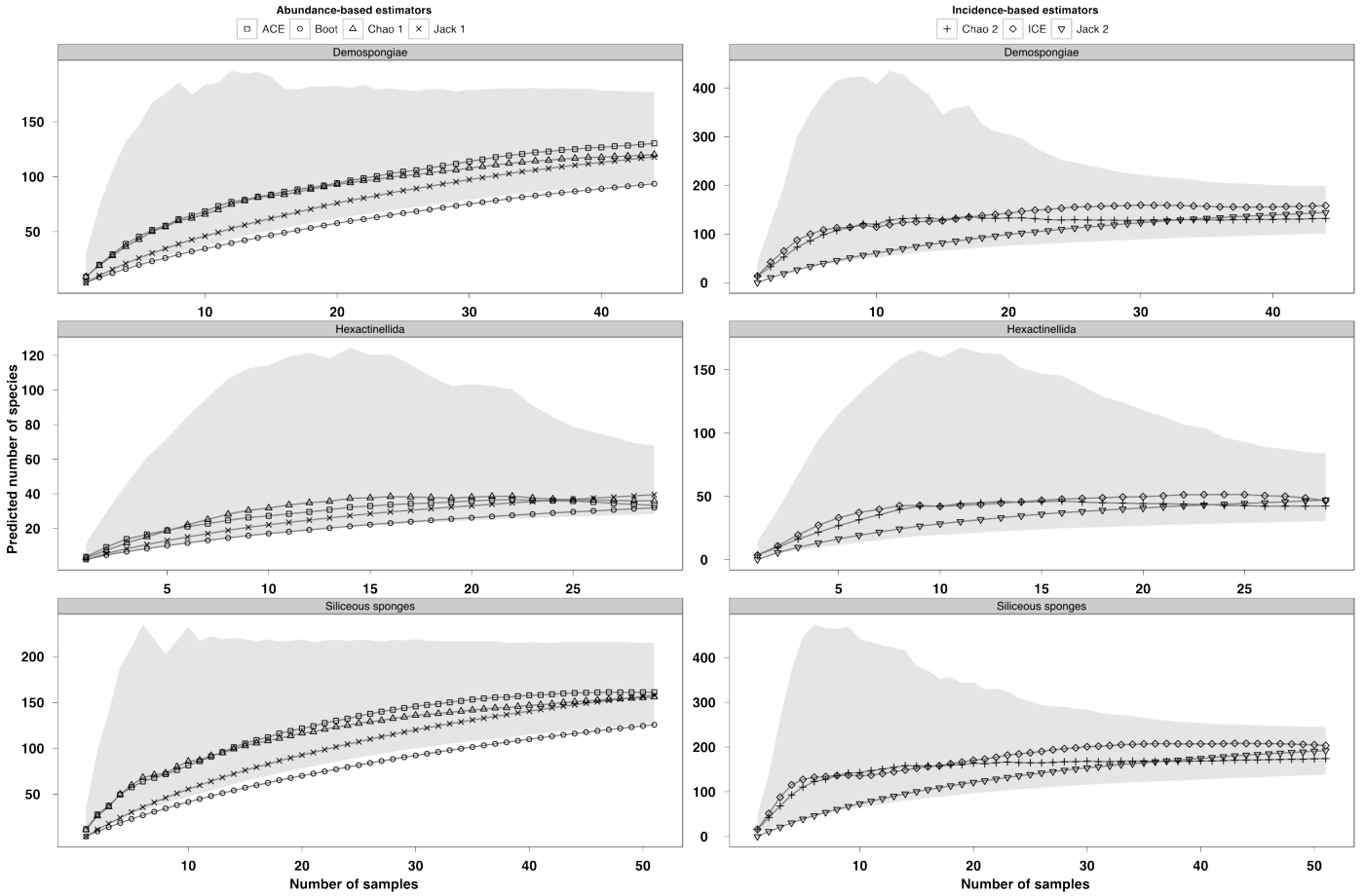

Figure 3.1: Non-parametric species richness estimation of Antarctic deep-sea sponges. Highlighted in grey is the confidence interval of the Chao 1 and Chao 2 simulation results. Siliceous sponges refer to Demosponges+Hexactinellids.

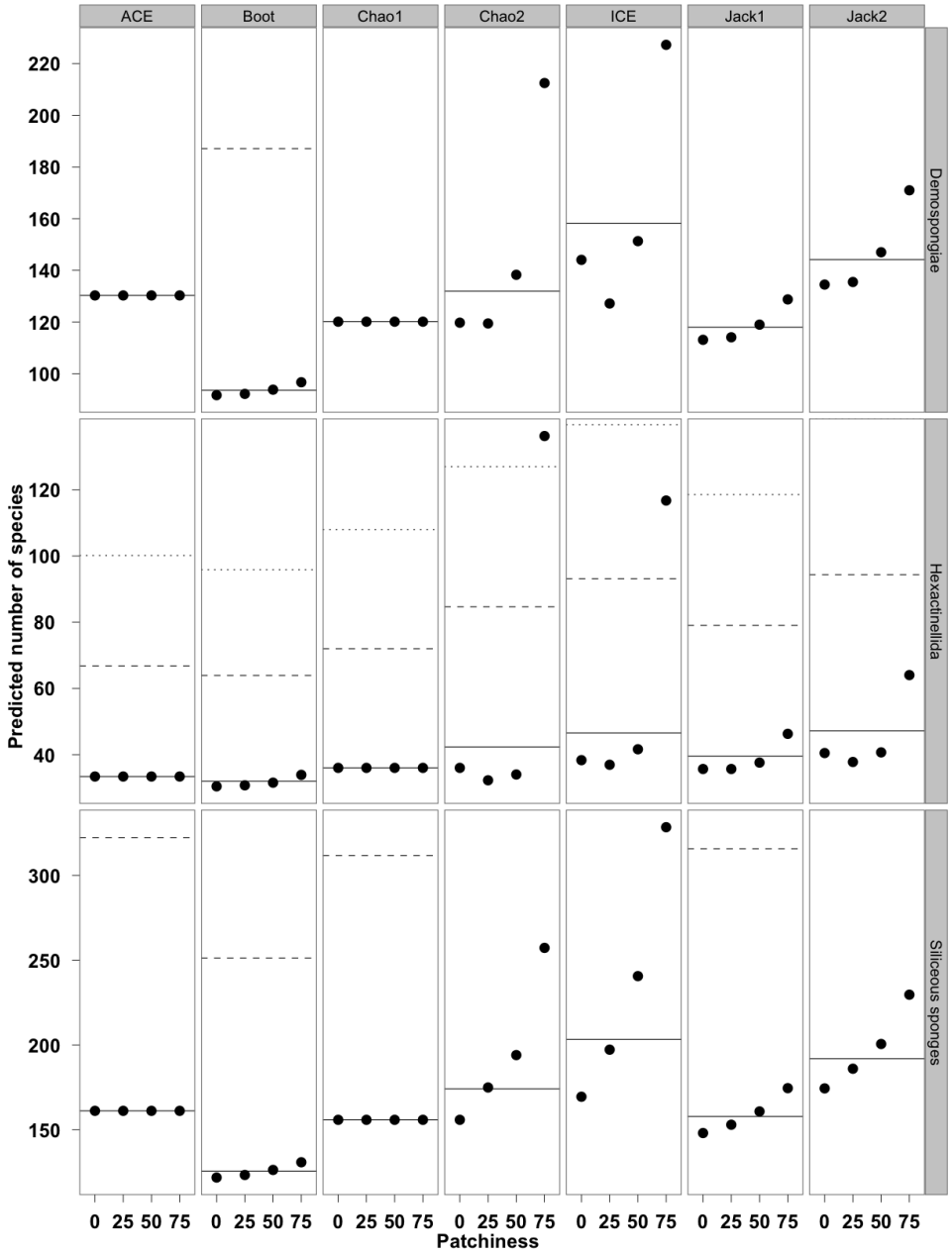

Figure 3.2: Effect of patchiness on the asymptotic species richness values obtained using different richness estimators. The straight, and dashed and pointed lines correspond to the species richness value obtained for the un-shuffled data matrices, and two and three times this value respectively.

lower for A=0.25 than for A=0; at A≥0.50, asymptotic species richness were higher than estimations using lower patchiness levels. As for demosponges, at intermediate patchiness (A=0.5) the number of predicted species was similar to the results of the un-shuffled dataset. When high levels of patchiness were assumed, all incidence-based estimators predicted 1.5 to 3 times more species than their immediate simulation with lower patchiness —Jack2 predicted 40 species under A=0.5 and 64 species under A=0.75, and Chao2 yielded 34 species under A=0.5 and 136 species when A=0.75 (Fig. 3.2). A total of 178 new samples are necessary to achieve a complete hexactinellid inventory, and 13 new samples will be needed to detect 75% of the predicted species.

3.3.3 All Siliceous Sponges

The accumulation curve for each simulation using all siliceous sponges are shown in Fig. 3.1. The predicted number of siliceous sponge species inhabiting Antarctic deep waters ranged between 125 and 203 species. The predicted values are higher than published expert-based estimations for Antarctic deep sea (~150 spp. Brandt et al., 2007a). Similar to the results of the demoponge and hexactinellid datasets, simulations assuming no patchiness (A=0) predicted less species than the un-shuffled dataset. Species richness values, similar to those obtained without shuffling were obtained when patchiness levels were low (A=0.25). If high patchiness (A=0.75) is assumed, predicted species richness ranges between 130 and 328. In general, ICE resulted in higher asymptotic species richness estimates while Boot consistently yielded the lowest values. The asymptotic species richness values obtained for the complete dataset under any given patchiness

value were congruent with those obtained by adding up the richness estimates obtained for demosponges and hexactinellids separately under the same simulation conditions. A total of 450 samples are still needed to collect all predicted siliceous sponge species. If only 75% of the predicted species is aimed 32 new samples are required.

3.4 Discussion

The Antarctic shelf sponge fauna is known to be rich (Barthel, 1992a; Sara et al., 1992). According to our results, the same case applies to deeper poriferan assemblages with ~200 species predicted to occur deeper than ~600m. Depending on the level of patchiness assumed, predicted deep-sea siliceous sponge richness varied between 174 and 328 species. Interestingly, the estimation results based on the un-shuffled matrix, which should reflect the spatial distribution of the species sampled, yielded values similar to those obtained using low levels of patchiness (A=0.25). This results are in accordance with the observation that sponge communities in Antarctica occur in patches (Gutt and Starmans, 2003). Moreover, globally low levels of patchiness are expected because sponges in the Antarctic shelf display a wide range of aggregation patterns with patchy species coexisting with species showing a more random distribution pattern (Gutt and Starmans, 2003). The results of the un-shuffled data matrix imply that estimates assuming A=0 are likely over-conservative. On the other hand, if high patchiness is assumed (A=0.75), the number of deep-sea species approaches the number of sponges species currently reported for Antarctica (352; McClintock et al., 2005) potentially duplicating poriferan richness in the region —a likely scenario.

Sponges are major scaffolding elements in the Antarctic shelf, harboring rich invertebrate communities (e.g. one single specimen of *Rossella racovitzae* from the Ross Sea shelf was found to have 5 brittle star species after only a superficial assessment) and providing refuge for juveniles and larvae of many marine organisms (Barthel, 1997; Kunzmann, 1996). It has been proposed that diversity in the Antarctic shelf can be locally driven by sponges: large hexactinellids can turn soft-bottoms into suitable settling surfaces for other sessile invertebrates (Barthel, 1992b). The significance of an elevated sponge species richness in Antarctic deep-waters for invertebrate richness in general remains a matter of speculation and deserves to be further investigated. Are sponge dominated communities in the Antarctic deep-sea more diverse than communities devoid of poriferans? Are sponge assemblages 'deep-sea oases' in an otherwise hostile environment for most mega-benthic invertebrates? At present, we can only put forward these questions, and point out that testing the potential key-role of deep-sea sponges in Antarctica and the Southern ocean in general remains to be done.

We have shown the use of extrapolation-based methods for the estimation of species richness in regions that are technically difficult to sample. Extrapolation methods can be reliable complements to exploration programs and provide guidelines for future sampling efforts. For instance, 32 new samples are expected to result in a coverage of 75% of all siliceous sponges species predicted to occur in the deep waters of the Weddell Sea. Depending on a number of conditions, approximately 10 deep-sea trawling stations can be done in a (successful) cruise to the Southern Ocean, and not all deployments yield sponges. It should be clear that a long-term research program would be necessary to

successfully collect enough deep-sea samples to further improve the knowledge on the rich sponge fauna inhabiting Antarctic waters. Finally, reports in other invertebrate groups show that Antarctic deep-sea communities can be as rich or even richer than those of shallower environments (Brandt et al., 2004). Our estimations are in agreement with this pattern, providing further support to the hypothesis that the cold Southern Ocean is a diversity hot-spot.

Chapter 4
Barcoding sponges: an overview based on comprehensive sampling*

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Abstract

Background: Phylum Porifera includes ~8,300 valid species distributed worldwide in aquatic ecosystems ranging from fresh-water bodies to coastal environments and deep-sea. The taxonomy and systematics of sponges is complicated, and morphological identification can be time consuming. DNA barcoding can provide sponge biologists with a simple and rapid method for the identification of samples of unknown taxonomic membership. The Sponge Barcoding Project (`http://www.spongebarcoding.org`), the first initiative to barcode a non-bilaterian metazoan phylum, aims to provide a comprehensive DNA barcode database for the Phylum Porifera.

Methodology/Principal Findings: ~7,500 sponge specimens have been extracted, and amplification of the standard COI barcoding fragment has been attempted for approximately 3,300 museum samples with ~25% mean amplification success. Based on this comprehensive sampling, we present the first report on the workflow and progress of the sponge barcoding project, and discuss some common pitfalls inherent to the barcoding of sponges.

Conclusion: A DNA-barcoding workflow capable of processing potentially large sponge collections has been developed and is routinely used for the Sponge Barcoding Project with success. Sponge specific problems such as the frequent co-amplification of non-target organisms have been detected and potential solutions are currently under development.

4.1 Introduction

Sponges (Phylum Porifera), are diverse, sessile, benthic metazoans, occurring in marine and fresh-water ecosystems worldwide. In marine habitats, from coral reefs to abyssal plains, sponges play important roles in biogeochemical cycling (Gatti, 2002) and in the spatial structuring of the seafloor (Gutt, 2007); sponges also participate in complex biotic interactions with diverse taxa (for a review see Bell, 2008). According to the World Porifera Database (van Soest et al., 2011), more than 8000 species are considered valid, with most belonging to the Class Demospongiae. From a taxonomic and systematic

point of view, Phylum Porifera is challenging because of the general paucity of characters useful for taxonomic and phylogenetic inference among sponges (Erpenbeck et al., 2006a). Furthermore, the relatively simple sponge body-plan and the ecological plasticity or evolutionary lability of the few characters available for identification make sponge taxonomy a field where uncertainty is commonplace (Cardenas et al., 2011; Dohrmann et al., 2006)

In recent years, DNA barcoding has been proposed as an aid to increase the speed of sponge identification (Wörheide and Erpenbeck 2007). The Sponge Barcoding Project (`http://www.spongebarcoding.org`; Wörheide et al., 2008) represents the first barcoding effort targeting non-bilaterian metazoans. The project aims to provide the most comprehensive repository of sponge barcodes, and to associate these barcodes with morphological annotations of the barcoded species. For this purpose a large number of specimens, including samples deposited in museum collections, needs to be processed (i.e. extracted, PCR-ed and sequenced) in a time- and cost-efficient manner. Furthermore, a number of difficulties intrinsic to working with sponges needs to be overcome. Barcoding sponges can be problematic due to the potentially large number of non-target macro- and microorganisms found in association with sponges (Erpenbeck et al., 2002). These organisms can get co-extracted, and either co-amplified or preferentially amplified during PCR causing sequences to be difficult to read or to belong to non-target organisms. Moreover, for defense purposes, sponges produce potent bioactive compounds that can inhibit enzymatic reactions such as PCR (Chelossi et al., 2004). Thus, a number of obstacles not usually found in other invertebrate groups need to be

Figure 4.1: Sponge families sampled and extracted for the Sponge Barcoding Project. Word size corresponds to frequency of the respective family in the sample. The word-cloud was generated using Wordle with frequency data available as Suplementary materials (available at http://www.wordle.net/)

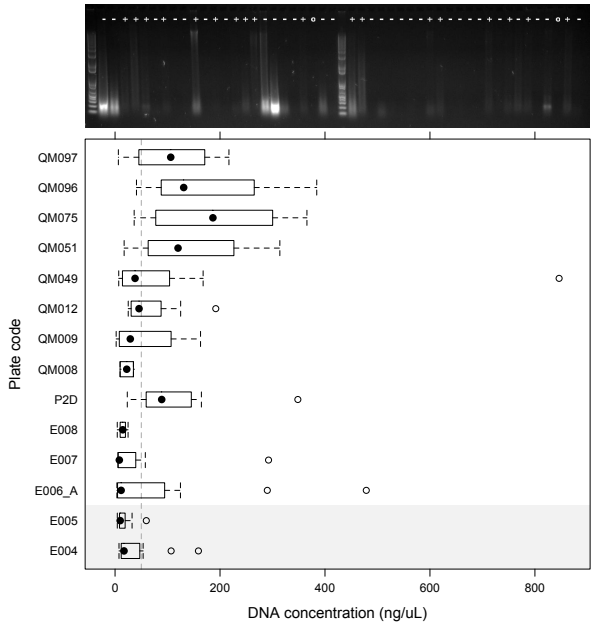

Figure 4.2: Yield and quality assessment of the DNA extracted using a modified Ivanova et al 2006 extraction protocol. Upper figure: DNA quality assessment and PCR success of 48 samples. + = PCR product obtained, - = no PCR product was obtained and o = negative (no-DNA) control. Lowere figure: Box-whisker plot of the DNA yield of 12 96-well plates. The dotted grey line represents a yield of 50ng/uL. Highlighted in grey two plates extracted using the Ivanova et al.'s original method.

tackled for successful sponge barcoding. Here, we summarize the results of an analytical pipeline established to barcode sponges and provide an overview of the current state-of-the-art on sponge barcoding that can serve other colleagues working on this challenging field.

4.2 Results

4.2.1 DNA yields

We obtained DNA extracts of 96 families in all three classes of Porifera (Fig. 4.1). The average DNA yield was 89±114 ng/uL (N=156), and the mean DNA concentration

of individual extraction plates ranged between 14±6 ng/uL and 191±117 ng/uL (Fig. 4.2). Within-plate variation in DNA concentration values was high, and concentration differences of up to two orders of magnitude were detected within single extraction plates. Agarose gel electrophoresis revealed that the purification method was capable of recovering high-molecular weight DNA, however variability was also high among samples (N=184) within plates. There was no apparent relationship between DNA quality, interpreted here as the presence of DNA of high molecular weight in the extracts, or DNA concentration and PCR success in 48 samples analyzed (Fig. 4.2).

4.2.2 PCR and sequencing success

Levels of COI amplification success ranged between 0% and 55% among 35 96-well plates analyzed (3,360 specimens). Mean amplification success was 27±17%, which is roughly equivalent to 26 positive samples per 96-well plate. When taxonomic groups (families) were analyzed (N=73) PCR success rates covered the entire 0%–100% range, however we noted that many taxa with extreme high success rates (e.g. 100%) were represented by only few specimens (Fig. 4.3). If the analysis is restricted to families with more than 30 processed specimens (N=28), PCR success levels ranged between 0% and ~50%. Among this group, the families Dysideidae, Plakinidae, Spongiidae and Thorectidae had PCR success rates that ranged between 0% and 2% while the PCR success rates for the remaining families (N=24) ranged between 10% and ~50%. PCR success rates were not independent from taxonomic membership ($\chi^2_{N=2580,df=24}$ = 178.90, $p < 0.001$). This result holds before and after the exclusion of families with extremely

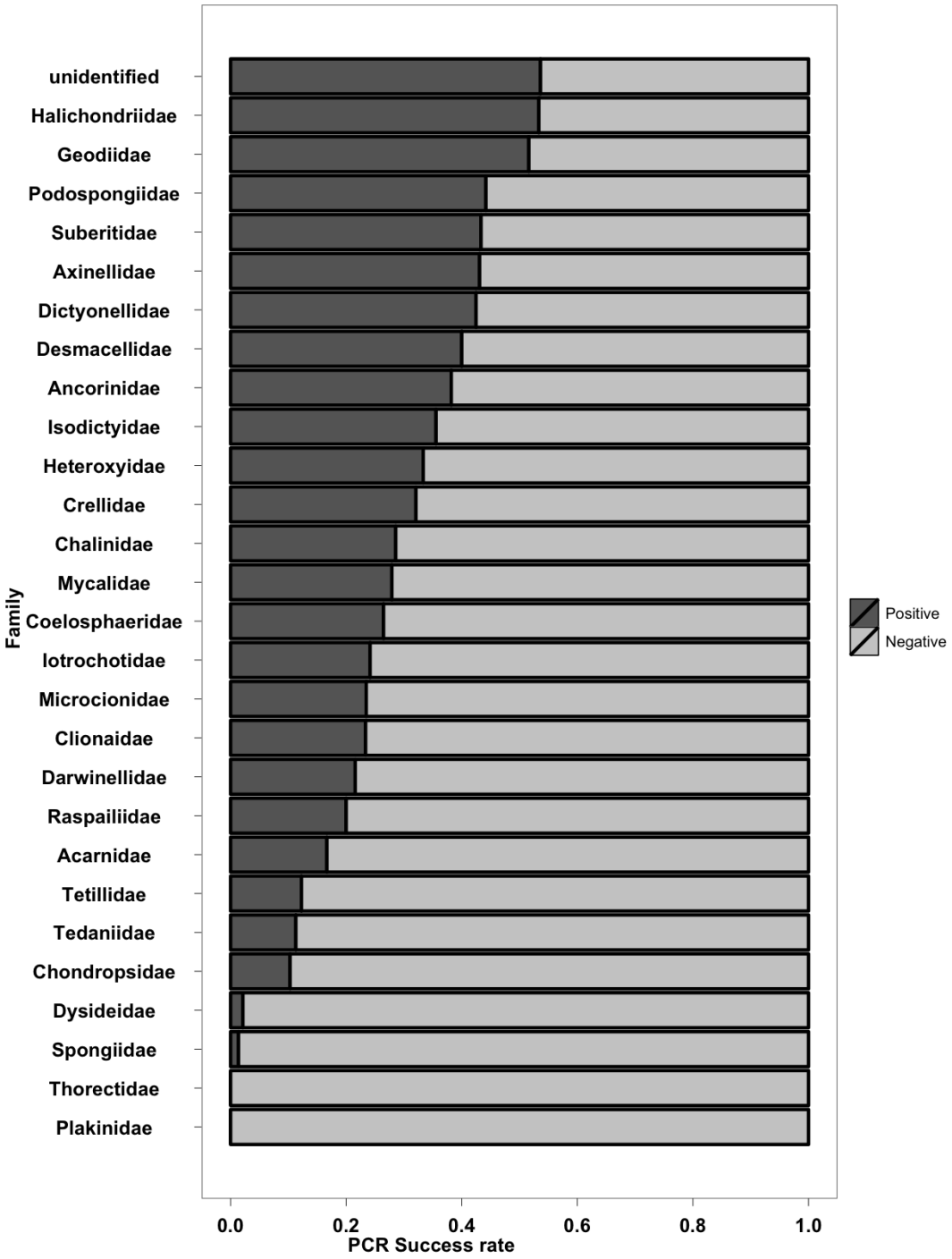

Figure 4.3: Amplification success rates of the standard barcoding COI partition in different sponge families. Grey and black colours represents failed and positive reactions, respectively.

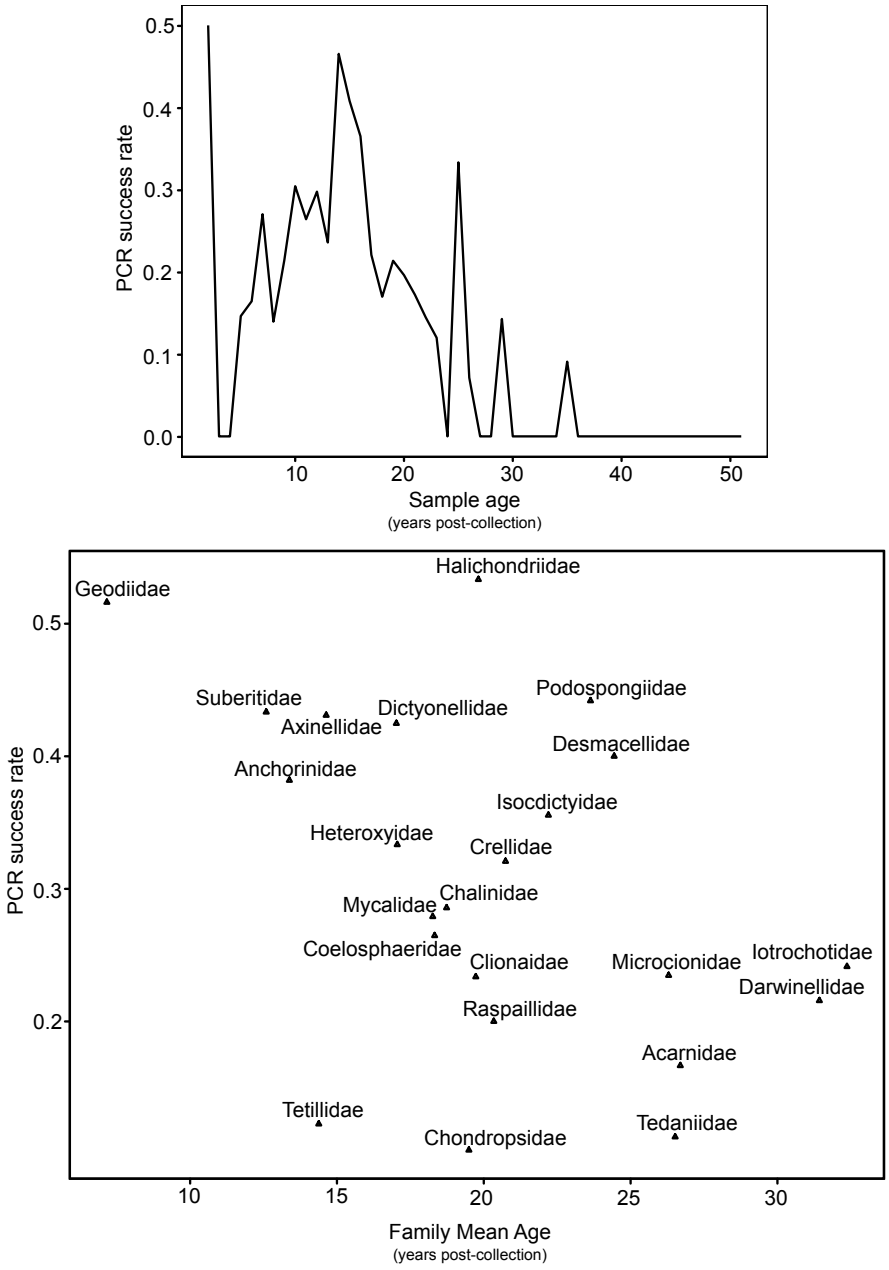

Figure 4.4: Relationship between COI amplification success rate and sample age. Upper figure: Mean amplification success rate irrespective of taxonomic membership by time in years. Lower figure: Scatter plot showing the relationship between family mean age and amplification success rate for selected families.

low (0%–2%, see above) PCR success rates. The effect of sample age within a given family, however, cannot be excluded as family mean age was related to PCR success in 23 families analyzed (r=-0.449, N=26, p=0.03; Fig. 4). The analysis of all extracted and amplified samples, independent of taxonomic membership, revealed that PCR success rates tend not to decrease significantly with sample age (r=-0.11, N=19,p=0.64) but remained ranging between ~14% and ~45% in samples of 5-23 years old (Fig. 4.4).

With regard to sequencing sucess, 40% of the sequences obtained corresponded to non-target organisms. Among these sequences, a considerable proportion belonged to bacterial strains likely to have been co-extracted with the sponge DNA. Despite the contaminant being co-amplified or preferentially amplified, DNA of sponge origin was present in the extracts (Table 4.1). Contrary to PCR success rates, sequencing success rates, defined as the proportion of sequences of sponge origin obtained for a given sponge family or plate, was independent from the taxonomic assignment of the sequenced sample ($G - test_{N=66,df=33}$ = 32.50, p=0.49; Fig. 4.5).

4.3 Discussion

We have presented a first assessment on the progress and technical aspects of the Sponge Barcoding Project. At present, two laboratory workers are capable of processing 576 samples (i.e. 6 96-well plates) a week using the analytical pipeline set for the project. In our experience, subsampling the sponge tissues for extraction is the limiting step in terms of the time needed to process a plate. Subsampling sponge tissue is a time consuming process and it is important that care is taken with this step to ensure that

Table 4.1: COI and 28S rDNA sequences obtained from 5 arbitrarily chosen samples corresponding to mixtures of DNA extracted from sponge tissues.

Sample ID	COI BLAST results	28S rDNA BLAST results
Myxillidae	Cnidaria	Porifera
cf. *Hemigelius bidens*	Bacteria	Porifera
Myxilla mariana	Bacteria	Porifera
Polymastia invaginata	Bacteria	Porifera
Rossella racovitzae	Bacteria	Porifera
Porifera undetermined	Bacteria	Porifera

surface contaminants are minimized and that tissues are prepared in small pieces to facilitate the extraction work-flow. After tissue has been subsampled, DNA extraction is completed within hours depending on the worker's experience. This means that DNA extraction, PCR, gel documentation and sequencing for 192 samples (i.e. two 96-well plates) can be done within two working days by one laboratory employee. This modest capacity allows a medium throughput facility to easily barcode large number of samples within short time. Moreover, because the DNA extraction yields are generally high —although this depends greatly on the tissue sample— the barcoding pipeline indirectly results in the establishment of a DNA-bank which can be further used for different purposes.

With respect to PCR success rates the values reported here correspond with published PCR success rates for archival moth specimens when Taq polymerase was used (i.e. ~50% for 2-year old specimens Hajibabaei et al., 2005). We did not observed a general drop in PCR success rates with age, however restricting the analysis to certain families (see Results) revealed a clear relation between PCR success rates and mean

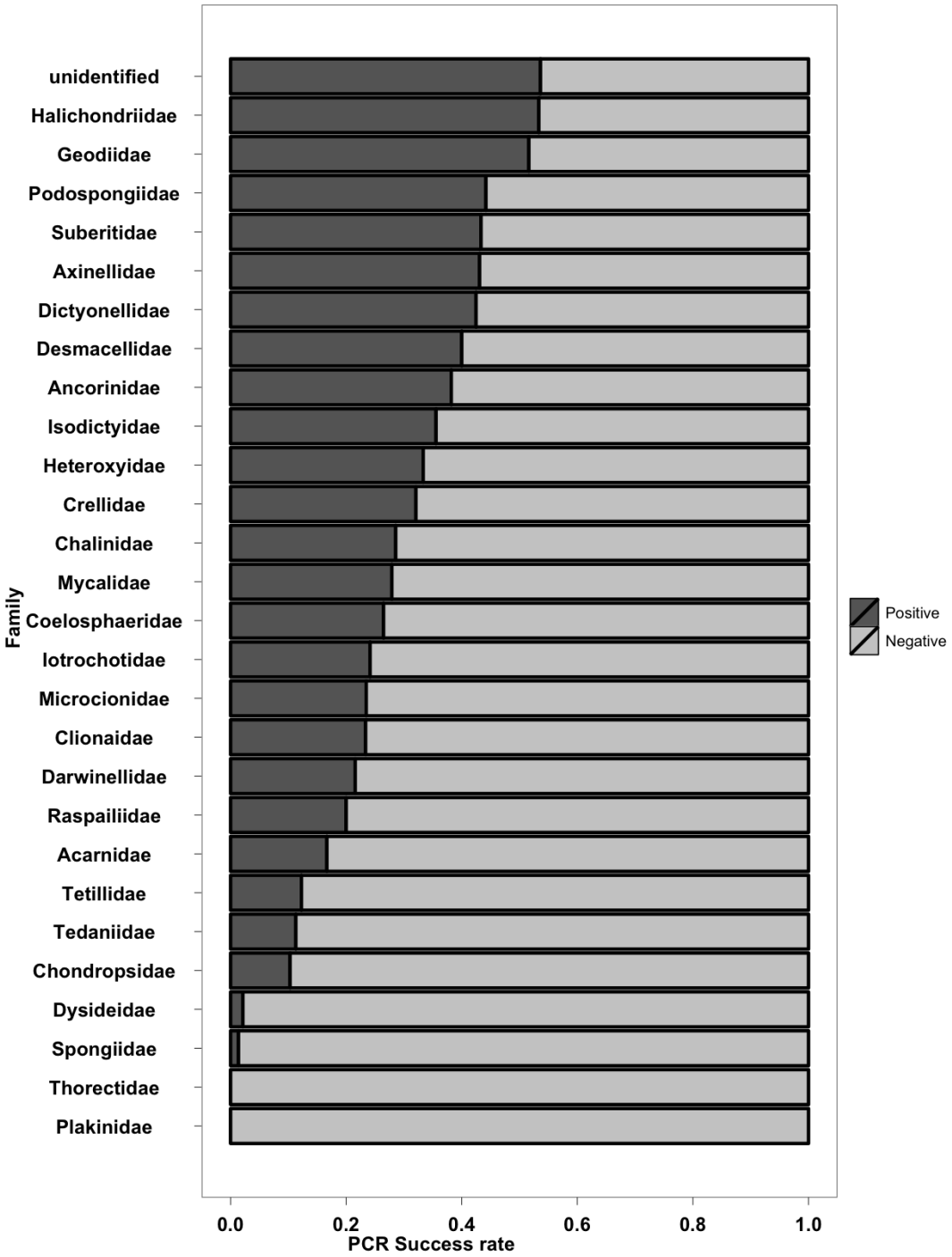

Figure 4.5: Sequencing success rates per sponge family. Grey and black colours represents sequences corresponding to non-target organisms and poriferans, respectively.

sample age. We have observed that the extraction protocol works particularly well with fresh tissue or with recently collected and properly fixed samples, however for poorly preserved material the method tend to give poor results. This should not be a surprise and is not a problem specific to the protocol, as commercial options also fail in this situation (pers. obs.). However, we have consistently observed low 260/230 absorbance ratios in our extractions. Low 260/230 have been related to the co-elution of thiocyanate salts (Ivanova et al. 2008); these strong protein denaturants could act synergistically with low-quality DNA to cause PCR failure in these cases.

In the case of samples yielding DNA of moderate to high quality, and for some taxonomic groups a family-specific effect cannot be ruled out as the cause of PCR failure. Our results revealed that PCR success and family membership are not independent. Although this result can be potentially confounded by the effect of sample age on PCR performance and by taxonomic collection bias during certain years, some families show contrasting PCR success rates despite their similar mean ages. For instance, the families Halichondriidae and Isodictyidae with approximately equal mean sample age (i.e. 20±24 and 22±26 years respectively) and sampling effort (N=45) had PCR success rates of 53% and 35%, respectively. This result suggests that a complex relation between sample age and taxonomic membership can affect the performance of the barcoding pipeline. The presence of secondary metabolites that could inhibit the PCR reaction is possible in sponges (see Erpenbeck and van Soest 2007), and family or genus specific mismatches in the priming site cannot be discarded. Further, it may be that morphological factors (which are correlated to taxon membership), such as tissue

density or perfusion rates, could influence the rate and quality of specimen fixation and therefore affect the preservation of DNA. We have observed that despite tissue subsampling has been standardized, it is particularly difficult to obtain homogeneous DNA concentrations within most DNA extraction plates. High intra-plate variation in DNA concentration hampers the high-throughput downstream processing of the samples, because samples that likely need to be diluted co-exist with low concentration samples that, likely, will not amplify after dilution. Increasing the volume of the buffer used in the digestion and binding steps of the DNA extraction protocol has helped to reduce intra-plate variability to some extent, but this remains problematic for the high-throughput barcoding of sponges.

Co-amplification or preferential amplification of non-target organisms represents a major obstacle for DNA barcoding (see also Siddall et al., 2009). We obtained non-target organisms in 40% of the sequenced samples which imply almost a duplication in the relative cost of a single sponge barcode. This problem is hard to solve because the complete isolation of contaminating tissues from sponge tissue is usually not possible, and because the phylogenetic origin of the "contaminants" can be diverse. Moreover, cloning is only possible in selected cases as this technique is not compatible with medium- or high-throughput sample processing. Here, we have demonstrated that a sponge DNA extract is actually a complex DNA mixture and can be better thought-of as a sponge's meta-genome. We have detected "contaminant" sequences belonging to bacteria, cnidarians, bryozoans, molluscs, ascidians, algae and poriferans themselves. Future work on the design of better primer sets or primer mixtures for sponges based on

an increased taxonomic sampling should help to improve the efficiency and selectivity of COI barcoding for Porifera.

Table 4.2: Modification to the genomic DNA protocol of Ivanova et al. 2006 used for sponge barcoding.

Protocol step	Ivanova et al. 2006	This study
Digestion	50 μl Lysis mix[1]	200μl Lysis mix
Binding	100 μl Binding mix[2]	400 μl Binding mix
First washing step	180 μl Protein wash buffer[3]	200 μl Protein wash buffer
Second washing step	750 μl Wash buffer[4]	750 μl Wash buffer
Elution	60 μl H_2O	50-100 μl H_2O

[1] Lysis mix: 100mM NaCl, 50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.5% SDS, Proteinase K 10% v/v.
[2] Binding buffer: 6M GuSCN, 20mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4, Triton X-100 4% v/v. The Binding mix is a 50% v/v solution of Binding Buffer in ethanol 96%.
[3] Protein wash buffer is a 30% v/v solution of Binding Buffer in ethanol 96%.
[4] Wash buffer: 50mM NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA pH 8.0, ethanol 60%.

4.4 Materials and Methods

4.4.1 DNA Extraction

A number of high-throughput methods for DNA extraction have been published (e.g. Boom et al., 1999; Boom et al., 1990). For the Sponge Barcoding Project, we looked for a centrifugation-based method available for 96-well plates. We selected the method proposed by Ivanova et al., 2006 for the Barcoding of Life, which is based on the selective binding of DNA molecules to a fibre-glass membrane in the presence of high concentrations of Guanidinium Thiocyanate. We optimized the published protocol (see Table 4.2) to increase the amount of tissue digested and the final DNA concentration (ng/uL). Using this modification, we have extracted a total ~7,400 sponge samples, mainly deposited at the Queensland Museum, Brisbane Australia, covering all poriferan classes and demosponges orders (see below). In order to evaluate the yields obtained,

the DNA concentration (ng/uL) of 12 randomly picked extracts per plate was determined using a Nanodrop 1000 spectrophotometer. In total, 14 plates were quantified.

4.4.2 Amplification and sequencing success of the standard barcoding fragment

The Sponge Barcoding Project focuses initially on sequencing the standard barcoding partition, located at the 5' end of the mitochondrial cytochrome oxydase subunit 1 (Folmer et al., 1994; Hebert et al., 2003), to comply with the current convention for metazoan barcoding (but see Erpenbeck et al., 2006b). We have used degenerate primers (Meyer et al., 2005), and reactions supplemented with BSA. The amplification program used was a standard three-step PCR with an initial denaturation step of 3 minutes at 94°C followed by 35-40 cycles of 30 seconds at 94°C, 30 seconds at 40°C and 1 minute at 72°C, and a final extension step of 5 minutes at 72°C. PCR products were visualized on a 1% agarose gel electrophoresis, and each reaction was categorized as "positive" or "negative". For sequencing, the same PCR primers were used after a standard ammonium acetate-ethanol clean-up (Sambrook et al., 1989). Reads were assembled and annotated as "contamination" or "sponge" according to the results obtained from BLAST (Johnson et al., 2008). For selected extracts tagged as "contamination", we amplified ca. 1.2kb of the nuclear 28S rDNA using primers NL4F+NL4R (Nichols, 2005), which tend to preferentially amplify poriferan DNA, to test for presence of sponge DNA in the extract. The annotated PCR and sequencing results were used to detect potential associations between PCR and sequencing success rates and taxonomic affiliation and year of collection.

Chapter 5
Diversity in a cold hot-spot: DNA-barcoding reveals patterns of evolution among Antarctic sponges*

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* This chapter will been submitted for stand-alone publication.

Abstract

Background: The ~350 demosponge species that have been described from the benthic communities inhabiting the Antarctic shelf represent a faunistic component distinct from that of neighbor regions. Sponges provide structure to the Antarctic benthos and refuge to other invertebrates, and can be dominant at certain localities. Despite their importance DNA barcodes from Antartic sponges are scarce.

Methodology/Principal Findings: We sequenced the standard barcoding COI region for a comprehensive sample of sponges collected during the National Institute of Water and Atmosphere's BioRoss and IPY field campaigns to the Ross Sea, and produced DNA-barcodes for ~50 demosponge species covering ~60% of the species collected and ~80% of the characteristic species present in the communities occurring on the Ross Sea shelf. Antarctic sponge communities are phylogenetically diverse matching the phylo-diversity of sponge communities in the Lusitanic and Mediterranean marine provinces in the Warm Temperate Northwest Atlantic. Additionally, DNA-barcoding revealed levels of *in situ* molecular evolution comparable to those present among Caribbean sponges. DNA-barcoding using the Segregating Sites Algorithm correctly assigned ~54% of the barcoded species.

Conclusion/Significance: A barcode library for Antarctic sponges was assembled and used to advance the systematic and evolutionary research of Antarctic sponges. We provide insights on the evolutionary forces shaping Antarctic's diverse sponge communities, and a barcode library against which future sequence data from other areas of Antarctica or depth strata can be compared, or sponge collections can be sorted to speed up the taxonomic identification and ecological research.

5.1 Introduction

Sponges are a conspicuous and, in some cases, dominant members of the rich invertebrate communities that inhabit the Antarctic shelf (Gutt and Starmans, 1998; Gutt, 2007). Large poriferans are key structural components of the Antarctic benthos, reaching high abundances and biomasses (Barthel, 1992a), serving as structural frameworks for other filter feeders, and providing refuge for the juvenile and adult stages of numerous organ-

isms (Barthel, 1992b, 1997; Gutt and Schickan, 1998; Schiaparelli et al., 2003). In terms of species richness, Antarctic sponge assemblages are comparable to those found in tropical ecosystems (McClintock et al., 2005). In addition, the ~350 sponge species reported for the Antarctic shelf constitute a distinct faunistic complex characterized by its high species-level endemism and generic cosmopolitanism, and by the alleged circumpolar distribution of most species (Sara et al., 1992). Of the ~350 sponge species identified from NIWA collections, 4 species have been recorded from Chatham Rise in southeastern New Zealand waters (*Suberites caminatus* Ridley & Dendy, 1886; *Craniella sagitta* (Lendenfeld, 1907), *Rossella antarctica* Carter, 1872, and *Tentorium papillatum*). *Polymastia invaginata* Kirkpatrick, 1908 has also been recorded from the Macquarie Ridge south of New Zealand.

The high endemicity levels observed among Antarctic sponges have been hypothesized to be the result of the continent's long geological history and relatively high geographic isolation (Sara et al., 1992). Antarctica separated from Gondwana ~140 million years ago and became progressively isolated from other land masses (Brandt et al., 2007). By the end of the Eocene, the opening of the Drake Passage broke Antarctica's last connection with South America and triggered the onset of the Antarctic Circumpolar Current (ACC) (Lawver and Gahagan, 2003). Climate change and the development of the ACC during the Oligocene (Lawver and Gahagan, 2003) further isolated the Antarctic biota, likely promoting speciation in the marine realm. *In situ* diversification in Antarctica has been documented for a number of marine invertebrates (Held, 2000; Held and Wagele, 2005; Linse et al., 2007; Brandão et al., 2010; O'loughlin et al., 2010),

and is likely in sponges which typically produce short-living planktonic larvae.

Notwithstanding their importance, Antarctic sponges have been largely neglected from molecular phylogenetic or DNA-barcoding studies of the phylum Porifera, due in part to the harshness of the environment and difficult of access for collection. Grant et al. (2009) found that only 2% of all sponge species reported for Antarctica had sequence data, and to date only a handful of studies have dealt with the molecular systematics of selected Southern ocean poriferans (see Chapters 1 and 2). The lack of Antarctic sponge sequences limits the evaluation of hypotheses regarding their macro-ecology and evolutionary history, DNA-barcoding (Hebert et al., 2003) can be used to rapidly sequence a representative set of Antarctic sponges. Here, we use a community-based approach (*sensu* Kress et al., 2009) to barcode the shelf and slope sponge associations occurring in the Ross Sea, Antarctica. Our barcode dataset provide first insights into the evolutionary processes that led to Antarctica's high sponge diversity and endemism, and reveal areas of taxonomic uncertainty in need of further research.

5.2 Results

5.2.1 Ross Sea sponge assemblages and depth stratification

Cluster analysis resulted in five groups of stations (cut height = 0.95), four of which were also present in the NMDS results (Fig. 5.1). These clusters were clearly related to the start trawling depth, and a generalized additive model (normal link) fitting this variable explained ~70% of the total deviance. In general terms, the main three clusters found corresponded to upper (Cluster 3; Depth=264±98m), middle (Cluster

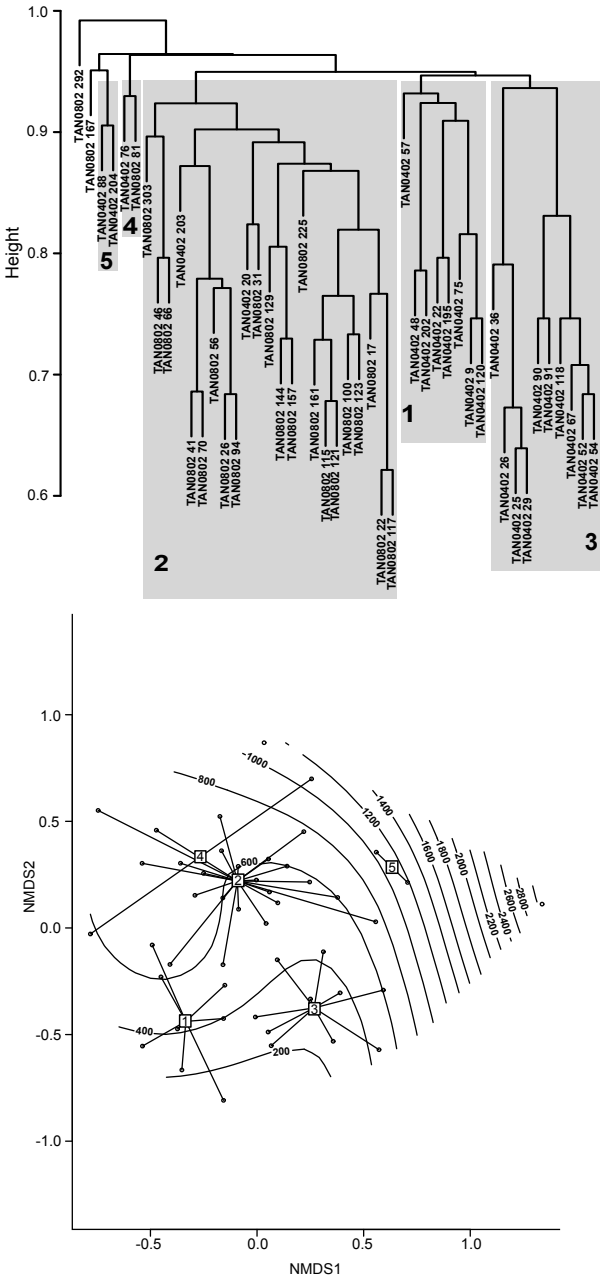

Figure 5.1: Cluster and Non-metric Multidimensional Scaling (NMDS) analyses (Jaccard distance) of the Ross Sea shelf and slope sponge communities. Depth (meters) contours in the NMDS were fitted using generalized additive models (normal link). Cluster groups at a height=0.95 are highlighted in gray in the cluster analysis and annotated in the NMDS plot.

Table 5.1: Characteristic species (in bold face) per station cluster. Characteristic species are those present in >25% of a cluster's member stations. Species for which a barcode was successfully sequenced are annotated.

	Cluster			
Species	1	2	3	Barcoded
Acanthorhabdus fragilis	0.37	0.34	.	YES
Anoxycalyx ijima	0.25	.	.	NO
Artemisina jovis	0.37	.	.	YES
Artemisina plumosa	.	.	0.7	YES
Cinachyra antarctica	.	0.26	.	YES
Cinachyra barbata	0.25	.	.	YES
Cinachyra vertex	0.62	.	0.4	YES
Clathria pauper	.	.	0.3	YES
Craniella microsigma	0.50	.	0.3	YES
Craniella sagitta	.	0.43	.	YES
Desmacidon maeandrina	.	.	0.6	NO
Gellius pilosus	.	.	0.3	YES
Homaxinella n.sp.1	0.37	0.30	.	YES
Inflatella belli	.	0.30	.	NO
Iophon spatulatum	.	0.34	.	YES
Isodictya cactoides	.	.	0.3	YES
Isodictya erinacea	.	0.26	0.5	YES
Latrunculia biformis	.	0.34	.	YES
Mycale acerata	.	0.30	0.3	NO
Phorbas glaberrimus	0.25	0.26	.	YES
Rossella antarctica	0.25	0.56	.	NO
Rossella fibulata	.	0.30	.	NO
Rossella nuda	.	.	0.3	NO
Rossella podogrosa	0.25	.	0.3	NO
Rossella racovitzae	.	0.39	.	NO
Rossella villosa	0.25	.	.	NO
Sphaerotylus capitatus	.	.	0.3	NO
Stylocordyla borealis	.	.	0.3	YES
Suberites caminatus	.	0.43	.	YES
Suberites papillatus	0.25	.	.	YES
Tedania massa	.	.	0.4	YES
Tedania oxeata	.	0.30	0.4	YES

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Tentorium papillatum[2]	.	0.56	.	NO
Tetilla leptoderma	.	0.26	.	NO

[1] *Rossella fibulata*, *Rossella nuda*, *Rossella podogrosa* and *Rossella villosa*, are likely synonimous (or subspecies) of *Rossella racovitzae* and were therefore not considered characteristic species of any cluster.

[2] *Suberites caminatus*, *Suberites papillatus* and *Tentorium papillatum* are, likely, synonimous. This will make them present in Cluster 1 and 2, and therefore not characteristic of any cluster.

1; Depth=443±302), and lower (Cluster 2; Depth=654±301) shelf sponge assemblages. The fourth and fifth clusters grouped, each, two stations that shared only one species and showed no relation with depth. The characteristic species for the shelf assemblages (i.e. Clusters 1-3) are shown in Table 5.1.

5.2.2 Phylogeny and phylogenetic diversity of Antarctic sponges

Antarctic sponges nested within several nominal demosponge orders, namely: Hadromerida, Halichondrida, Haplosclerida, Poecilosclerida and Spirophorida (Fig. 5.2). The families Polymastidae and Suberitidae, currently in Hadromerida, did not formed a monophylum. Polymastidae included two main clades corresponding to the genera *Polymastia* and *Tentorium* (referred in the figures and tables as *'Suberites'*). Interestingly, one Genbank sequence attributed to *Polymastia* was sister to a specimen of *'Suberites caminatus'* from the Ross Sea, and a sequence belonging to a specimen classified as *Sphaerotylus antarcticus* sampled in the Ross Sea was included within a clade corresponding to sequences of *Polymastia isidis* from this same area. Within the family Suberitidae, all specimens of *Homaxinella* n. sp. 1 formed a highly supported clade that included a specimen classified as *Iophon spatulatus* (Poecilocleride). This clade was

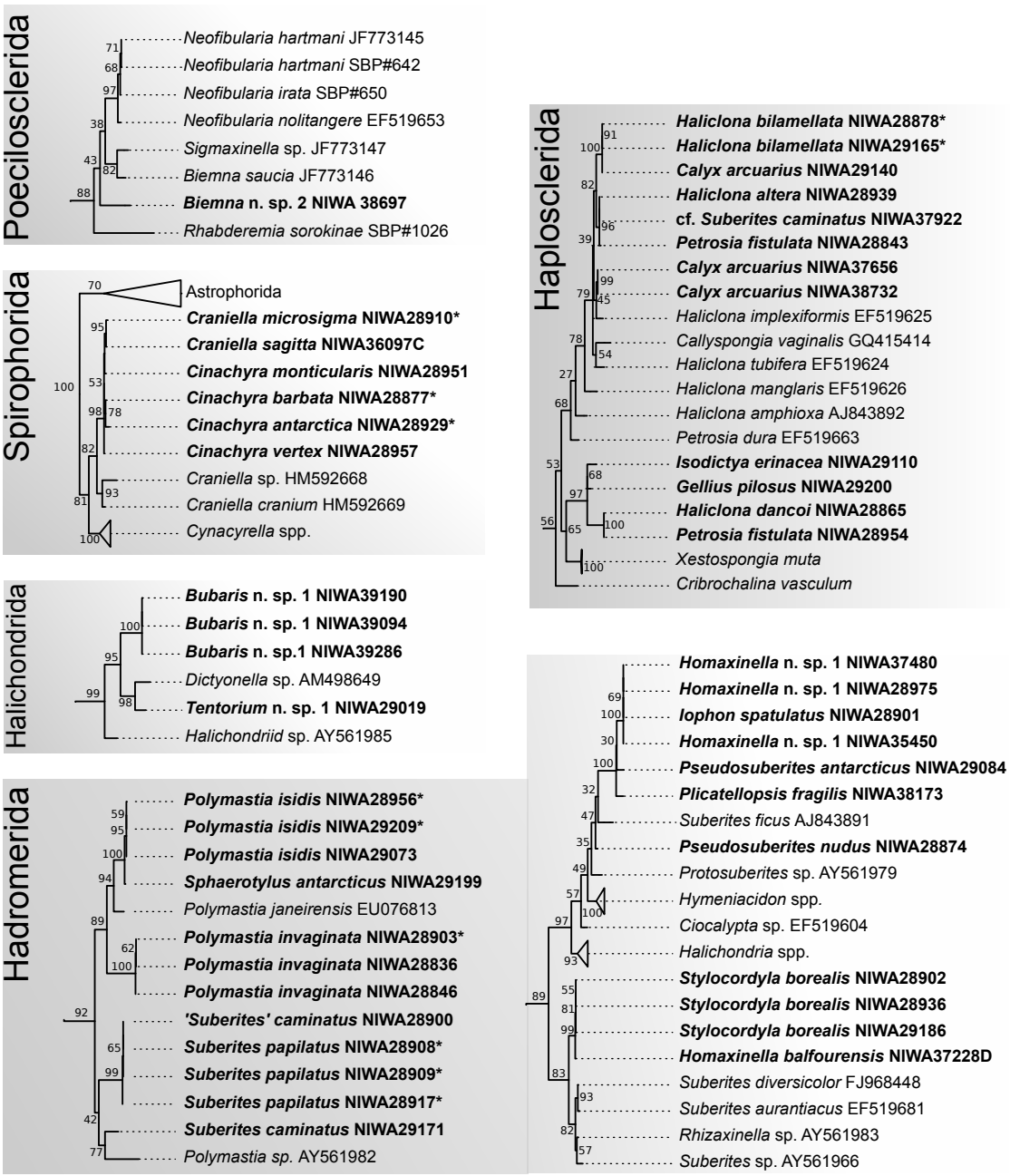
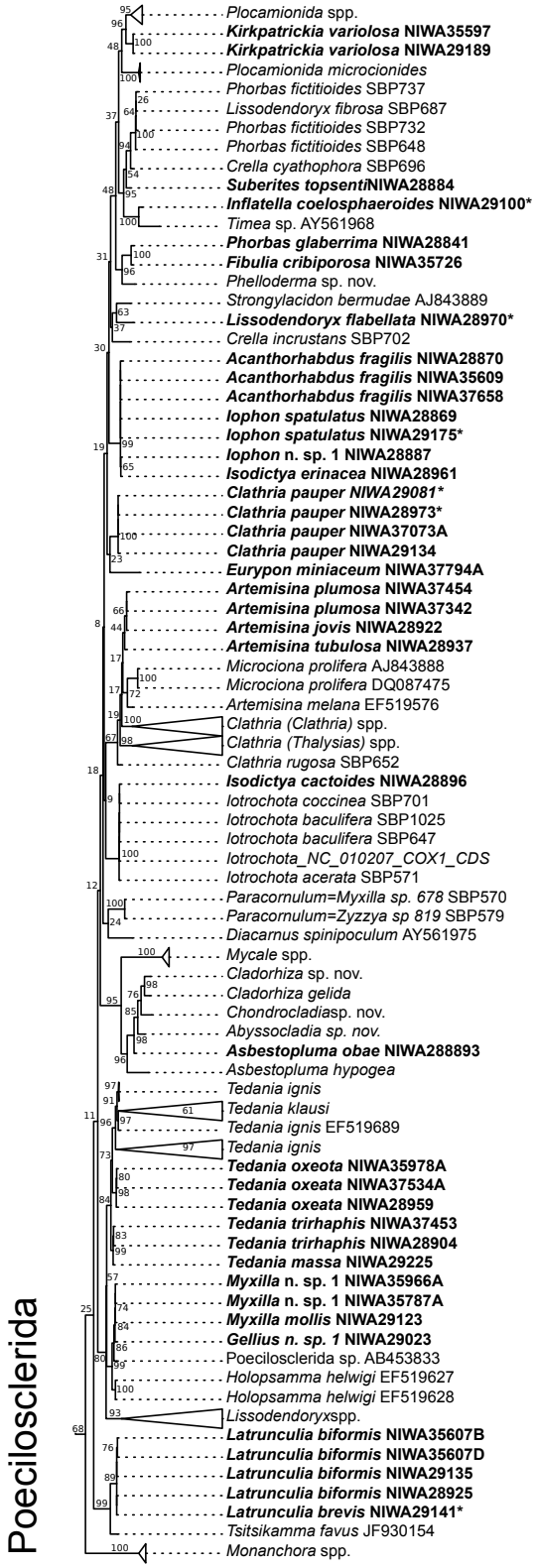


Figure 5.2: COI maximum likelihood phylogeny of Antarctic sponges (in bold face). Subtrees containing Antarctic sponges were pruned from the full tree. Bootstrap support is given near each branch of the tree. Exclusive Antarctic clades are annotated with an asterisk (*).


Poecilosclerida
Plocamionida spp.
Kirkpatrickia variolosa NIWA35597
Kirkpatrickia variolosa NIWA29189
Plocamionida microcionides
Phorbas fictitioides SBP737
Lissodendoryx fibrosa SBP687
Phorbas fictitioides SBP732
Phorbas fictitioides SBP648
Crella cyathophora SBP696
Suberites topsentiNIWA28884
Inflatella coelosphaeroides NIWA29100*
Timea sp. AY561968
Phorbas glaberrima NIWA28841
Fibulia cribiporosa NIWA35726
Phelloderma sp. nov.
Strongylacidon bermudae AJ843889
Lissodendoryx flabellata NIWA28970*
Crella incrustans SBP702
Acanthorhabdus fragilis NIWA28870
Acanthorhabdus fragilis NIWA35609
Acanthorhabdus fragilis NIWA37658
Iophon spatulatus NIWA28869
Iophon spatulatus NIWA29175*
Iophon n. sp. 1 NIWA28887
Isodictya erinacea NIWA28961
Clathria pauper NIWA29081*
Clathria pauper NIWA28973*
Clathria pauper NIWA37073A
Clathria pauper NIWA29134
Eurypon miniaceum NIWA37794A
Artemisina plumosa NIWA37454
Artemisina plumosa NIWA37342
Artemisina jovis NIWA28922
Artemisina tubulosa NIWA28937
Microciona prolifera AJ843888
Microciona prolifera DQ087475
Artemisina melana EF519576
Clathria (Clathria) spp.
Clathria (Thalysias) spp.
Clathria rugosa SBP652
Isodictya cactoides NIWA28896
Iotrochota coccinea SBP701
Iotrochota baculifera SBP1025
Iotrochota baculifera SBP647
Iotrochota_NC_010207_COX1_CDS
Iotrochota acerata SBP571
Paracornulum=Myxilla sp. 678 SBP570
Paracornulum=Zyzzya sp 819 SBP579
Diacarnus spinipoculum AY561975
Mycale spp.
Cladorhiza sp. nov.
Cladorhiza gelida
Chondrocladiasp. nov.
Abyssocladia sp. nov.
Asbestopluma obae NIWA288893
Asbestopluma hypogea
Tedania ignis
Tedania klausi
Tedania ignis EF519689
Tedania ignis
Tedania oxeota NIWA35978A
Tedania oxeata NIWA37534A
Tedania oxeata NIWA28959
Tedania trirhaphis NIWA37453
Tedania trirhaphis NIWA28904
Tedania massa NIWA29225
Myxilla n. sp. 1 NIWA35966A
Myxilla n. sp. 1 NIWA35787A
Myxilla mollis NIWA29123
Gellius n. sp. 1 NIWA29023
Poecilosclerida sp. AB453833
Holopsamma helwigi EF519627
Holopsamma helwigi EF519628
Lissodendoryxspp.
Latrunculia biformis NIWA35607B
Latrunculia biformis NIWA35607D
Latrunculia biformis NIWA29135
Latrunculia biformis NIWA28925
Latrunculia brevis NIWA29141*
Tsitsikamma favus JF930154
Monanchora spp.

related with high support to two Antarctic species of the genus *Plicatellopsis*, which did not formed a monophylum in the ML tree. The genus *Pseudosuberites* and specimens of *Stylocordyla borealis* (Stylocordilidae) were included in Suberitidae. In the Order Halichondrida, Ross Sea specimens of *Bubaris* n. sp. 1 (Bubaridae) formed a highly supported monophylum sister to a clade that included, with high support, a GenBank sequence attributed to *Dyctionella* and a Ross Sea specimen identified as *Tentorium* n. sp. 1. Antarctic marine haplosclerids were distributed in four clades which included (1) the species *Haliclona bilamellata* and one specimen of *Calix arcuarius*, (2) *Petrosia fistulata* and *Haliclona altera* together with a specimen determined as cf. *'Suberites caminatus'*, (3) a second group of *C. arcuarius* specimens and *Haliclona implexiformis*, and (4) a clade composed of *Haliclona dancoi*, *P. fistulata*, *Gellius pilosus* and *Isodyctia erinacea*, a poecilosclerid sponge. Members of Spirophorida, specifically species of *Cinachyra* and *Craniella*, from the Ross Sea formed a highly supported clade. The genus *Craniella* was not monophyletic but its species were split in two clades. Finally, the order Poecilosclerida was not monophyletic. Species of the families Desmacellidae and Rhabderemidae were not related to chelae-bearing poecilosclerids, which formed a monophylum. Among Antarctic poecilosclerids, *Kirkpatrickia variolosa* was sister to species of *Plocamionida*. *Phorbas glaberrimus* was not related to other species of *Phorbas* available in Genbank but to *Fibulia cribiporosa* from the Ross Sea, and *Lissodendoryx flabellata* was not related to other species of the genus included in the phylogenetic analysis. Species of *Iophon* formed a highly supported clade with *Acanthorhabdus fragilis* and *Isodictya erinacea*, this last species was not related to *Isodictya cactoides* which was included in a clade with

species of *Iotrochota*. All specimens of *Clathria pauper* formed a highly supported monophylum that was not related to other members of the genus *Clathria*. Within this genus, the Antarctic species of *Artemisina* formed a clade and were not related to *Artemisina melana* from Genbank. The genus *Tedania* was monophyletic, with *Tedania oxeata* more closely related to *Tedania ignis*+*Tedania klausi* than to other Antarctic species (i.e. *Tedania trirhaphis* and *Tedania massa*). Antarctic *Myxilla* formed a highly supported clade sister to a specimen identified as *Gellius* n. sp. 1 from the Ross Sea. Finally, the genus *Tsitsikamma* and the Antarctic species of *Latrunculia* formed a highly supported monophylum.

The rarified inclusive phylogenetic diversity for seven sampled MPs is shown in Figure 5.3. The NBS was the least diverse MP sampled while the TNWA showed the highest PD_I values. The Continental High Antarctic (CHA) MP, represented here by the Ross Sea barcoded sponges, had intermediate PD_I values comparable to the L and MS provinces. Interestingly, the amount of phylogenetic diversity attributable to *in situ* evolution (Fig. 5.3) within an area was maximal for the CHA and the TNWA, where PD_E accounted for ~35% of the PD_I of both areas. PD_E was minimal for NBS and NES (11% and 17% respectively) and reached intermediate values (17-27%) for the L, MS and WTNWA.

5.2.3 DNA-barcoding assignment power

The SSA correctly assigned ~54% of the species in the Ross Sea shelf sample. Among the remaining species, ~47% were found to be either potential misidentifications or groups of specimens which taxonomy deserves to be revised (see Discussion) and 53% can be

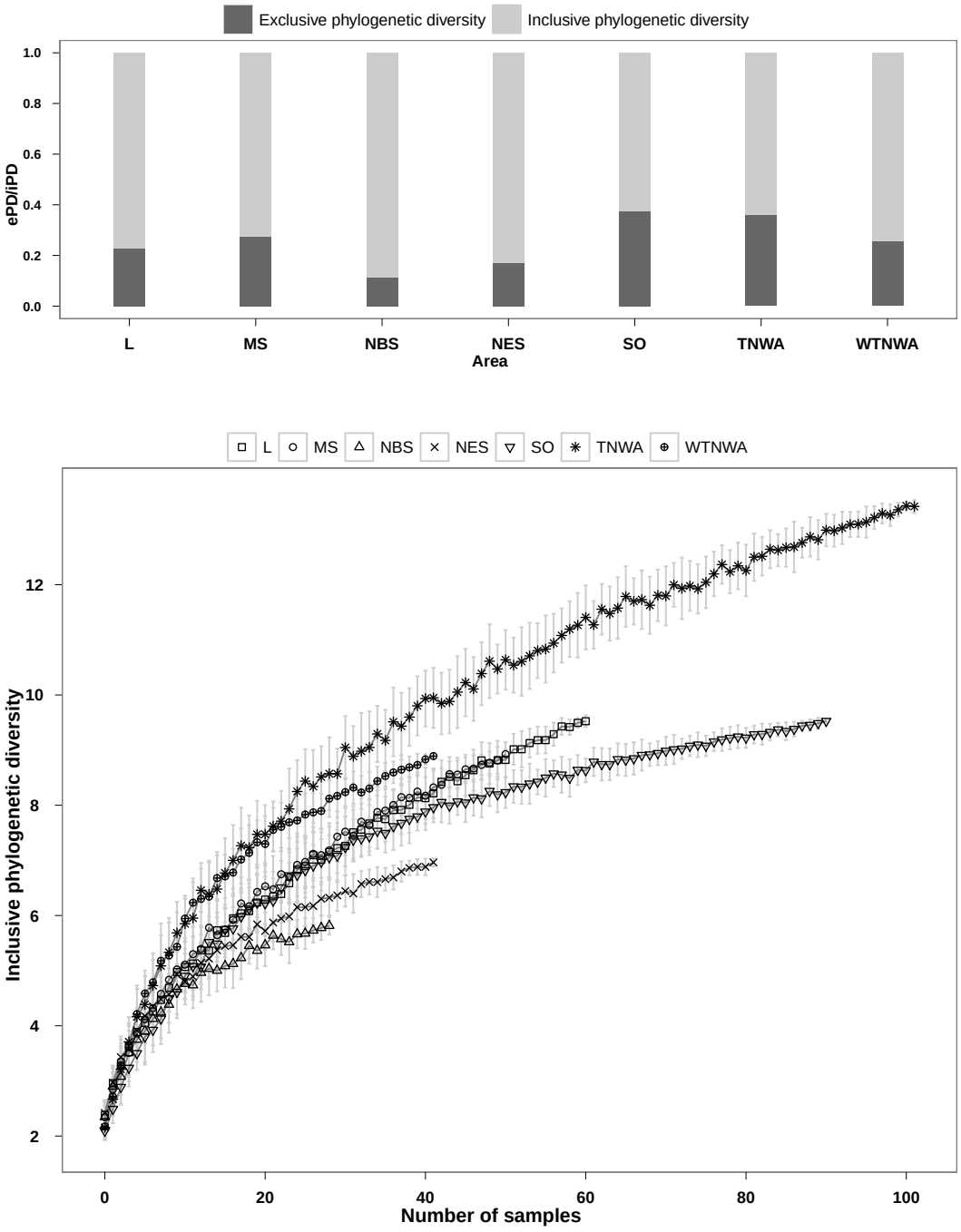


Figure 5.3: Sponge phylogenetic diversity of seven marine provinces in the Atlantic and Southern Oceans. A. Rarified inclusive phylogenetic diversity. B. Exclusive to Inclusive phylogenetic diversity ratio.

attributed to the incompleteness of the candidate species database. Figure 5.4 shows the standardized assignment risk resulting from the leave-one-out cross validation SSA trials. In general, the assignment risk using the standard COI barcoding fragment remained low to moderate within families or orders and increased between them. In the case of specimens for which only one sequence was available, SSA was able to assign the queries to related genera in the same family (e.g. *Cinachyra* as *Craniella*, *Iophon* as *Acantorhabdus*, and *Plicatellopsis* as *Homaxinella*) or clade (e.g. *Fibulia* as *Phorbas* and vice versa)(Table 5.2).

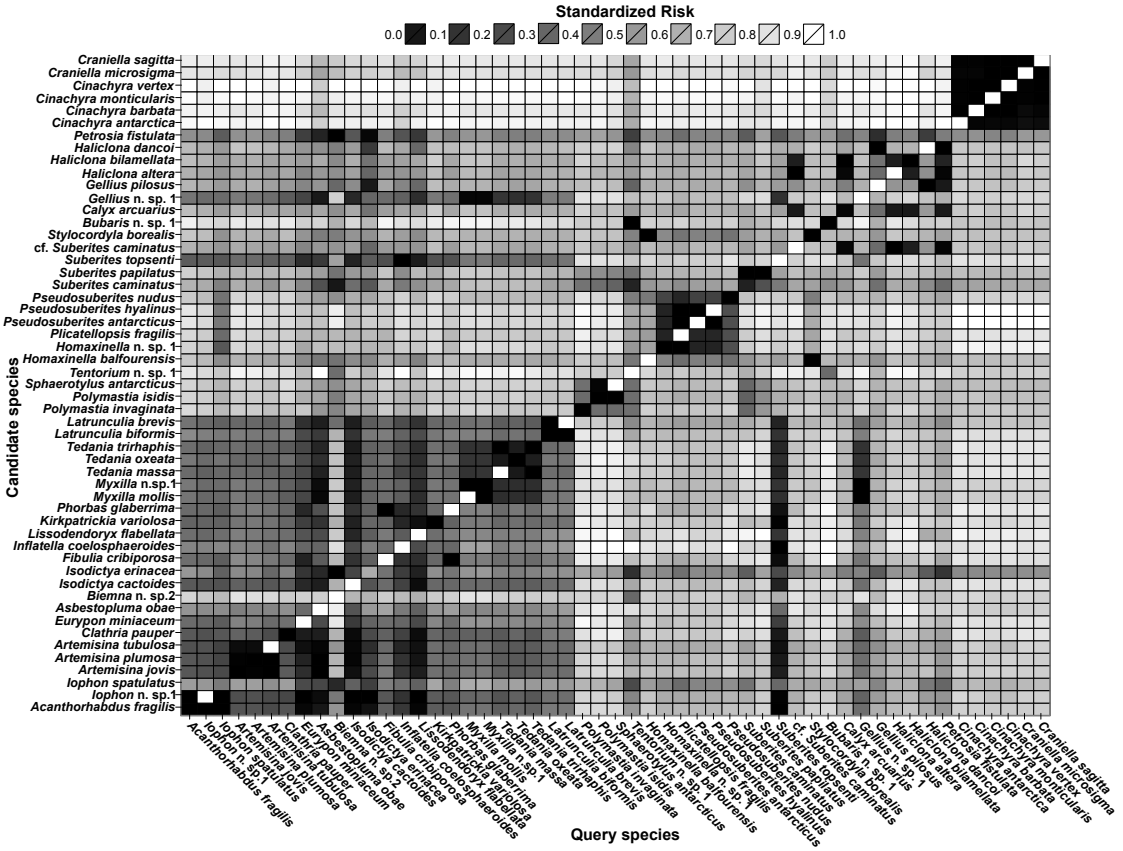


Figure 5.4: Classification accuracy, measured as standardized assignment risk, of the Segregating Sites Algorithm for Antarctic sponges

Table 5.2: Minimum risk species assignment obtained in the leave-one-out SSA trials. Correctly assigned species are in bold face. If a query had two minimum risk assignments, the candidate species are separated by |.

Query Species	Minimum Risk Assignment
Acanthorhabdus fragilis	*Acanthorhabdus fragilis*
Artemisina jovis	*Artemisina jovis*
Artemisina plumosa	*Artemisina plumosa*
Artemisina tubulosa	*Artemisina plumosa* | *Artemisina jovis*
Asbestopluma obae	*Artemisina plumosa* | *Artemisina jovis*
Biemna n. sp. 2	*Petrosia fistulata*
Bubaris n. sp. 1	*Bubaris* n. sp. 1
Calyx arcuarius	*Haliclona bilamellata*
cf. *Suberites caminatus*	*Haliclona altera*
Cinachyra antarctica	*Cinachyra barbata*
Cinachyra barbata	*Cinachyra monticularis* | *Cinachyra vertex*
Cinachyra monticularis	*Craniella sagitta*
Cinachyra vertex	*Craniella sagitta*
Clathria pauper	*Clathria pauper*
Craniella microsigma	*Craniella sagitta*
Craniella sagitta	*Craniella microsigma*
Eurypon miniaceum	*Iophon* n. sp. 1
Fibulia cribiporosa	*Phorbas glaberrima*
Gellius n. sp. 1	*Myxilla* n. sp. 1
Gellius pilosus	*Haliclona dancoi*
Haliclona altera	cf. *Suberites caminatus*
Haliclona bilamellata	*Haliclona bilamellata*
Haliclona dancoi	*Gellius pilosus*
Homaxinella balfourensis	*Stylocordyla borealis*
Homaxinella n. sp. 1	*Homaxinella* n. sp. 1
Inflatella coelosphaeroides	*Suberites topsenti*
Iophon n. sp. 1	*Acanthorhabdus fragilis*
Iophon spatulatus	*Iophon* n. sp. 1
Isodictya cactoides	*Iophon* n. sp. 1
Isodictya erinacea	*Iophon* n. sp. 1
Kirkpatrickia variolosa	*Kirkpatrickia variolosa*
Latrunculia biformis	*Latrunculia brevis*
Latrunculia brevis	*Latrunculia biformis*
Lissodendoryx flabellata	*Iophon n. sp. 1*

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Myxilla mollis	*Myxilla* n. sp. 1
Myxilla n. sp. 1	*Myxilla mollis*
Petrosia fistulata	*Haliclona dancoi*
Phorbas glaberrima	*Fibulia cribiporosa*
Plicatellopsis fragilis	*Homaxinella* n. sp. 1
Polymastia invaginata	*Polymastia invaginata*
Polymastia isidis	*Polymastia isidis*
Pseudosuberites antarcticus	*Pseudosuberites hyalinus*
Pseudosuberites hyalinus	*Pseudosuberites antarcticus*
Pseudosuberites nudus	*Pseudosuberites nudus*
Sphaerotylus antarcticus	*Polymastia isidis*
Stylocordyla borealis	*Stylocordyla borealis*
Suberites caminatus	*Suberites papilatus*
Suberites papilatus	*Suberites papilatus*
Suberites topsenti	*Inflatella coelosphaeroides*
Tedania massa	*Tedania trirhaphis*
Tedania oxeata	*Tedania oxeata*
Tedania trirhaphis	*Tedania trirhaphis*
Tentorium n. sp. 1	*Bubaris* n. sp. 1

5.3 Discussion

We have presented the results of the first DNA-barcoding campaign directed towards sequencing a comprehensive sample of sponges collected in the Ross Sea, Antarctica. Ross Sea sponges were found to be part of three distinct shelf assemblages that were highly correlated with their depth of occurrence. These assemblages are, likely, variations of (Gutt, 2007) broadly defined Suspension feeders community (SFC), which occur around Antarctica from ~30m down to the shelf break. The existence of distinct, identifiable, sponge assemblages within the SFC showing a strong relationship with bathymetry indicates that Antarctic SFCs might be more heterogeneous than previously thought. Clear bathymetric distribution patterns have been also documented for hexactinellids

inhabiting the slope and abyssal realms of the Weddell Sea (Gocke and Janussen, 2011), and appear to be also present in Antarctic shelf demosponges.

In terms of the coverage reached by our barcoding campaign, 81% (N = 21) of the characteristic demosponge species (N = 26) of the three shelf assemblages found were successfully barcoded (Table 5.1), and 32 additional (non-characteristic) species were sequenced to account for 60% of the demosponge species collected during NIWA's BioRoss and IPY expeditions. In this respect, DNA-barcoding was successful in rapidly gathering information about Antarctic sponge communities that can be used for phylogenetic inference (Fig. 5.2) and phylo-diversity comparisons (Fig. 5.3), or for sorting large collections and complement taxonomic work. For instance, the inferred COI gene-tree (Fig. 5.2) clearly pointed potential misidentifications as well as specimens in need of deeper taxonomic examination, such as a specimen labelled *S. antarcticus* which formed a monophyletic clade with specimens of *P. isidis* and, consequently, had no sphaerotyles diagnostic of *Sphaerotylus* species (Boury-Esnault, 2002). Similarly, a specimen identified as cf. *'S. caminatus'* was included in a clade together with *H. altera* and *P. fistulata* and, consistently, had no tylostyles among its spicule complement but oxeas. Also within Haplosclerida, the COI ML phylogeny revealed a rather diverse, highly supported clade joining *I. erinacea*, *G. pilosus*, *H. dancoi* and *P. fistulata* together. In this clade, the specimen of *I. erinacea* had no chelae but sigma microscleres in agreement with the description of *Gellius* (Desqueyroux-Faundez and Valentine, 2002a), and *P. fistulata* had sigmas despite being described as lacking microscleres (Desqueyroux-Faundez and Valentine, 2002b). It is interesting to note that Burton (1929) (cited in Desqueyroux-Faundez and Valen-

tine, 2002b) considered *P. fistulata* a juvenile of *H. dancoi* because of their morphological similarity, thus it is likely that these species are confused on a regular basis. In contrast to the examples above, all the barcoded specimens of *C. arcuarius* had toxas and oxeas in accordance with the species description (Desqueyroux-Faundez and Valentine, 2002b). The oxeas of these specimens were somewhat different, being either stout or more delicate, and pointing to the need of further taxonomic work on these specimens. Two more cases worth noting, are the exclusion of *C. pauper* from *Clathria* suggested by de Laubenfels (1936) and the inclusion of *Suberites (Laxosuberella) topsenti* in Poecilosclerida foreseen by MK who annotated this species as "related to *Semisuberites*" because its "tylostyles are mycalostyles", both hypotheses are here corroborated. Table 5.3 summarizes the potential misidentifications or contaminations detected during the analysis of the barcode dataset.

Sponge taxonomy and nomenclature are complicated and can easily obfuscate the study of diversity patterns among sponges. It has been suggested that the diversity of Antarctica's sponge communities is comparable to that of tropical ecosystems (McClintock et al., 2005). The analysis of the phylogenetic diversity of the Ross Sea collection revealed PD_I values comparable to those observed in subtropical MPs (i.e. L and MS) and higher than those of some MPs in tropical realms (e.g. NBS). This is remarkable given that the Ross Sea represents only one of the six Marine Ecoregions of the World (MEOW) grouped within the Continental High Antarctic MP, whereas the Lusitanic and Mediterranean Sea MPs include three and seven MEOWs each. Thus, it is very likely that the inclusion of a more comprehensive sample of sponges from other ME-

Table 5.3: Examples of potential misidentifications or contaminations detected during the Ross Sea DNA-barcoding campaign. Cases in which an unexpected placement in the COI ML gene-tree was obtained are also included.

Species	Observations
Calyx arcuarius	Two group of specimens showing stout or more delicate oxeas.
Fibulia cribiporosa	The species appears to be correctly assigned. Acanthostyles were not present among the specimen's megascleres as expected if the barcode resulted from a contamination with tissue from *P. glaberrimus*.
Gellius n. sp. 1	Likely a contamination: arcuate chelae observed in spicule preparation in agreement with the topological position obtained.
Homaxinella balfourensis	Likely a misidentification. The remarcks on this specimen clearly state "lollipop sponge" (=*Stylocordyla borealis*) but the database annotate the specimen as *H. balfourensis*.
Isodictya cactoides	No birotula spicules were found in an spicule preparation of this specimen. However, more samples need to be sequenced to corroborate this barcode.
Iophon spatulatus	The specimen had no chelae, consequently, it is likely a misidentification.
Petrosia fistulata	Likely a mixture of specimens one of which is compatible with *H. dancoi*. Specimens need to be revised.
Phorbas glaberrimus	This specimen had arcuate chelae and acanthostyles in accordance with its generic assignment.
Sphaerotylus antarcticus	No sphaerotyles were observed in the spicule preparation.
Suberites topsenti	No chelae were found in an spicule preparation as expected if the barcode was the result of tissue contamination.
cf. *Suberites caminatus*	No tylostyles but oxeas, likely a misidentification.

OWs within the Continental High Antarctic MP will results in Antarctic PD_I values comparable to those of tropical MPs (e.g. TNWA).

The high PD_E/PD_I ratio observed for the Ross Sea sample implies that a high proportion of substitution changes in the standard COI barcoding fragment can be attributed to *in situ* (within the Southern Ocean) evolutionary change (Lewis and Lewis, 2005). This pattern of evolution can be the result of Antarctica's long age and high isolation (Sara et al., 1992; Lawver and Gahagan, 2003), especially considering that the rates of evolution of Antarctic organisms are expected to be generally low (Held, 2001; Clarke, 2008). It also favors, albeit indirectly, the hypothesis of an ancient (Gondwanan) origin followed by *in situ* diversification of Antarctic sponges. Our COI gene-tree included some Antarctic exclusive clades (Fig. 5.2) in Haplosclerida and Hadromerida as expected under a model of *in situ* diversification, however other groups (e.g. *Tedania* in Poecilosclerida or *Polymastia* in Hadromerida) revealed that more complex historical processes likely played a role in shaping Antarctic demosponge diversity patterns. A more extensive species sample, ideally covering various localities in Antarctica as well as neighboring regions, would be necessary to evaluate the biogeographic significance of the evolutionary pattern here detected (i.e. high *in situ* evolutionary change).

DNA-barcoding can be used to sort and classify new collections from a particular geographic region provided that there is a reliable candidate species database available for this task. Here, using the standard barcoding (COI) fragment and the SSA we were able to correctly identified ~54% of the sampled species. Considering that DNA-barcoding can be easily done for many taxonomic groups simultaneously by technical staff trained

in general molecular biology techniques, this method has the potential for reducing a taxonomist's work load allowing specialists to focus only on specimens not accurately classified through barcoding. These specimens can either be species absent from the original candidate species database or groups with a complex taxonomy in need of detailed revisionary work. Additionally, DNA-barcoding can be used in conjunction with traditional morphological studies to simultaneously provide access to genotypic and phenotypic data for a (potentially) large number of specimens, promoting integrative taxonomic research and cross validation between both identification approaches. A more heretical alternative would be to use DNA-barcodes without considering nomenclature for ecological or evolutionary research. We have done this with success for the analysis of sponge phylo-diversity, and there is no reason why the same approach cannot be used for the study of other aspects of ecology or organismic biology in sponges or other organisms.

5.4 Materials and Methods

5.4.1 Sample collection and characterization of the Ross Sea sponge assemblages

Sponges were collected mainly by dredging during the National Institute of Water and Atmosphere's (NIWA) BioRoss (2004) and IPY (2008) expeditions to the Ross Sea, Antarctica. Sponges were sorted and frozen on board, and latter transferred to and stored in 70% ethanol. They were taxonomically determined and accessioned into NIWA's Invertebrate Collection (NIC), Wellington, New Zealand. The identified collec-

tions were used to build a species by station presence-absence matrix. This matrix only included stations with at least 5 sponge species. We used the presence-absence matrix in cluster analysis (average linkage) and Non-metric multidimentional scaling (NMDS) using the Jaccard distance in both analyses. Station clusters were determined at a cutoff height of 0.95, and the characteristic species set for each cluster was determined using a constancy table; a species was considered characteristic if it was present in at least, 25% of the stations grouped in a cluster and was absent (or occurred in less than 25% of the stations) from other clusters. Furthermore, using the NMDS results, we assessed the relationship between depth and faunistic composition of the sampled stations using Generalized Additive Models with a normal link.

5.4.2 Molecular methods

Genomic DNA was extracted from pieces of pre-frozen, 70% ethanol-preserved sponge tissue using a modified (Ivanova et al., 2006) extraction protocol (see Chapter 4). The standard barcoding fragment was initially amplified using primers dgLCO1490 and dgHCO2198 (Meyer et al., 2005), however, using this primer set we mainly obtained sequences from non-target organisms, especially bacteria. In order to reduce the amplification of non-target organisms a new forward primer (SpongeCOI-F1: 5'-**ACATTTTGCTGCCGGTCA**GATAGGDACWGCNTTTA-3'), supplemented with an M13 tail (in bold face), was designed to specifically exclude bacteria. Using this new primer we exclusively amplified sponge DNA. We used a standard three-step PCR consisting of an initial denaturation step of 94°C for 3 minutes, 35-40 cycles consisting

each of a denaturation step of 94°C for 30 seconds, an annealing step of 40°C for 30 seconds and an extension step of 72°C for 1 minute, and, finally, a last extension step of 72°C for 5 minutes. For samples that proved difficult to amplify samples we used a semi-nested approach in which a first, short PCR reaction (15-20 cycles; PCR program as above) using the primers dgLCO1490 and dgHCO2198 was re-amplified using primers SpongeCOI-F1+dgHCO2198 (35-40 cycles; PCR program as above). Negative controls were used for both DNA extractions and PCR reactions to monitor potential contaminations. If the semi-nested PCR approach was used, the negative control from the first PCR was also re-amplified. PCR products were excised from a 1.5% agarose gel (TAE) and purified using a modified freeze-squeeze method. Sequencing was carried out in both directions using primer M13-20F and dgHCO2198 and the Big Dye Terminator version 3.1 (Applied Biosystems) chemistry. Sequencing reactions were cleaned-up using a standard ethanol-ammonium acetate precipitation following the BigDye Terminator 3.1 manual protocol and analyzed on an ABI 3730 Genetic Analyzer at the Sequencing Service of the Department of Biology, LMU München. Trace files were assembled in CodonCode Aligner (CodonCode Corporation) and the sponge origin of the sequences was confirmed using BLAST. Sequences will be deposited at EMBL upon submission for stand-alone publication[1].

[1]The final taxonomic assignment of the specimens is currently being revised (M. Kelly) based on the results of this Chapter. In order to avoid later corrections to the deposited sequences the submission to EMBL has been delayed.

5.4.3 Phylogenetic methods and phylogenetic diversity

Newly obtained COI sequences and sponge COI sequences available at the NCBI Genbank (Query = Porifera AND COI; Query date: 16 Sept. 2011) were aligned in Seaview (Gouy et al., 2010) using the Muscle algorithm (v3.8.31 Edgar, 2004) with default settings. The resulting alignment (available at `http://dx.doi.org/10.5282/ubm/data.48` as a mase file) was used to infer a bootstrapped Maximum Likelihood (ML) tree (1000 fast pseudo-replicates Stamatakis et al., 2008) using RAxML 7.2.8 (Stamatakis, 2006). We used the GTR model of sequence evolution and accounted for among-site rate variation using a discrete gamma distribution with 4 categories. Bayesian phylogenetic analyses were done under the same model settings as above but were not used further because the estimation of branch-lengths in a Bayesian context is problematic (Brown et al., 2010), and most of our analyses (see below) use the branch-lengths of the COI gene-tree.

The ML topology was used to estimate inclusive and exclusive phylogenetic diversity (denoted PD_I and PD_E, respectively Lewis and Lewis, 2005) for the Ross Sea shelf sponge samples and for several Marine Provinces (MPs; *sensu* Spalding et al., 2007) that have been extensively sampled for molecular studies (e.g. Erpenbeck et al., 2007). We annotated each species in the ML tree as present/absent in the Northern European Seas (NES), the Lusitanian (L), the Mediterranean Sea (MS), and the Warm Temperate Northwest Atlantic (WTNWA) MPs in the Temperate Northern Atlantic Marine Realm, and the Tropical Northwest Atlantic and North Brazil Shelf MPs in the Tropical Atlantic Marine Realm. Species lists for these MPs were obtained from the World Porifera Database. Direct PD_I comparisons between MPs are difficult due to the distinct sampling efforts

historically allocated to them. We resorted to rarefaction (Sanders, 1968) to make comparisons between MPs with different sampling efforts possible. In brief, if N leaves on the ML tree are known to occur in a given MP, we subsampled $k = 1, \ldots, N$ leaves and estimated the PD_I of the taxon set k. Because there are $\frac{n!}{k!(n-k)!}$ ways to sample k taxa from a set of N taxa, we used Monte Carlo sampling to approximate the phylogenetic diversity for any given $k < N$ (Simberloff, 1972). For a fixed number of pseudoreplicates, k terminals were randomly chosen and the complement set k' was pruned from the ML tree. The inclusive phylogenetic diversity of the sample k ($PD_{I,k}$) was determined as the difference between the total length of the ML tree and the length of the k'-pruned tree. The rarified phylogenetic diversity values were used for inter-MP comparisons. In addition, for all MPs we estimated the $\frac{PD_E}{PD_I}$ ratio for the total sample of taxa available for a given MP (i.e. N).

5.4.4 DNA-barcoding

In order to assess the assignment power of the COI barcode in the case of Antarctic sponge species, we used leave-one-out cross-validation and the Segregating Sites Algorithm (SSA; Lou and Golding, 2010). The SSA uses a Bayesian approach to assign sequences of unknown taxonomic affiliation to a set of sequences of known taxonomic origin. Each COI sequence obtained from Ross Sea sponges was used as a query against a dataset consisting of the remaining sequences, and the assignment risk of the query to each species in the candidate species dataset was determined. A species assignment was considered correct if the minimum risk candidate species matched the queried species

or, in the case of species for which only one sequence was available, if the minimum risk candidate species matched the genus of the query species. To facilitate comparisons, after each leave-one-out trial the assignment risk (R) of the query sequence, i, to each sequence, j, in the dataset ($R_{i,j}$) was range standardized using the formula $R^s_{i,j} = \frac{(R_{i,j} min(R))}{(max(R)+min(R))}$.

Concluding remarcks

The taxonomy of the important glass sponge genus *Rossella* was clarified using two different monophyly tests in conjunction with a thorough topological analysis. The most appropriate taxonomy for the Southern Ocean *Rossella*, likely, contains only two species, implying that a single species or species complex is responsible for a considerable proportion of the biomass produced by the Antarctic megabenthos, highlighting the need for a better understanding on the causes of the somewhat striking morphological variation observed within *Rossella racovitzae*, and pointing to the need of revising and further testing the classification of this species complex.

Based on its morphology, *Abyssocladia* has been related to the Family Cladorhizidae and to the genus *Phelloderma* (Phellodermidae), depending on expert criterion and on contrasting interpretations of the morphological evolution in poecilosclerid sponges. Here, using two independent molecular markers to test both hypotheses the affinities of *Abyssocladia* with Cladorhizidae, and the monophyly of this family were corroborated. The monophyly of Cladorhizidae provides evidence for the independent evolution of chelae microscleres, long thought to accurately reflect the evolutionary history of poecilosclerid sponges and used as the basis for the classification of this Order. The homoplasic evolution of chelae suggests that the taxonomy of Poecilosclerida deserves to be revised based on molecular and morphological evidences.

The deep waters of Antarctica are difficult to sample, and the number of species that occur in the vast abyssal plains of the Southern Ocean remain unknown. In this

study, lower bounds for the number of sponge species occurring in the waters of the Weddell Sea, Western Antarctica, are provided. These results are dependent on the spatial distribution of the taxa involved in the extrapolation procedure increasing the number of predicted species as assumed the level of patchiness in the spatial distribution of the sampled taxa increased. In addition, the results of the species richness prediction provide guidelines for future sampling and highlight the need of dedicated research programs to achieve the levels of sampling required to have a complete list of taxa occurring in deep Southern Ocean habitats.

DNA barcoding, the use of standard DNA markers for species identification, can provide a reliable system for the rapid assessment of the molecular phylogenetic diversity of biological communities world wide. Here, an analytical workflow for the processing of sponges for DNA barcoding was established and used for the barcoding of Antarctic sponges communities inhabiting the shelf and slope of the Ross Sea. The barcoding campaign serve to demonstrate that Ross Sea sponge assemblages are as diverse as their counterparts in temperate ecosystems and that *in situ* evolution has accounted for a high number of mutation steps in Antarctic lineages. These results suggest that the high diversity of sponges in Antarctica are the product of a long history of isolation in the continent's waters, and serve as a clear case-study on the use of DNA barcoding for integrative taxonomic research among poriferans.

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Appendixes

Other contributions by the author of this dissertation

Precious coral and rock sponge gardens on the deep aphotic fore-reef of Osprey Reef (Coral Sea, Australia)

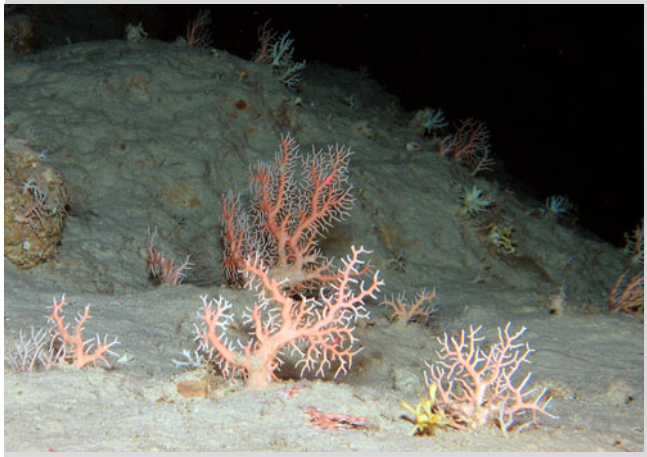

Fig. 1 Garden of precious corals (*Corallium* sp.) at North Horn (Osprey Reef, 265 m depth). Size of colonies about 30 cm (centre foreground). *Photo*: Marum

Fig. 2 Garden of rock sponges ('Lithistida', the dirty round 'balls'), red ophiuroids, a crinoid and cold-water corals (*Madrepora* sp., *left* and *right* side of image) at North Horn (Osprey Reef, 375 m depth). *Photo*: Marum

In December 2009, the Deep Down Under Expedition (www.deepdownunder.de) explored the deep fore-reef slopes of the Queensland Plateau's western reefs in the Coral Sea to a depth of 850 m. Due to its rich biodiversity and heritage value, the Coral Sea was declared a Conservation Zone (Coral Sea Conservation Zone, CSCZ) by the Australian Government in May 2009. However, while the uniqueness and importance of Coral Sea shallow-water reefs have been recognized, knowledge of sub-photic benthic communities is deficient (see also Bongaerts et al. 2011). An inspection-class remotely operated vehicle (ROV 'Cherokee', Marum, Bremen) was deployed at Osprey (13°50S 146°32E) and Bougainville (15°29S 147°05E) Reefs to explore deep aphotic benthic communities. For the first time in tropical eastern Australia, gardens of precious corals (*Corallium* sp.; Fig. 1) as well as rock sponges ('Lithistida') and cold-water corals (*Madrepora* sp.) (Fig. 2) were discovered on the walls of Osprey Reef near North Horn, in depths of 265 and 375 m, respectively. Several new species of glass sponges that have reef-building potential were also recently described from deep Osprey Reef (Dohrmann et al. 2011). The discovery of these previously unknown and unique communities on the deep Coral Sea fore-reefs underpins the importance of the CSCZ as an exceptional biodiversity resource that warrants continued protection, scientific exploration and documentation.

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ERRATUM

Erratum to: Precious coral and rock sponge gardens on the deep aphotic fore-reef of Osprey Reef (Coral Sea, Australia)

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Erratum to: Coral Reefs (2011)
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During the course of video/still image analysis, some pictures unfortunately appear to have been mixed up. The coral colonies shown in Fig. 1 have not been sampled, and their identity is not confirmed. However, numerous *Corallium* sp. colonies were found at the same site in greater depth (602–627 m), as shown in Fig. S1 (attached), and their identity was confirmed by DNA barcoding. We would like to emphasize that the main message of our Reef Site article, the first discovery of precious corals on the aphotic reef slope of Osprey Reef, is still unchanged. We thank Rob Beaman (JCU) for pointing this out to us and Sarah Adolf (LMU) for assistance with DNA barcoding.

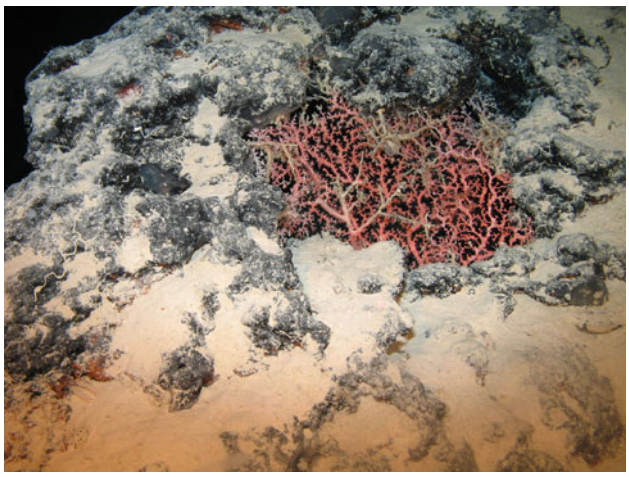

Fig. S1 *Corallium* sp. in 627 m depth at Osprey Reef (Coral Sea) (*reddish* colony in the *center*, *horizontal* size approx. 40 cm)

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Molecular paleobiology of early-branching animals: integrating DNA and fossils elucidates the evolutionary history of hexactinellid sponges

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Abstract.—Reconciliation of paleontological and molecular phylogenetic evidence holds great promise for a better understanding of the temporal succession of cladogenesis and character evolution, especially for taxa with a fragmentary fossil record and uncertain classification. In zoology, studies of this kind have largely been restricted to Bilateria. Hexactinellids (glass sponges) readily lend themselves to test such an approach for early-branching (non-bilaterian) animals: they have a long and rich fossil record, but for certain taxa paleontological evidence is still scarce or ambiguous. Furthermore, there is a lack of consensus for taxonomic interpretations, and discrepancies exist between neontological and paleontological classification systems. Using conservative fossil calibration constraints and the largest molecular phylogenetic dataset assembled for this group, we infer divergence times of crown-group Hexactinellida in a Bayesian relaxed molecular clock framework. With some notable exceptions, our results are largely congruent with interpretations of the hexactinellid fossil record, but also indicate long periods of undocumented evolution for several groups. This study illustrates the potential of an integrated molecular/paleobiological approach to reconstructing the evolution of challenging groups of organisms.

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Introduction

The fossil record provides the only tangible evidence of the temporal distribution of taxa, but it is well appreciated that these data are often incomplete. Furthermore, taxonomic interpretation of fossils can be difficult when key characters are not preserved, leaving much room for speculation. Molecular sequences provide an independent source of data that can be brought to bear on evolutionary questions. However, in order to use these data to elucidate the timing of evolutionary events by estimating dates of clade divergence, external information, typically taken from the fossil record, is required. Therefore, synthesizing paleontological and molecular data for a more holistic understanding of evolutionary history is both one of the most promising and challenging lines of modern paleobiological research (Brochu et al. 2004; Magallón 2004; Donoghue and Benton 2007; Peterson et al. 2007). However, taxa vary greatly in their suitability for such integrated analyses.

For diverse groups with still no appreciable fossil record, e.g. Platyhelminthes or Placozoa, analyses integrating paleontological data will probably never be possible. The evolution of other important groups for which there are only scattered fossil occurrences, usually as partly isolated Lagerstätten, e.g., Nematoda or Medusozoa, may also be prohibitively difficult to address with synthetic analyses combining fossil and molecular data because first fossil occurrences of subclades are not likely to correspond closely to their evolutionary origin (Cartwright and Collins 2007). A further difficulty is posed for groups that have a relatively rich fossil record, but whose phylogenetic histories are difficult to reconstruct with morphological data due to paucity of informative characters and high levels of homoplasy. Sponges (Porifera) are perhaps the most notorious example of such a taxon (see Hooper and van Soest 2002). Molecular systematics has greatly contributed to resolve relationships of extant sponges (Erpenbeck and Wörheide 2007), but has also generally indicated that traditional sponge classification and taxonomy is based on characters that do

not accurately reflect evolutionary history. Since morphology-based classification is already difficult for extant taxa, it is even more problematic for fossil sponges. Paleontologists naturally have to rely on morphological characters for taxonomic assignment, and the task is made more difficult because the poriferan fossil record is biased towards groups with fused or articulated skeletons, leaving substantial gaps for taxa that more readily disintegrate after death (Pisera 2006). This difficult situation, however, is less severe in the glass sponges (Hexactinellida), which have recently been shown to have an evolutionary history, as elucidated by molecular sequence data, that is largely consistent with the distribution of morphological features across its traditional taxa (Dohrmann et al. 2008, 2009). However, the fossil record presently provides incomplete or ambiguous evidence regarding the origin and evolution of extant hexactinellid subtaxa (see next section). Furthermore, the poor concordance between paleontological and neontological systematics greatly complicates matters (Krautter 2002; Reiswig 2006).

Here we use glass sponges to illustrate how a molecular paleobiological approach (Peterson et al. 2007) can enhance our understanding of the evolution of such "problematic" animal groups. We estimate divergence times of crown-group Hexactinellida from the largest molecular phylogenetic dataset assembled to date for this class of sponges (Dohrmann et al. 2011b), using a relaxed molecular clock approach (see e.g. Welch and Bromham 2005; Yang 2006 for reviews) and fossil-based age constraints for calibration. We then compare the dated phylogeny with the fossil record and discuss implications of congruencies and discrepancies between the two sources of evidence, thereby coming to an enhanced appreciation of hexactinellid evolution.

Fossil Record and Systematics of Glass Sponges.—Hexactinellids were important components of deep- and at times also shallower-water benthic ecosystems throughout the Phanerozoic, often associated with reef communities (e.g. Finks 1960; Mehl 1992; Brunton

and Dixon 1994; Leinfelder et al. 1994; Krautter et al. 2001; Carrera and Botting 2008). Their rich fossil record (see Krautter 2002; Pisera 2006) dates back to the late Neoproterozoic (Steiner et al. 1993; Gehling and Rigby 1996; Brasier et al. 1997). The two extant subclasses, Hexasterophora and Amphidiscophora, whose monophyly is strongly supported by both morphological and molecular data (Mehl 1992; Dohrmann et al. 2008), appear in the early Paleozoic, as indicated by isolated microscleres (Mostler 1986). Since the oldest hexasters (the defining autapomorphy of Hexasterophora) are known from the lowermost Ordovician, the hexasterophoran and amphidiscophoran stem-lineages must have already evolved during the Cambrian (Mostler 1986; Mehl, 1996). However, given that the earliest (late Ediacaran and early Cambrian) bodily preserved hexactinellid fossils (e.g. Steiner et al. 1993; Gehling and Rigby 1996; Brasier et al. 1997; Wu et al. 2005; Xiao et al. 2005) bear no resemblance to any specific extant subtaxon, it is likely that they represent stem-group members, implying that the origin of the crown-group (i.e., the split between the two subclasses), does not predate the Ediacaran/Cambrian boundary. Thus, while the origin of Hexactinellida from a common ancestor with demosponges certainly occurred in Precambrian times, their crown-group likely evolved rapidly as part of the Cambrian radiation (cf. Zhang and Pratt 1994; Reitner and Mehl 1995; Xiao et al. 2005; see also Erwin 2011).

Following a high Paleozoic diversity, most groups dominant in that Era had disappeared by the end of the Permian (Mostler 1990; Mehl 1996; Mehl-Janussen 1999; Krautter 2002). Because the taxonomically important microscleres are rarely preserved *in situ* (but see e.g. Kling and Reif 1969; Rigby et al. 2007 for notable exceptions), and Paleozoic skeletal architectures differ greatly from Mesozoic and modern forms (Mehl and Mostler 1993; Mehl 1996), relationships of these taxa to extant hexactinellids remain largely elusive. Thus, fossils assigned to modern families are mostly confined to the Mesozoic-Cenozoic. The fossil record of Amphidiscophora is poor (Mehl 1992, 1996), and the earliest crown-group member (family Hyalonematidae) was described from the Late Cretaceous (Mehl and Hauschke 1995).

Likewise, there is no conclusive evidence for Paleozoic Lyssacinosida, a hexasterophoran order characterized by largely unfused (lyssacine) skeletons (Mehl 1992, 1996). Although most Paleozoic glass sponges are lyssacine, assignment of early Paleozoic or even late Neoproterozoic fossils, solely based on this single character, to the Lyssacinosida (e.g. Krautter 2002) is highly questionable because this type of skeletal organization also characterizes Amphidiscophora and is therefore likely plesiomorphic (Mehl 1992). The modern families of Lyssacinosida (Rossellidae, Euplectellidae, Leucopsacidae) are definitely present by the Late Cretaceous (Salomon 1990; Brückner and Janussen 2005; Brückner 2006), but due to their limited fossilization potential the earlier history of Lyssacinosida remains obscure.

The earliest unambiguous evidence for crown-group Hexasterophora is the occurrence of dictyonal frameworks – rigid skeletons produced by fusion of hexactine megascleres – in the Late Devonian (e.g. Rigby et al. 1981, 2001; Rigby 1986; Mehl and Mostler 1993; Mehl 1996). These structures are diagnostic for the "Hexactinosida", a hexasterophoran order that underwent major radiations during the Mesozoic (Mehl 1992; Mehl and Mostler 1993; Pisera 1999) and is still abundant and diverse today (see Hooper and van Soest 2002; Leys et al. 2007). Curiously, dictyonal skeletons are not documented from the Carboniferous, Permian, and Early Triassic (Pisera and Bodzioch 1991; Mehl and Mostler 1993; Mehl 1996), which is hypothesized to be a preservational artefact (Mehl 1996; Rigby et al. 2001). Although molecular data (Dohrmann et al. 2008, 2009) suggest that the "Hexactinosida" are paraphyletic with respect to Lyssacinosida, the majority of hexactinosidans form a highly supported clade, the Sceptrulophora (Mehl 1992; Dohrmann et al. 2011a), which is the sister group of the remaining hexasterophorans (Dohrmann et al. 2008, 2009). This taxon is characterized by the possession of sceptrules, a scepter-like spicule type that occurs in various forms, mostly scopules or clavules (cf. Dohrmann et al. 2011a). In contrast, sceptrules are lacking in the Dactylocalycidae, which were resolved as the sister group of Lyssacinosida in

molecular studies (Dohrmann et al. 2009, 2011b). Spicule fragments interpreted as sceptrules have been reported from late Cambrian and Ordovician strata (e.g. Bengtson 1986; Webby and Trotter 1993; Dong and Knoll 1996; Kozur et al. 1996; Zhang and Pratt 2000). However, their poor preservation and the next appearance of sceptrules in the Triassic (Donofrio 1991; Krainer and Mostler 1991) raise doubts about the homology of the Paleozoic and Mesozoic-Recent forms.

Another important hexasterophoran taxon is the Lychniscosida, species-poor in today's oceans (Reiswig 2002a), but once highly diverse and reef-building. Lychniscosidans appeared in the Middle Jurassic (Pisera and Bodzioch 1991; Mehl 1992; Mehl and Mostler 1993; Pisera 1999) and also have dictyonal skeletons. However, their skeletons probably evolved convergently since they are built from lantern-like hexactins rather than simple hexactins (Mehl 1992). Lychniscosidans have not been sampled yet for molecular systematics, so the hypothesis that this taxon is nested within Lyssacinosida (Mehl 1992) remains to be tested.

In general, hexactinellid diversity gradually declined after a Late Cretaceous peak, which might be related to restrictions of shelf habitats (Mehl 1992) and/or changes in ocean chemistry (Maldonado et al. 1999).

Methods

We based our study on a DNA sequence data set (~4600 bp) consisting of concatenated nuclear 18S and partial 28S ribosomal DNA (rDNA), partial mitochondrial 16S rDNA and partial mitochondrial cytochrome oxidase subunit I (COI) from 50 hexactinellid species. For details on the molecular methods and phylogenetic analysis of this data set see Dohrmann et al. (2011b). In brief, we applied independent substitution models to COI, 16S, 18S single-stranded regions (loops), 28S loops, and 18S+28S double-stranded regions (stems), including an RNA model to account for coevolution of paired sites for the latter (cf. Savill et al. 2001).

This analysis was conducted in a maximum likelihood (ML) framework using the software RAxML (Stamatakis 2006).

For calibration, we constrained the ages of eight internal nodes and the root node, using nine minimum and one maximum constraints in total, as detailed below. Analyses without data, i.e. sampling only from the prior distribution, showed that the prior mean divergence times of the internal nodes significantly differed from the posterior estimates (results not shown), confirming that this calibration set was suitable, allowing the data to dominate the results. The calibrations used are as follows (see also Fig. 1; stratigraphy follows Gradstein et al. [2004] throughout this paper):

- For the age of the root (= origin of crown-group Hexactinellida, or the Amphidiscophora/Hexasterophora-split) we used a minimum of 488 Ma (million years ago) (early Tremadocian: first hexasters [Mostler 1986]). Although the fossil record generally only provides minimum ages for taxa, at least one maximum age constraint is required to produce meaningful results from relaxed molecular clock analyses (see Warnock et al. 2011). Therefore, we also used a maximum of 542 Ma (Ediacaran/Cambrian-boundary) for the root age (see Introduction for justification). In preliminary analyses (results not shown) we also applied soft bounds (Yang and Rannala 2006) in order to relax this assumption. However, this still resulted in a Cambrian estimate for the root age while producing younger ages for many internal nodes. The reason for this is probably that all minimum constraints were also relaxed since PhyloBayes currently does not support application of soft bounds only to specific nodes or only to maximum constraints. As we have very little doubt about the validity of our minimum constraints, we therefore considered it more appropriate to use hard bounds for the final analysis.

- The age of crown-group Sceptrulophora was constrained to be at least 237 Ma, according to the earliest unambiguous finds of sceptrules in the Middle Triassic (lower Ladinian) (Krainer and Mostler 1991). Although the Late Devonian dictyonal frameworks (see Introduction) were assigned to extant sceptrulophoran families by Rigby et al. (2001), this interpretation has to be viewed with caution (see Results and Discussion). Instead, these fossils might represent stem-group Sceptrulophora, so we did not use them to calibrate this node.

- Aphrocallistidae, Farreidae, and Tretodictyidae (all Sceptrulophora), as well as Hyalonematidae (Amphidiscophora) are known with certainty from fossils that clearly exhibit crown-group morphology from the Late Cretaceous Campanian stage (see, e.g. Schrammen [1912] for the sceptrulophoran families, and Mehl and Hauschke [1995] for Hyalonematidae). Thus, we assigned minimum ages of 83.5 Ma (base of the Campanian) to the crown-nodes of these clades. However, in case of the Tretodictyidae we assigned the constraint to the node that separates *Hexactinella carolinensis* and *Tretodictyum tubulosum*, since the third included species, *Psilocalyx wilsoni*, exhibits a rather peculiar morphology (Reiswig 2002b; Reiswig and Kelly 2011; Dohrmann et al. 2011a) that differs from the Late Cretaceous fossils, which more closely resemble the other two genera (in fact, some of these fossils were actually assigned to *Hexactinella* or *Tretodictyum*, although this is rather speculative due to non-preservation of microscleres).

- Crown-group members of the lyssacinosidan families Rossellidae and Leucopsacidae are known from bodily preserved fossils since the Late Cretaceous Coniacian stage (Brückner and Janussen 2005; Brückner 2006), and the earliest unambiguous crown-euplectellid was described from the Cenomanian (Salomon 1990). Although several of these fossils were assigned to extant genera that are included in our molecular data set, the lack of microscleres

renders these interpretations somewhat speculative (Brückner 2006). Thus, we assigned minimum ages of 89.3 Ma (Rossellidae, Leucopsacidae) and 99.6 Ma (Euplectellidae) to the crown-nodes of these families in order to test if those generic assignments were consistent with the molecular age estimates.

Using these constraints, branch lengths of the ML tree topology (Fig. 1) were re-estimated from the sequence data in units of absolute time, employing the Bayesian Markov chain Monte Carlo (BMCMC) framework provided by the PhyloBayes (v. 3.2f) package (Lartillot et al. 2009). Since the models used to infer the tree topology are not implemented in PhyloBayes, we used the CAT model (Lartillot and Philippe 2004) with GTR exchange rates (CAT-GTR), as the manual recommends for nucleotide data. Among-site rate variation was modelled with the Dirichlet process of Huelsenbeck and Suchard (2007), and among-lineage rate variation was accounted for by employing a log-normal autocorrelated relaxed molecular clock model (see Lepage et al. 2007 for details and justification). We ran two chains simultaneously (sampling every 1000th point) and checked for convergence using the *tracecomp* application of the PhyloBayes package. Chains were stopped when minimum effective sizes/maximum discrepancies of model parameters had achieved values >100/<0.1, as recommended in the manual. Node age summary statistics were then extracted with *readdiv*, discarding the first 25% of sampled points as burn-in. Additionally, node ages were extracted from each tree in the post burn-in sample using ETE v. 2.1 (Huerta-Cepas et al. 2010; script available in Supplementary Material); the posterior distribution of the node ages was then plotted in R (http://www.r-project.org/) and used to evaluate alternative hypotheses concerning the systematic position of a number of hexactinellid fossils of ambiguous taxonomic affinities. In brief, the assignment of a fossil to a clade on the chronogram constrains the age that fossil can have in order to be congruent with the chronogram. Thus, for every fossil assigned to a clade on the chronogram it is possible to evaluate whether the

fossil's age lies within the 95% credibility interval (CrI) of the node's estimated age and reject the assignment when the fossil's age does not fall into this interval.

Results and Discussion

The BMCMC analyses took several weeks to reach convergence, and the two runs produced very similar results. We base our discussion on the chronogram shown in Fig. 2, which is derived from the arbitrarily chosen "chain 1". The means, standard errors, and 95% CrIs of age estimates derived from both chains are summarized in Supplementary Table S1. For selected nodes discussed below, we also show histograms of the posterior distributions of estimated ages (Figs. 3-7).

The root age estimate of ~518 Ma (Cambrian Series 2) is consistent with the notion of a strong mid-Cambrian radiation of Hexactinellida (Mehl 1996; Krautter 2002). However, one has to bear in mind that we constricted the root age *a priori* to the Cambrian (see Methods), so the influence of the prior on this estimate might be strong (sampling only from the prior distribution gave a mean of ~516 Ma [results not shown]). Therefore, a more precise estimate of the age of crown-group Hexactinellida might require further study.

The overall distribution of nodes (i.e., cladogenetic events or splits) through time in our chronogram is broadly consistent with paleontological views on Phanerozoic hexactinellid diversification (see Introduction): a "deep" Paleozoic radiation (13 splits) followed by a peak Mesozoic diversification (29 splits) and a marked Cenozoic decline (only 7 splits, which are confined to two families). Age estimates for the origin of most families' crown-groups are considerably older than unambiguous fossil evidence suggests (gray areas in Fig. 2); however, the estimated age ranges in many cases are consistent with more contentious assignment of older fossils to the respective families, as discussed in more detail below.

One striking result of our analysis is the huge difference in the estimated ages of the crown groups of the two subclasses: while the Hexasterophora already radiated some 40 million years (myr) after the origin of their stem lineage, the Amphidiscophora crown radiation was delayed until the Late Triassic, suggesting the extinction of deeply branching amphidiscophoran lineages. In contrast, our results suggest an Early Ordovician origin of crown-group Hexasterophora, in good congruence with the first appearance of hexasters in the fossil record (Mostler 1986; Mehl 1996). Intriguingly, paleontological data indicate that major transitions in siliceous sponge morphology and ecology occurred during that time, including colonization of nearshore siliciclastic settings by hexactinellids (Carrera and Botting 2008). Thus, our estimate suggests that the early crown-group diversification of Hexasterophora might have been connected to these events.

Because Sceptrulophora is the sister group to all other Hexasterophora, the crown-group origin of this subclass coincides with the origin of total-group Sceptrulophora. Although the mean age estimate of ~476 Ma (Floian; upper Early Ordovician) post-dates Paibian (late Cambrian) and Tremadocian (lower Early Ordovician) fragmentary microfossils interpreted as scopules (Dong and Knoll 1996; Kozur et al. 1996), these stages lie within the 95% CrI (Fig. 3, Table S1). Thus, we cannot reject the hypothesis that these spicules really came from early stem-group sceptrulophorans. In contrast, our results clearly reject an alleged early Paleozoic occurrence of clavules (e.g. Bengtson 1986; Webby and Trotter 1993; Dong and Knoll 1996; Kozur et al. 1996; Zhang and Pratt 2000): these spicules are restricted to the Farreidae (see Dohrmann et al. 2011a), and our results suggest a late Paleozoic origin of this clade (~343 Ma at most; Fig. 4, Table S1). Thus, we suggest that at least the Paleozoic "clavules", which are in fact morphologically rather different from those of extant Farreidae, represent either different spicule types such as anchorate basalia, or are convergently evolved spicules unrelated to the modern forms ("paraclavules").

Our mean estimate of the age of crown-group Sceptrulophora (377 Ma; Frasnian) almost perfectly matches the age of the oldest known dictyonal frameworks (~380 Ma; Rigby et al. 2001), suggesting that their world-wide appearance in Late Devonian strata was the result of extensive radiations that gave rise to the modern sceptrulophoran lineages. Although assignment of some of these fossils to the Euretidae (Rigby et al. 2001) is incompatible with our mean estimate of ~350 Ma (Early Mississippian) for the origin of the lineage leading to Euretidae n. gen., their age lies within the standard error bounds for that node (Table S1). However, since monophyly of Euretidae is questionable from a morphological point of view and currently not resolved by molecular data (see Dohrmann et al. 2011a), one should be cautious to "shoe-horn" fossils of simple dictyonal skeletons into that family. According to our results, extensive cladogenesis within Sceptrulophora also occurred during the Carboniferous and Permian, which is intriguing because neither dictyonal skeletons nor sceptrules are documented from these periods (Mehl 1996). Thus, our results suggest that the early radiation of crown-Sceptrulophora continued in habitats that were either cryptic (Mehl 1996) or are simply not preserved due to a bias towards terrestrial sedimentary outcrops from the late Paleozoic (Smith and McGowan 2007).

Crown-Tretodictyidae are estimated to date back to the Early Mississippian (~351 Ma), which is considerably older than the first unambiguous records of this family from the Late Cretaceous. This result might suggest that the monospecific genus *Psilocalyx* belongs to a very ancient lineage of Tretodictyidae. However, no fossil record of this genus is known, and inclusion of additional tretodictyid genera, which might break up the long branch leading to *Psilocalyx* in the molecular phylogeny, will be required to further test this hypothesis. In contrast, the split between *Hexactinella* and *Tretodictyum*, which we used for calibration (see Methods), is estimated to be only ~17 myr older (Albian; late Early Cretaceous) than those fossils (e.g. Schrammen 1912). This is inconsistent with assignment of putative tretodictyids from the Early (Mostler 1990) and Middle (Mehl and Fürsich 1997) Jurassic to these genera,

rather suggesting a stem-lineage membership of the *Hexactinella* + *Tretodictyum* clade (Fig. 5). In contrast to the Tretodictyidae, the crown-groups of Farreidae and Aphrocallistidae are estimated to have originated much later, in the Early Cretaceous, although their stem-lineages already separated in the mid-Permian. Farreoid skeletons and clavules were reported already from the Middle Triassic (Donofrio 1991; Krainer and Mostler 1991; Mehl and Mostler 1993), so our results suggest that these autapomorphies (see Dohrmann et al. 2011a) evolved very early in the farreid stem-lineage. Other than that it is unclear at present which pre-Cretaceous fossil taxa might be good candidates for stem-group Farreidae and Aphrocallistidae.

Speculations about Ordovician stem-group Rossellidae (Botting 2004) are clearly incompatible with our chronogram, since we estimated an early Permian origin for this lineage (Late Devonian at most; Fig. 6, Table S1). Instead, according to our results the sponges described by Botting (2004) fall on the lineage leading to a late Silurian (~420 Ma) Dactylocalycidae/Lyssacinosida split (assuming they are indeed hexasterophorans). Since these fossils show a lyssacine skeletal organization, this supports the hypothesis that dictyonal frameworks of Dactylocalycidae are not homologous to those of Sceptrulophora but evolved independently from a lyssacine condition. However, the CrIs for this node include the age of Botting's (2004) fossils (Fig. 6, Table S1), so we cannot completely rule out that they were stem-group Lyssacinosida. The inferred Silurian age of Lyssacinosida also further discourages classification of older lyssacine hexactinellids in this order (see Introduction).

According to our estimate, Euplectellidae diverged from its sister lineage by the Middle Devonian (~390 Ma), followed by crown-group radiation around the Carboniferous/Permian boundary (~300 Ma). This is much older than, and therefore consistent with, assignment of Early Triassic (Rigby and Gosney 1983) and Early Jurassic (du Dresnay et al. 1978) fossils to the Euplectellidae, although these interpretations have been questioned (Pisera and Bodzioch 1991). Our results are also consistent with assignment of Early Jurassic isolated spicules to

the recent families of Lyssacinosida (Mostler 1989, 1990), although the claim that extant genera were already present at that time should be viewed with caution. Mostler (1989, 1990) lists the following: *Aulosaccus*, *Caulophacus*, *Crateromorpha*, *Rossella* (Rossellidae), *Leucopsacus* (Leucopsacidae), and *Bolosoma* (Euplectellidae). Among these, only *Bolosoma* and *Leucopsacus* could have been present in the early Jurassic with reasonable certainty if our results are accurate (Fig. 7, Table S1). In contrast, although the upper bounds of the CrIs for the stem nodes of *Caulophacus*, *Crateromorpha*, and *Rossella* reach back to ~200 Ma, the rossellid genera are estimated to be younger (Fig. 7, Table S1). However, even in case of *Bolosoma*, increased taxon sampling might break up the long branch leading to the single species sampled here and therefore lead to much younger estimates. Nonetheless, the hypothesis that the spicules reported by Mostler came from crown-group members of the extant lyssacinosidan families is corroborated by our study.

Finally, although the classification of several Coniacian (Late Cretaceous) fossils from Denmark (Brückner and Janussen 2005; Brückner 2006) in extant genera is somewhat tentative due to non-preservation of microscleres (Brückner 2006), our results are consistent with assignments to *Rossella* (Rossellidae), *Regadrella*, *Docosaccus*, and *Acoelocalyx* (Euplectellidae). Interestingly, Rossellidae is the only hexasterophoran family that shows post-Cretaceous cladogenesis in our chronogram. This might indicate that large parts of the diversity of this most speciose hexactinellid family are the product of relatively recent radiations, in contrast to other taxa. However, increased taxon sampling among the remaining families might reveal a more homogenous pattern of diversification across the Hexactinellida.

Conclusions

By integrating molecular and fossil data, we further illuminated the evolutionary history of Hexactinellida, a group of non-bilaterian animals that significantly contributed to benthic

communities, including reefs, throughout the Phanerozoic. While our results corroborate some attempts by paleontologists to classify hexactinellid fossils, especially from the Mesozoic record, within recent taxa, in other cases we could reject some rather speculative hypotheses of systematic affinities using probability distributions of molecular node age estimates for the respective clades. This demonstrates how molecular chronograms can help narrowing down the possibilities in face of ambiguously interpretable fossils, which is particularly relevant in studies of taxonomically difficult groups (see also Waggoner and Collins 2004; Peterson et al. 2008). On the other hand, our dated phylogeny revealed extensive periods of missing fossil records for many clades, thereby providing a framework for more targeted efforts by paleontologists to recover older fossils of the respective taxa. We hope this work will stimulate future paleobiological research and re-evaluation of the hexactinellid fossil record, and also encourage researchers working on other non-bilaterian animal groups to apply molecular paleobiological approaches.

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Table S1. Means, standard errors (SE), and lower (Inf95) and upper (Sup95) bounds of 95% Bayesian credibility intervals for estimated node ages from both PhyloBayes chains (1, 2). Node numbers correspond to those indicated in Fig. 2.

Node	Mean1	Mean2	SE1	SE2	Inf95_1	Inf95_2	Sup95_1	Sup95_2
0	517.58	518.65	15.09	14.92	489.57	491.13	541.26	541.37
1	475.72	476.55	21.71	21.78	429.33	433.59	515.93	518.60
2	420.09	421.15	27.13	26.21	362.25	365.04	469.78	469.53
3	389.60	389.05	31.40	28.70	322.33	330.63	449.06	444.05
4	322.60	324.97	33.05	31.48	255.80	264.30	385.68	388.33
5	294.69	295.60	32.85	30.74	235.44	233.48	363.27	357.93
6	218.24	218.69	32.25	31.01	154.15	158.78	283.58	278.04
7	170.62	173.14	32.02	31.65	108.17	115.17	230.99	242.40
8	153.80	156.53	31.68	31.92	94.45	98.17	219.02	228.71
9	144.66	146.92	31.10	31.30	86.50	87.84	213.37	216.97
10	125.70	125.83	32.40	31.87	67.67	69.76	194.83	202.70
11	40.79	38.85	20.38	20.51	13.12	11.86	92.11	92.13
12	27.33	26.84	14.99	15.69	8.24	8.17	66.87	70.70
13	28.52	30.36	13.91	16.35	11.32	10.84	65.15	77.33
14	130.18	132.94	32.95	32.43	71.74	75.94	198.54	199.76
15	59.20	60.91	24.37	26.07	20.01	22.50	118.13	121.34
16	196.05	195.30	32.66	32.56	135.16	132.57	265.61	257.12
17	132.14	132.90	31.64	31.11	75.44	72.96	199.14	194.79

18	35.03	35.15	19.60	20.39	9.24	9.55	86.77	90.62
19	13.86	13.59	10.43	10.23	2.69	2.72	45.52	42.37
20	112.01	110.79	31.93	31.04	55.44	53.98	184.49	175.28
21	164.03	161.78	38.71	38.06	98.27	95.24	251.37	239.28
22	300.20	299.51	37.37	34.75	222.46	230.44	372.53	366.87
23	272.83	270.3	39.42	36.61	189.83	202.08	349.37	342.43
24	191.40	185.47	40.35	37.66	104.72	113.74	268.03	264.43
25	160.27	153.44	40.80	38.61	85.26	75.78	244.60	239.01
26	124.36	118.07	38.20	36.39	52.00	52.67	207.29	202.49
27	77.96	73.36	35.76	33.74	22.71	20.03	160.29	162.06
28	89.48	83.32	39.13	37.48	25.50	24.51	176.26	169.00
29	248.05	245.60	41.05	39.31	157.33	169.87	323.41	317.36
30	91.00	88.53	36.54	35.73	28.44	30.01	173.38	167.53
31	216.77	214.60	45.94	44.45	124.68	128.55	310.15	306.12
32	376.97	376.83	33.36	33.57	309.93	310.01	446.59	444.56
33	349.55	350.05	35.17	35.04	282.72	286.23	418.08	422.58
34	324.14	324.05	34.66	34.73	263.80	255.62	393.56	399.77
35	274.39	274.03	32.50	31.19	216.90	218.36	342.47	342.93
36	140.56	139.73	35.73	33.16	87.79	89.61	222.62	216.91
37	93.80	93.29	30.42	29.21	47.89	49.08	168.01	155.55
38	123.61	121.64	35.11	32.99	71.99	69.86	206.27	191.75
39	131.03	127.38	32.25	30.94	87.08	84.82	210.17	201.71
40	77.62	74.67	28.93	28.21	35.53	35.32	143.08	144.38
41	106.50	102.59	31.76	30.02	59.53	58.72	177.12	168.83
42	350.89	351.15	33.56	34.33	287.98	283.24	419.52	426.28
43	100.92	100.09	15.10	15.02	84.22	83.89	142.93	140.24
44	217.75	215.65	48.14	45.16	135.74	137.02	320.91	316.02
45	124.13	122.51	34.88	32.57	85.01	84.75	212.08	202.14

46	45.42	44.63	28.60	26.11	11.78	10.91	126.17	109.41
47	104.36	103.38	48.35	49.20	33.34	29.86	231.21	219.40
48	68.35	67.36	37.20	38.21	17.65	16.94	158.43	173.20

Fig. 1. Maximum likelihood molecular phylogeny of Hexactinellida, based on combined rRNA and COI genes (Dohrmann et al. 2011b). Clade support values are bootstrap proportions; scale bar indicates expected number of substitutions per site. Black dots indicate calibration nodes; ages in million years ago (Ma). See text for explanation and references.

Fig. 2. Time-calibrated molecular phylogeny of Hexactinellida plotted on stratigraphic chart. Calibration nodes indicated by black dots (see Fig. 1). Numbers on the right side of nodes are mean age estimates in million years ago (Ma); bold numbers on the left correspond to the node numbers in Table S1 (first column). Gray areas illustrate the implied temporal extent of missing fossil records for the crown-groups of the hexactinellid families (where "missing" refers to the absence of undisputed fossils; see text). Ordov., Ordovician; Sil., Silurian; Pg., Paleogene; Ng., Neogene.

Fig. 3. Frequency distribution for the posterior node age estimate of total-group Sceptrulophora (= crown-group Hexasterophora). Dashed lines = mean and 95% Bayesian credibility interval (CrI), dotted lines = standard error (SE). The shaded area indicates the time window from which putative Paleozoic scopules have been reported. See text for further explanation.

Fig. 4. Frequency distribution for the posterior node age estimate of total-group Farreidae. Dashed lines = mean and 95% CrI, dotted lines = SE. The shaded area indicates the time

window from which putative Paleozoic clavules have been reported. See text for further explanation.

Fig. 5. Frequency distribution for the stem (light gray histogram) and crown (dark gray histogram) posterior node age estimates of the *Hexactinella* + *Tretodictyum* - clade (Tretodictyidae). Dashed lines = mean and 95% CrI, dotted lines = SE. The shaded area indicates the time window from which putative Jurassic members of this clade have been reported; the star on the time line indicates the calibration constraint (≥83.5 Ma). See text for further explanation.

Fig. 6. Frequency distribution for the posterior node age estimates of total-group Lyssacinosida (dark gray histogram) and total-group Rossellidae (light gray histogram). Dashed lines = mean and 95% CrI, dotted lines = SE. The shaded area indicates the approximate age of putative Ordovician stem-group rossellids. See text for further explanation.

Fig. 7. Frequency distribution for the posterior node age estimates of several lyssacinosid genera. Dashed lines = mean and 95% CrI, dotted lines = SE. The shaded area indicates the approximate age of Early Jurassic isolated microscleres attributed to these taxa. See text for further explanation. *"*Rossella s.s.*" refers to the Southern Ocean members of this genus; the North Atlantic *Rossella nodastrella* groups with *Aulosaccus mitsukurii* in the molecular phylogeny (node 15) and should be removed from *Rossella* in future revisions of this taxon (see Dohrmann et al. 2008).

1

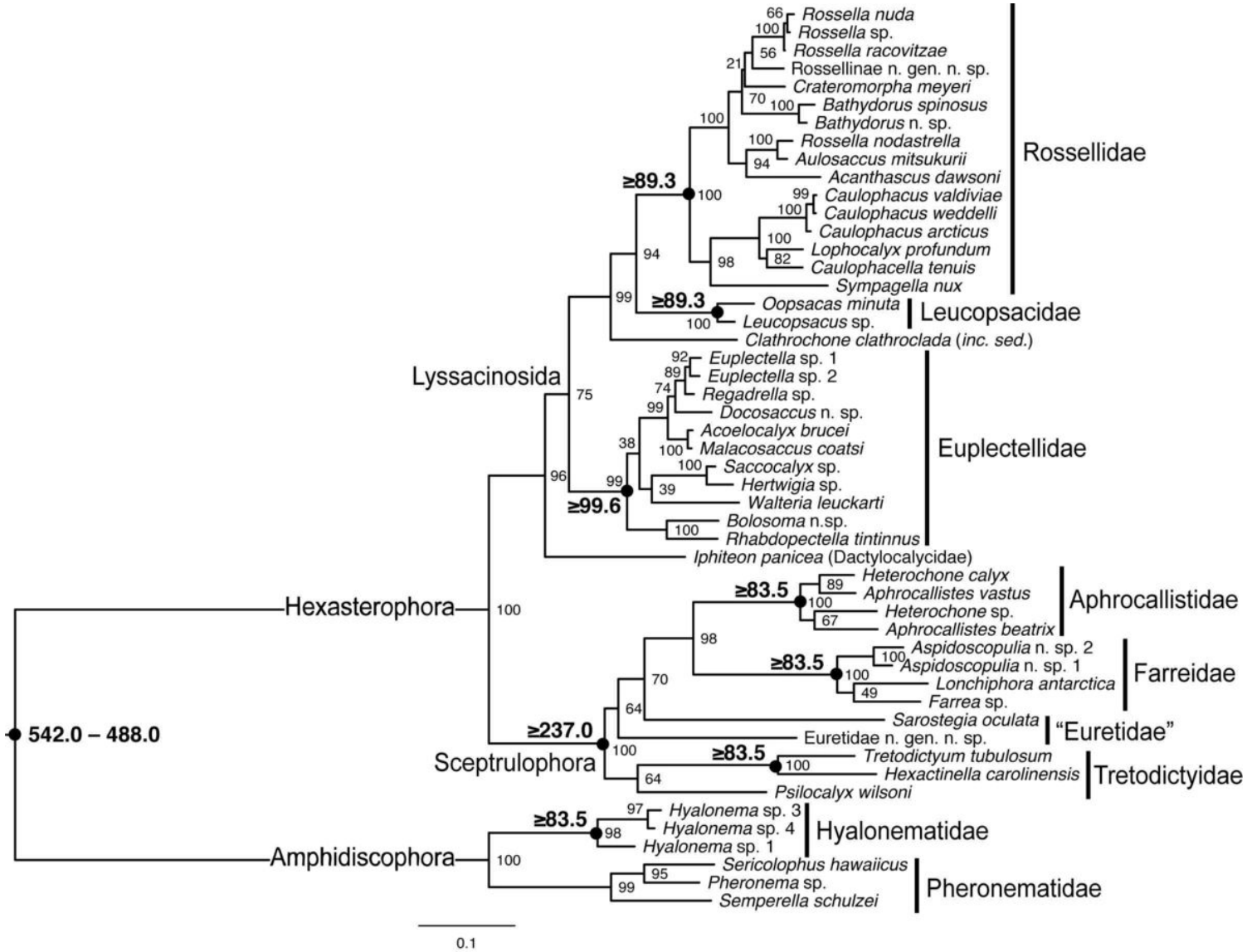
2

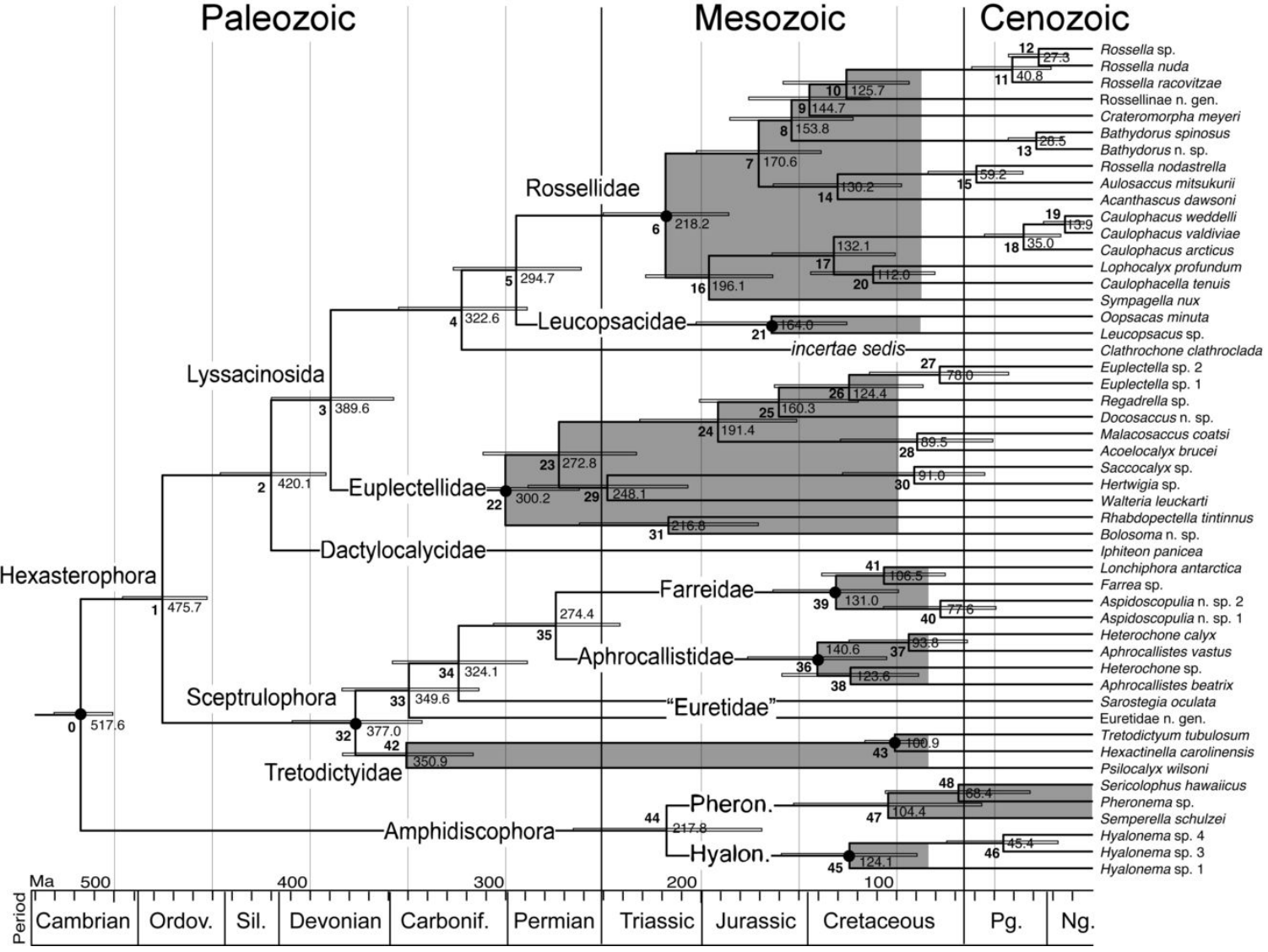
3

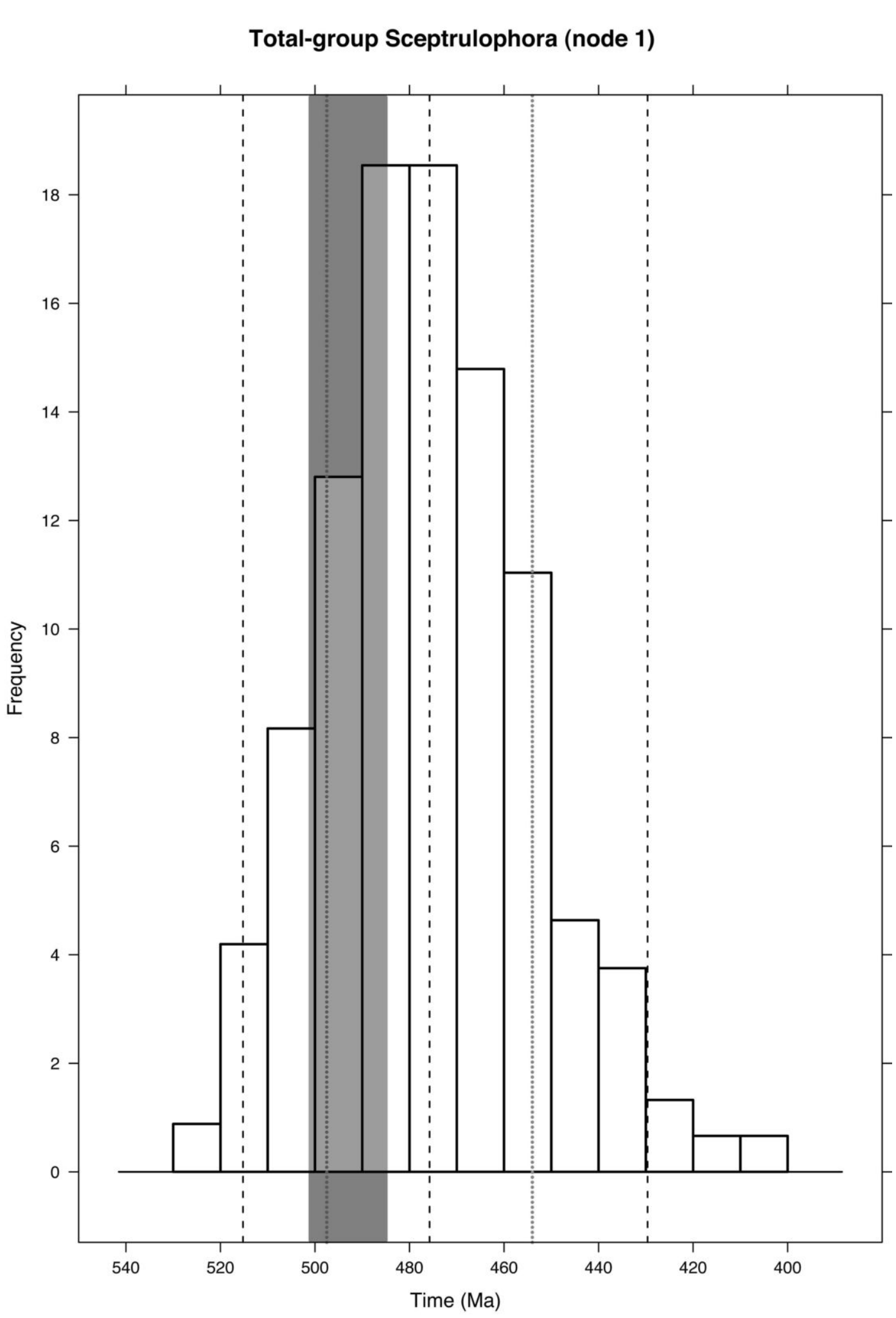
4

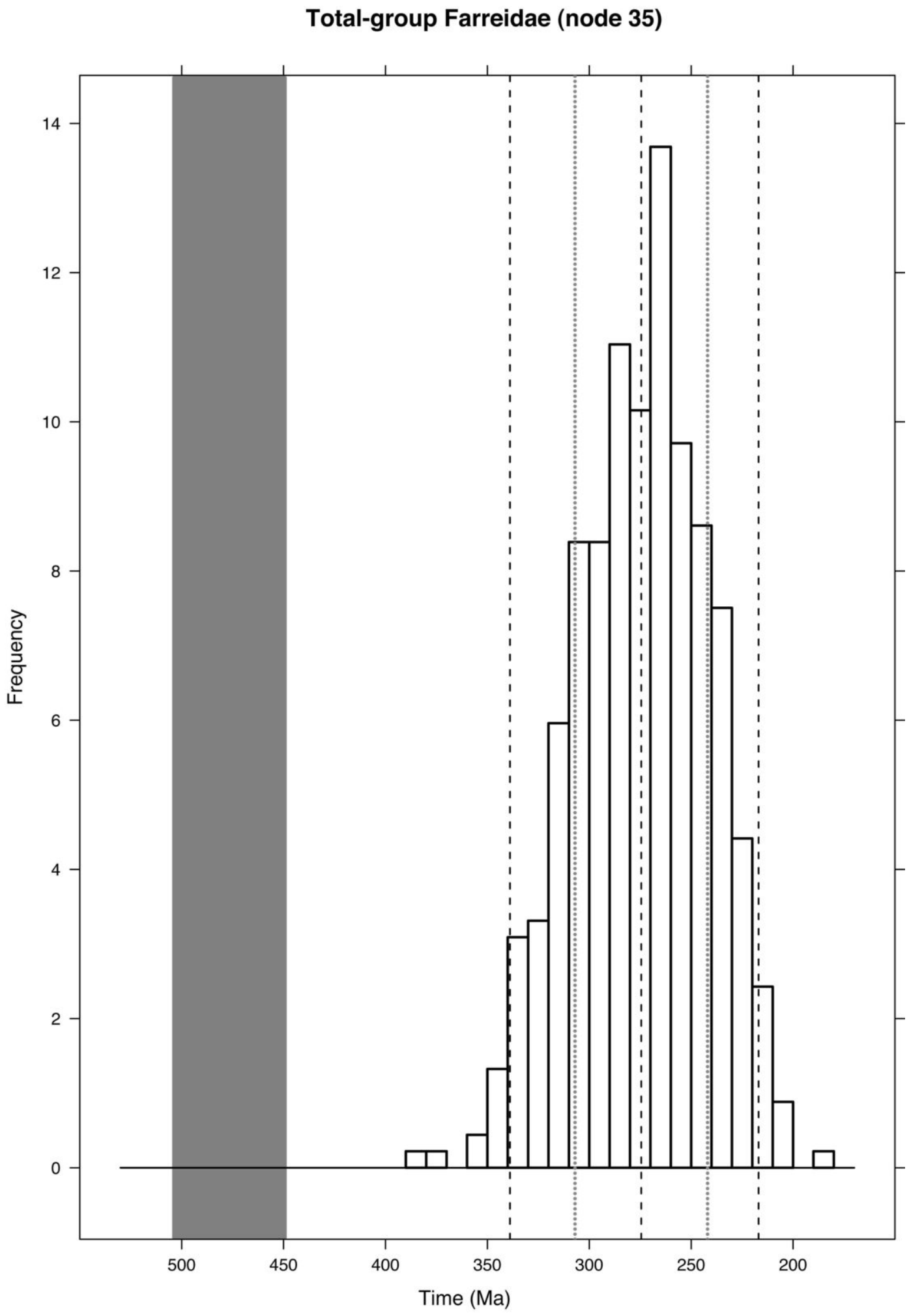
5

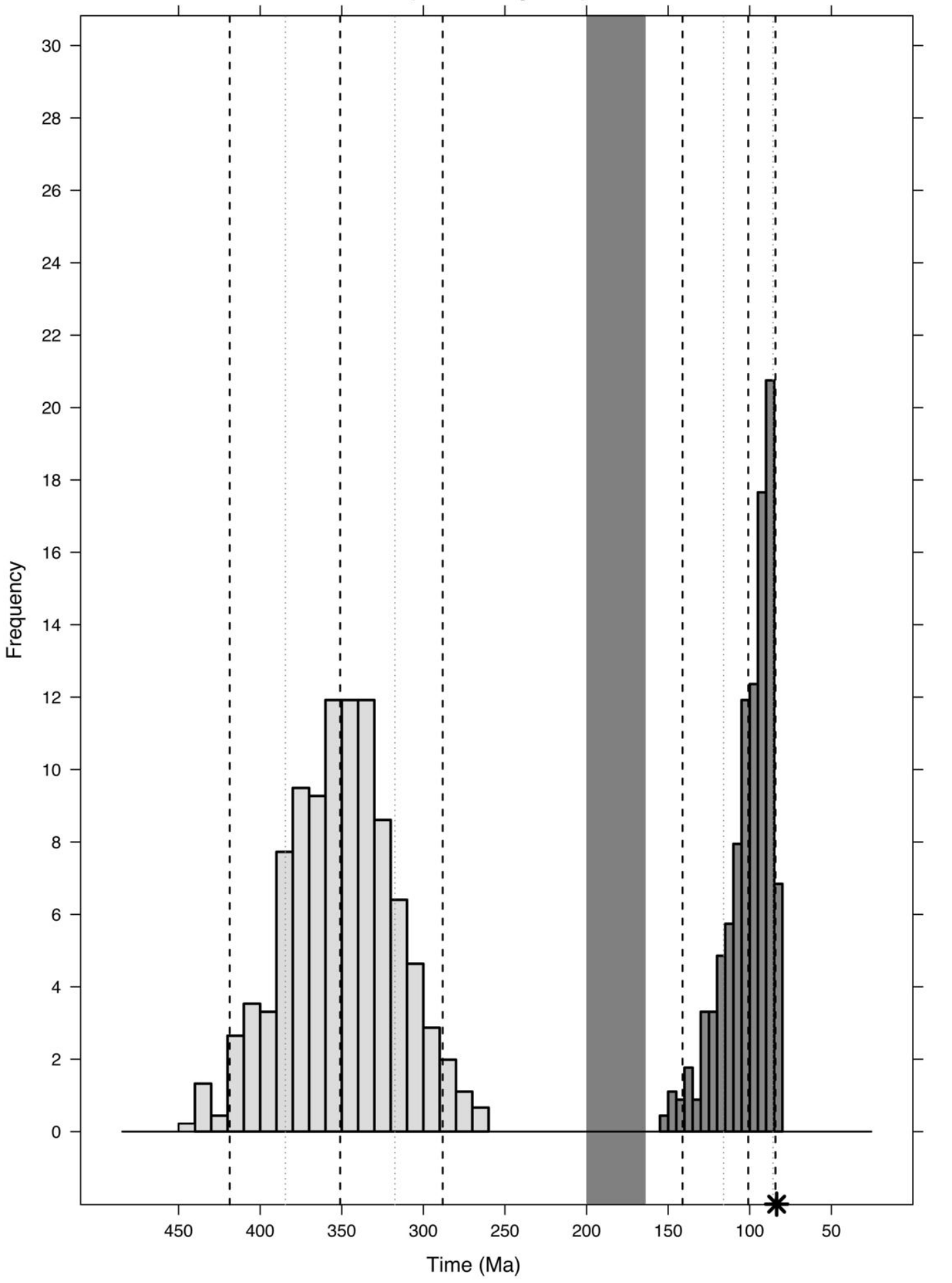
6

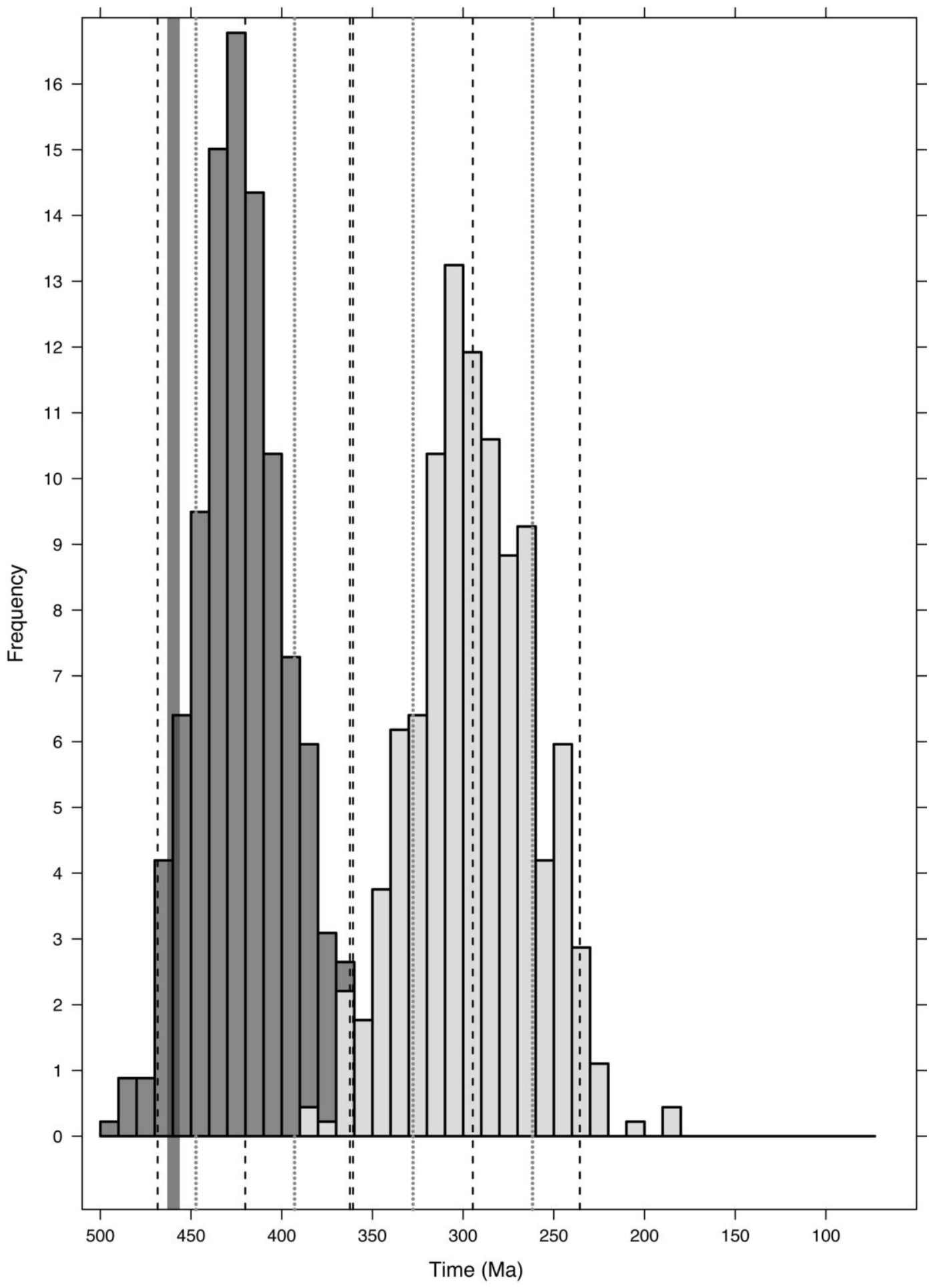

Rossella nuda
Rossella sp.
Rossella racovitzae
Rossellinae n. gen. n. sp.
Crateromorpha meyeri
Bathydorus spinosus
Bathydorus n. sp.
Rossella nodastrella
Aulosaccus mitsukurii
Acanthascus dawsoni
Caulophacus valdiviae
Caulophacus weddelli
Caulophacus arcticus
Lophocalyx profundum
Caulophacella tenuis
Sympagella nux
Rossellidae
≥89.3
Oopsacas minuta
Leucopsacus sp.
Leucopsacidae
Clathrochone clathroclada (inc. sed.)
Lyssacinosida
Euplectella sp. 1
Euplectella sp. 2
Regadrella sp.
Docosaccus n. sp.
Acoelocalyx brucei
Malacosaccus coatsi
Saccocalyx sp.
Hertwigia sp.
Walteria leuckarti
≥99.6
Bolosoma n.sp.
Rhabdopectella tintinnus
Euplectellidae
Iphiteon panicea (Dactylocalycidae)
Heterochone calyx
≥83.5
Aphrocallistes vastus
Heterochone sp.
Aphrocallistes beatrix
Aphrocallistidae
Aspidoscopulia n. sp. 2
≥83.5
Aspidoscopulia n. sp. 1
Lonchiphora antarctica
Farrea sp.
Farreidae
Sarostegia oculata
Euretidae n. gen. n. sp.
"Euretidae"
Hexasterophora
542.0 – 488.0
≥237.0
Sceptrulophora
≥83.5
Tretodictyum tubulosum
Hexactinella carolinensis
Psilocalyx wilsoni
Tretodictyidae
≥83.5
Hyalonema sp. 3
Hyalonema sp. 4
Hyalonema sp. 1
Hyalonematidae
Amphidiscophora
Sericolophus hawaiicus
Pheronema sp.
Semperella schulzei
Pheronematidae
0.1

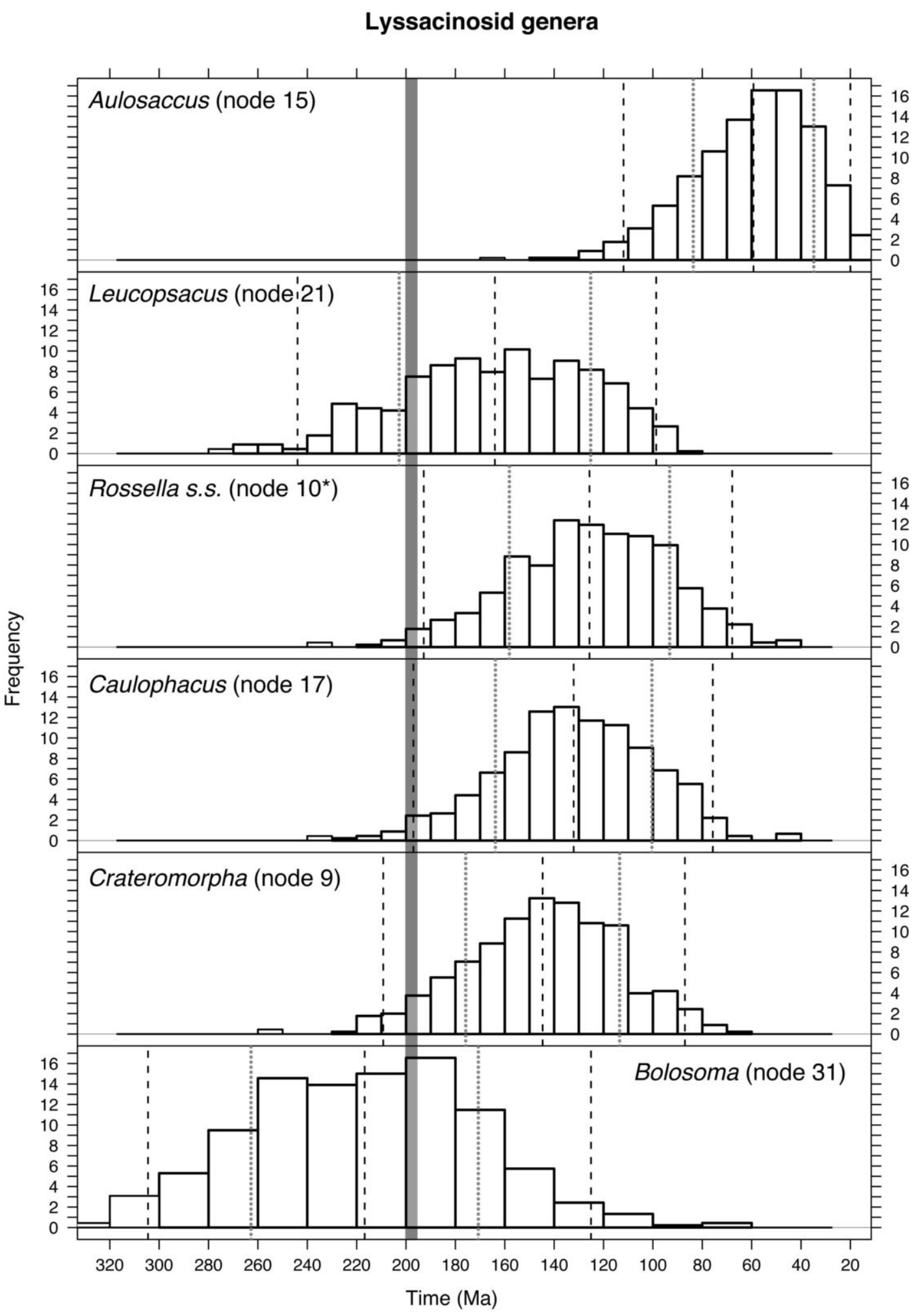

Paleozoic
Mesozoic
Cenozoic
Rossellidae
Leucopsacidae
Lyssacinosida
Euplectellidae
Dactylocalycidae
Hexasterophora
Farreidae
Aphrocallistidae
Sceptrulophora
"Euretidae"
Tretodictyidae
Amphidiscophora
Pheron.
Hyalon.
Rossella sp.
Rossella nuda
Rossella racovitzae
Rossellinae n. gen.
Crateromorpha meyeri
Bathydorus spinosus
Bathydorus n. sp.
Rossella nodastrella
Aulosaccus mitsukurii
Acanthascus dawsoni
Caulophacus weddelli
Caulophacus valdiviae
Caulophacus arcticus
Lophocalyx profundum
Caulophacella tenuis
Sympagella nux
Oopsacas minuta
Leucopsacus sp.
incertae sedis
Clathrochone clathroclada
Euplectella sp. 2
Euplectella sp. 1
Regadrella sp.
Docosaccus n. sp.
Malacosaccus coatsi
Acoelocalyx brucei
Saccocalyx sp.
Hertwigia sp.
Walteria leuckarti
Rhabdopectella tintinnus
Bolosoma n. sp.
Iphiteon panicea
Lonchiphora antarctica
Farrea sp.
Aspidoscopulia n. sp. 2
Aspidoscopulia n. sp. 1
Heterochone calyx
Aphrocallistes vastus
Heterochone sp.
Aphrocallistes beatrix
Sarostegia oculata
Euretidae n. gen.
Tretodictyum tubulosum
Hexactinella carolinensis
Psilocalyx wilsoni
Sericolophus hawaiicus
Pheronema sp.
Semperella schulzei
Hyalonema sp. 4
Hyalonema sp. 3
Hyalonema sp. 1
0 517.6
1 475.7
2 420.1
3 389.6
4 322.6
5 294.7
6 218.2
7 170.6
8 153.8
9 144.7
10 125.7
11 40.8
12 27.3
13 26.5
14 130.2
15 59.2
16 196.1
17 132.1
18 35.0
19 13.9
20 112.0
21 164.0
22 300.2
23 272.8
24 191.4
25 160.3
26 124.4
27 78.0
28 89.5
29 248.1
30 91.0
31 216.8
32 377.0
33 349.6
34 324.1
35 274.4
36 140.6
37 93.8
38 123.6
39 131.0
40 77.6
41 106.5
42 350.9
43 100.9
44 217.8
45 124.1
46 45.4
47 104.4
48 68.4
Ma
500
400
300
200
100
Period
Cambrian
Ordov.
Sil.
Devonian
Carbonif.
Permian
Triassic
Jurassic
Cretaceous
Pg.
Ng.


Total-group Sceptrulophora (node 1)
Frequency
0
2
4
6
8
10
12
14
16
18
540
520
500
480
460
440
420
400
Time (Ma)


Total-group Farreidae (node 35)
Frequency
0
2
4
6
8
10
12
14
500
450
400
350
300
250
200
Time (Ma)


Stem (node 42) and crown (node 43) of the *Hexactinella* + *Tretodictyum* - clade (Tretodictyidae)
Frequency
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
450
400
350
300
250
200
150
100
50
Time (Ma)


Total-group Lyssacinosida (node 2) and total-group Rossellidae (node 5)
Frequency
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
500
450
400
350
300
250
200
150
100
Time (Ma)


Lyssacinosid genera
Aulosaccus (node 15)
Leucopsacus (node 21)
Rossella s.s. (node 10*)
Caulophacus (node 17)
Crateromorpha (node 9)
Bolosoma (node 31)
Frequency
Time (Ma)
320 300 280 260 240 220 200 180 160 140 120 100 80 60 40 20
0 2 4 6 8 10 12 14 16

Supplementary materials for Chapter 1

Collection details of sequenced specimens

Species	Expedition/Station/ Voucher number	Coordinates (Latitude;Longitude)	Depth (meters)
Rossella racovitzae	Systco/48-1/SMF11729	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella cf. fibulata	Systco/48-1/SMF11733	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella cf. vanhoeffeni	Systco/48-1/SMF11736	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella nuda	Systco/48-1/SMF11715	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella levis	Systco/48-1/SMF11728	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella fibulata	Systco/48-1/SMF11732	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella antarctica	Systco/48-1/SMF11734	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella antarctica	Systco/48-1/SMF11735	70° 23.94′ S; 8° 19.14′ W	602.1
Rossella racovitzae	ANTXIII-8/697-1/SMF11731	63° 15.38′ S; 59° 3.94′ W	143.7
Rossella nuda	ANTXIII-8/700-4/SMF11730	65° 56.08′ S; 60° 20.28′ W	211.1
Rossella racovitzae	STN54AEV393GD4075 (SVR68)	? (Terra Adelie)	?

Tree files

Best ML trees (in nexus format) for the monophyly constraints inferred with RAxML 7.2.8 and used for the pLRTm and AU-Test, and the ML and Bayesian topologies with support values and branch lenghts, and partition specific ML trees are available as an electronic appendix at `http://dx.doi.org/10.5282/ubm/data.48`

Supplementary materials for Chapter 2

Tree files

A nexus file with the Supplemetary trees is available at `http://dx.doi.org/10.5282/ubm/data.48`.

Supplementary materials for Chapter 4

Number of samples extracted per taxonomic groups. These data was used to construct the word-cloud presented as figure 1 in the text

Taxonomic group	Number of samples extracted
(Calcarea)	1
(Lyssacinosida incertae sedis)	1
Acanthochaetetidae	15
Acarnidae	49
Agelasidae	41
Alectonidae	12
Ancorinidae	547
Aphrocallistidae	3
Aplysinellidae	95
Aplysinidae	23
Astroscleridae	35
Axinellidae	566
Baeriidae	1
Callyspongiidae	177
Calthropellidae	9
Chalinidae	93
Chondrillidae	5
Chondropsidae	84
Cladorhizidae	6
Clathrinidae	10
Clionaidae	230
Coelosphaeridae	55
Corallistidae	30
Crambeidae	20
Crellidae	62
Darwinellidae	107
Dendoricellidae	2
Desmacellidae	62
Desmacididae	15
Desmanthidae	13
Dictyodendrillidae	14
Dictyonellidae	411
Dysideidae	509
Esperiopsidae	42
Euretidae	11
Farreidae	39

continues in the next page. . .

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Geodiidae	99
Grantiidae	6
Guitarridae	3
Halichondriidae	126
Halisarcidae	12
Hamacanthidae	1
Hemiasterellidae	6
Heteropiidae	2
Heteroxyidae	116
Hyalonematidae	19
Hymedesmiidae	35
Ianthellidae	315
Iotrochotidae	83
Ircinidae	81
Isodictyidae	58
Isoraphiniidae	2
Jenkinidae	1
Latrunculiidae	17
Lelapiidae	2
Leucaltidae	12
Leucettidae	236
Levinellidae	23
Macandrewiidae	1
Metaniidae	7
Microcionidae	760
Minchinellidae	5
Mycalidae	126
Myxillidae	7
Niphatidae	139
Pachastrellidae	15
Petrosiidae	328
Pheronematidae	6
Phloeodictyidae	85
Phymatellidae	19
Placospongiidae	6
Plakinidae	63
Pleromidae	12
Podospongiidae	53
Polymastiidae	13
Pseudoceratinidae	35
Raspailiidae	384
Rhabderemiidae	8
Rossellidae	12
continues in the next page. . .	

. . . comes from last page. . .	
Scleritodermidae	19
Siphoniidae	1
Soleneiscidae	22
Spirastrellidae	13
Spongiidae	384
Spongillidae	20
Suberitidae	71
Sycettidae	37
Tedaniidae	77
Tethyidae	30
Tetillidae	198
Theonellidae	4
Thorectidae	707
Timeidae	1
Trachycladidae	16
unassigned	2
unidentified	257
Verticillititiidae	16
Total Result	8610

Author contributions

Chapter 1. Nuclear and mitochondrial markers support two species of *Rossella* (Hexactinellida: Lyssacinosida, Rossellidae) in the Southern Ocean

Sergio Vargas designed the study, generated the sequences for the species of *Rossella*, performed the analyses and wrote the manuscript. Martin Dohrman participated in the study design, provided DNA alignments for the study, and helped editing the manuscript. Christian Göcke identified the specimens of *Rossella* used for the study. Dorte Janussen collected and identified the specimens of *Rossella* used for the study, and participated in the initial study design. Gert Wörheide participated in the design of the study. All authors contributed to draft versions of the manuscript and approved the final version of the manuscript.

Chapter 2. Chelae clash: molecular phylogeny of *Abyssocladia* (Cladorhizidae: Poecilosclerida) and *Phelloderma* (Phellodermidae: Poecilosclerida) suggests a diversification of chelae microscleres in cladorhizid sponges

Sergio Vargas designed the study, generated the sequences, performed the analyses and wrote the manuscript. Dirk Erpenbeck provided DNA alignments for the study. Kathryn Hall and John Hooper provided samples and identified specimens used for the study. Dorte Janussen collected and identified the specimens of Cladorhizidae and Phellodermidae that served as the basis for the study and participated in the initial study design. Gert Wörheide participated in the design of the study. All authors contributed to draft versions of the manuscript and approved the final version of the manuscript.

Chapter 3. Counting in the abyss: sponge species richness estimation in deep Antarctic waters

Sergio Vargas designed the study, performed the analyses and wrote the manuscript. Christian Göcke identified the specimens and generated the dataset used for richness estimation. Dorte Janussen collected and identified the specimens used to build the dataset herein analysed. Gert Wörheide participated in the design of the study. All authors contributed to draft versions of the manuscript and approved the final version of the manuscript.

Chapter 4. Barcoding sponges: an overview based on comprehensive sampling

Sergio Vargas implemented the DNA extraction protocol and tested and modified it as reported here, designed the study, generated part of the sequences, performed the

analyses and wrote the manuscript. Astrid Schuster collaborated during the implementation and test of the extraction protocol, provided data on DNA concentration, and participated in the generation of DNA barcodes for the Queensland Museum collection. Katharina Sacher, Gabrielle Büttner, Simone Schätzle and Benjamin Läuchli generated data used for the analyses. Kathryn Hall and John Hooper provided samples and identified specimens used for the study. Dirk Erpenbeck contributed to the implementation of the barcoding workflow, and to the design of the study. Gert Wörheide participated in the design of the barcoding workflow and in the study. All authors contributed to draft versions of the manuscript and approved the final version of the manuscript.

Chapter 5. Diversity in cold hot-spot: DNA-barcoding reveals patterns of evolution among Antarctic sponges

Sergio Vargas designed the study, generated the sequences, performed the analyses and wrote the manuscript. Michelle Kelly taxonomically identified the material and provided information on the systematics of the sponges, Kareen Schnabel collected part of the material and helped during subsampling of the collection. David Bowden collected part of the material and took part in the organization of the cruises. Gert Wörheide participated in the design of the study. All authors contributed to draft versions of the manuscript and approved the final version of the manuscript.

Curriculum vitae

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Current position

2009–present Research Associate, Dept. of Earth & Environmental Sciences, Palaeontology & Geobiology, LMU München, Germany.
Head researcher, Prof. Gert Wörheide, LMU München.

Formal Education

1998–2002 Bachellor Degree in Biology. Universidad de Costa Rica.

2003–2007 Magister Scientiae Degree in Biology. Universidad de Costa Rica. Honors Thesis.

2009–present Promotion. Dept. of Geo- and Environmental Sciences, Geobiology and Palaeontology, LMU München, Germany.

Working Experience

1999–2000 Research assistant. HERMA Project, Centro de Investigaciones Marinas y Limnológicas, UCR. Lab. assistant, CARICOMP project. Field and lab. assistant, Bahía Culebra monitoring program.
Head researcher: Dr. Jorge Cortés Ph.D. CIMAR, UCR.

2001–2002 Research assistant. Rice biotechnology group, Centro de Investigaciones en Biología Celular y Molecular, UCR. Statistical analysis and database maintenance.
Head researcher: Dr. Ana Mercedes Espinoza Ph.D. CIBCM, UCR.

2002–2003 Research assistant. Instituto Clodomiro Picado, UCR. Molecular biology and evolution of snake venom PLA2 toxins.
Head researcher: Dr. Alberto Alape Ph.D. ICP, UCR.

2004 Scientific staff: Lab. director assistant. La Selva Biological Station, OTS. Lab. administrative maintenance, academic support for undergraduate and graduate

students and courses.
Lab. director, La Selva Biological Station: Mahmood Sasa Ph.D. ICP, UCR.

Sept. 2006–Jan. 2007 Short-term student fellow. Molecular phylogenetics of the eastern Pacific genus *Heterogorgia*, Verrill and related taxa. ITZ, Tierärzliche Hochschule Hannover. Hannover, Germany.
ITZ director: Prof. Dr. Bernd Schierwater, TiHo, Hannover.

2004–2008 : Research assistant. Sistematics and biogeography of eastern Pacific octocorals.
Head researcher: Hector M. Guzman Ph.D. Smithsonian Tropical Research Institute, Panamá.

Teaching Experience

2003 Teaching Assistant. Course: Molecular Techniques in Tropical Ecology. Organization for Tropical Studies. Professor in charge: Dr. Mahmood Sasa Ph.D., ICP, UCR.

2004 Teaching Assistant. Course: Undergraduate Semester Abroad Program (USAP). Organization for Tropical Studies/Duke University. Professor in charge: Dr. Erika Deinert, OTS; Dr. Mahmood Sasa Ph.D., ICP, UCR.

2005–2006 Teaching Assistant. Course: Statistics. Graduate School in Microbiology, Universidad de Costa Rica. Professor in charge: Dr. Mahmood Sasa Ph.D., ICP, UCR.

Peer reviewed published articles[2,3]

Gert Wörheide, **Sergio Vargas**, Carsten Lüter & Joachim Reitner. 2011. Precious corals and sponge rock gardens on the deep aphotic fore-reef of Osprey Reef (Coral Sea, Australia). ***Coral Reefs***, 30: 901.

Sergio Vargas, Michael Eitel, Odalisca Breedy & Bernd Schierwater. 2010. Molecules match morphology: mitochondrial DNA supports Bayer's *Lytreia-Bebryce-Heterogorgia* (Alcyonacea: Octocorallia) clade hypothesis. ***Invertebrate Systematics***, 24: 23–31.

Sergio Vargas, Odalisca Breedy & Hector M. Guzman. 2010. The phylogeny of *Pacifigorgia* (Coelenterata, Octocorallia, Gorgoniidae): A case of study of the use of continuous characters in the systematics of the Octocorallia. ***Zoosystema***, 32: 5–18. **MSc.**

[2]MSc=Results from my Masters thesis
[3]Peer reviewed abstracts and talks can be found at `http://www.marinemolecularevolution.org/publications`

Sergio Vargas, Odalisca Breedy, Francisco Siles & Hector M. Guzman. 2010. How many kinds of sclerite?: Towards a morphometric classification of gorgoniid microskeletal components. ***Micron***, 41: 158–164. **MSc. On the cover!**

Odalisca Breedy, Hector M. Guzman & **Sergio Vargas**. 2009. A revision of the genus *Eugorgia* Verrill, 1868 (Coelenterata: Octocorallia: Gorgoniidae). ***Zootaxa***, 2151: 1–46.

Sergio Vargas, Hector M. Guzman & Odalisca Breedy. 2008. Distribution patterns of the genus *Pacifigorgia* (Octocorallia, Gorgoniidae): Track analysis and parsimony analysis of endemicity. ***Journal of Biogeography***, 35: 241–247. **MSc.**

Griselda Arrieta–Espinoza, Elena Sanchez, **Sergio Vargas**, Jorge Lobo, Tania Quesada & Ana M. Espinoza. 2005. The Weedy Rice Complex in Costa Rica. I. Morphological Study of Relationships Between Commercial Rice Varieties, Wild Oryza Relatives and Weedy Types. ***Genetic Resources and Crop Evolution***, 52: 575–587.

Accepted manuscripts

Dirk Erpenbeck, Kathryn Hall, Gabriele Büttner, Katharina Sacher, Simone Schätzle, Astrid Schuster, **Sergio Vargas**, John N.A. Hooper & Gert Wörheide. The phylogeny of halichondrid demosponges: Past and present re-visited with DNA-Barcoding data. ***Organisms Diversity and Evolution***.

Sergio Vargas, Martin Dohrmann, Christian Göcke, Dorte Janussen & Gert Wörheide. Nuclear and mitochondrial markers support two species of *Rossella* (Hexactinellida: Lyssacinosida, Rossellidae) in the Southern Ocean. Under revision in ***Molecular Phylogenetics and Evolution***.

Submitted manuscripts

Sergio Vargas, Astrid Schuster, Katharina Sacher, Gabrielle Büttner, Simone Schätzle, Benjamin Läuchli, Dirk Erpenbeck & Gert Wörheide. Barcoding sponges: an overview based on comprehensive sampling. ***PLoS ONE***

Martin Dohrmann, **Sergio Vargas**, Dorte Janussen, Allen G. Collins & Gert Wörheide. Molecular paleobiology of early-branching animals: integrating DNA and fossils elucidates the evolutionary history of hexactinellid sponges.***Palaeobiology***.

Manuscripts in preparation[4]

Miscellaneous information

Operating Systems GNU-Linux/Unix.

[4]Manuscripts in preparation can be found at `http://www.marinemolecularevolution.org/publications`

Programming languages Perl, Python, Java, C/C++.

Software packages

- Statistics: R, SPSS, STATISTICA, SYSTAT.
- Systematics: PAUP, NONA/PIWE, RAxML, POY, MrBayes, TNT.

Languages

- English (TOEFL: 643; TWE: 4)
- German [ZD (B1): 247 (max. 300)].

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