Cladorhizid sponges from hydrothermal vents and cold seeps in the NE Atlantic Ocean

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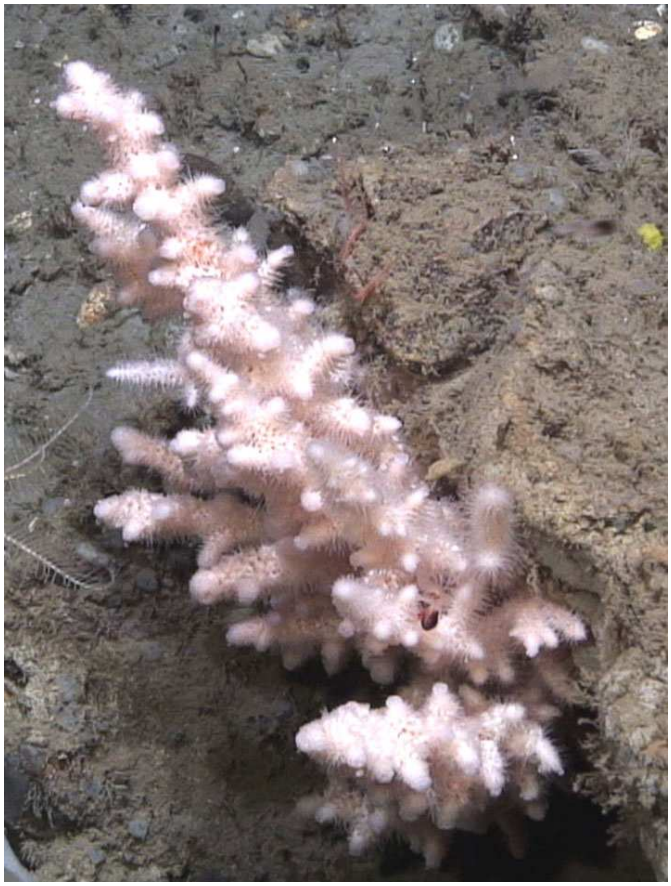

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Summary

Sponges are known to frequently contain a great number of symbiotic organisms. Symbiosis is also a characteristic trait of vent and seep animals worldwide. Sponges in the deep sea family Cladorhizidae, otherwise also known for their surprising ability to capture invertebrate prey, are among the most common sponges found in proximity to vents, and in one reported case methanotrophic symbiotic bacteria have been described in a cladorhizid species at a mud volcano. The phylogenetic relationships within the Cladorhizidae and between the cladorhizids and sister families are uncertain. New species of Cladorhizidae are still discovered, even in comparatively well studied areas such as the North Atlantic, and their affinity to vent systems make them likely candidates for harboring methanotrophic symbionts.

The 2006-2009 R/V "G.O. Sars" cruises to methane rich habitats in the Norwegian and Arctic Seas have yielded a number of cladorhizid specimens. This thesis contains a taxonomic inventory of these specimens using traditional morphological methods, and the results of sequencing the Folmer COI, and ITS partitions of the identified species. Lastly, the *pmoA* partition of the gene coding for particulate methane monooxygenase in known methanotrophic bacteria, was sequenced as a method to determine the presence of any methanotrophic symbionts from the specimens sampled.

Morphological results showed that the R/V "G.O. Sars" material contained six *Asbestopluma* and three *Cladorhiza* species. With the exception of two species affiliated to *A. lycopodium*, all were previously described species from the North Atlantic and Arctic. Though obtaining sequences proved challenging due to contamination from other organisms, cloning allowed the separation of cladorhizid and contaminant sequences, and it proved possible to get COI and ITS sequences from nearly all morphologically identified species. ITS sequences proved too divergent for phylogenetic analysis; however Folmer COI sequences had a level of resolution applicable both to place the Cladorhizidae within the broader Poecilosclerida group, close to Mycalidae, and to resolve internal relationships within the Cladorhizidae. The COI results suggest that *Asbestopluma* might be paraphyletic, though additional data are needed to draw more robust conclusions. Sequencing of the *pmoA* partition met with mixed results. It proved possible to prove the presence of the gene in small quantities in some samples, but contamination and lack of ambient controls made it difficult to draw any strong conclusions as to the origin of the sequences.

Sammendrag

Svamper er kjent for å ofte inneholde store mengder symbiotiske organismer. Symbiose er også et karakteristisk trekk ved vent- og seep-dyr fra ulike steder på jorden. Svamper fra dyphavsfmilien Cladorhizidae, ellers kjent for sin overraskende evne til å fange små evertebater, er blant de vanligste svampene i vent-nære områder, og i ett beskrevet tilfelle har metanotrofe symbiotiske bakterier blitt påvist i en cladorhizide fra en slamvulkan. Fylogenetiske forhold innad i Cladorhizidae og mellom cladorhizidene og beslektede familier er usikre. Nye arter Cladorhizidae blir fremdeles oppdaget selv i forholdsvis godt utforskede områder som i Nord-Atlanteren, og deres tilknytning til vent-systemer gjør dem til gode kandidater for påvisning av metanotrofe symbionter.

Blant utbyttet av F/F «G.O. Sars»-toktene i 2006-2009 til metanrike områder i Norskehavet og Arktis finnes flere cladorhizider. Denne oppgaven inneholder en artsbestemmelse av disse prøvene ved bruk av tradisjonelle, morfologiske metoder, og resultatene fra sekvensering av Folmer COI- og ITS-partisjonene fra identifiserte arter. I tillegg ble *pmoA*-partisjonen av genet som koder for metan monooksygenase i kjente metanotrofe bakterier sekvensert som en metode for å påvise hvorvidt metanotrofe symbionter var tilstede i noen av prøvene.

Morfologiske resultater viste at F/F «G.O. Sars»-materialet inneholdt seks *Asbestopluma*- og tre *Cladohiza*-arter. Med unntak av to sannsynlige arter knyttet til *A. lycopodium,* var alle prøvene tidligere beskrevne arter fra Nord-Atlanteren og Arktis. Sekvensering viste seg å være utfordrende på grunn av kontaminasjon fra andre organismer, men kloning gjorde det mulig å skille cladorhizide og andre sekvenser, og det viste seg mulig å få COI- og ITS-sekvenser fra nesten alle de morfologisk identifiserte artene. ITS-sekvensene viste seg å være for ulike for å bruke i fylogenetisk analyse, men Folmer COI-sekvensenes grad av oppløsning viste seg å være i stand til både å plassere Cladorhizidae nær Mycalidae innenfor Poecilosclerida, og vise interne forhold innad blant cladorhizidene. COI-resultatene indikerte at *Asbestopluma* sannsynligivs er parafyletisk. Sekvenseringen av *pmoA*-partisjonen ga blandede resultater. Det viste seg å være mulig å påvise små mengder av genet i noen prøver, med kontaminasjon og mangel på kontrollprøver gjorde det vanskelig å trekke noen konklusjoner angående opphavet til sekvensene.

Acknowledgements

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Writing this thesis has been challenging, but also fun and interesting. I would first of all like to thank my supervisors, Christoffer Schander and Hans Tore Rapp, for initial ideas for exploring this fascinating group of sponges, and giving me the opportunity to write this thesis. I am especially grateful for having been given the occasion to work with such a wide range of methods, both morphological and molecular, and having had the opportunity to do field work aboard the R/V "G.O. Sars" during the 2009 H_2DEEP cruise. I have always received good guidance, have always felt welcome when asking for advice on my thesis, and greatly appreciate the thorough and timely feedback I have received on my work.

Several people have helped me with different aspects of this thesis, giving invaluable aid and advice. I would like to especially thank Kenneth Meland, who not only spent a great amount of time teaching me most of the molecular lab methods in this thesis, but also proved an invaluable source of advice on lab issues, sequence results, and phylogeny; and graciously agreed to look through and provide input on this part of the thesis. Paco Cárdenas provided additional molecular advice, gave assistance on the more sponge specific aspects of the phylogeny, provided pictures and articles, and helped with the translation of French sources. Solveig Thorkildsen and Bernt Rydland Olsen helped me learn and perform molecular cloning, which proved to be invaluable in obtaining good molecular results.

Alexander Plotkin kindly helped me both to obtain a couple of the more hard to get Koltun sources, and also helped translate both species descriptions and source titles from the original Russian. Additional translation was provided by Grigori Merkin. I would also like to thank Rolf Birger Pedersen and Haflidi Haflidason for providing information about survey sites and maps for use in this thesis.

I would like to thank my mother for taking the time to go through the language of the thesis. I would like to thank my fellow students that I have had the pleasure of studying with these last couple of years: Audun, Frøydis, Joar, Kaja, and Margrethe. Last but not least, I would like to thank my wife, Kjersti, for putting up with me during the time it took to write this thesis.

Contents

1. Introduction

1.1 Background

The Porifera, or the sponges, is one of the major recognized animal phyla, containing approximately 8,300 extant species currently described (van Soest et al. 2008). Though a number of freshwater species are known, the majority of sponges are marine, and they constitute an important part of marine ecosystems worldwide.

Sponges are defined as sedentary filter-feeders characterized by their unique possession of an aquiferous system, an arrangement of afferent and efferent canals conveying water through chambers lined with a single layer of flagellated choanocyte cells. A unidirectional current created by the action of choanocyte flagella brings suspended microorganisms and other nutrients to these chambers. Choanocytes and other cells remove nutrients and oxygen from the water and eject it through a main exhalant orifice (Figure 1.1). A number of cell types reside in an extracellular matrix in the interior of the sponge, the mesohyl, and are characterized by a high degree of mobility and plasticity. This matrix provides support for the sponge, typically through an inorganic spicule based skeleton, collagen fibrils and spongin fibers (Bergquist 1978; Hooper et al. 2002).

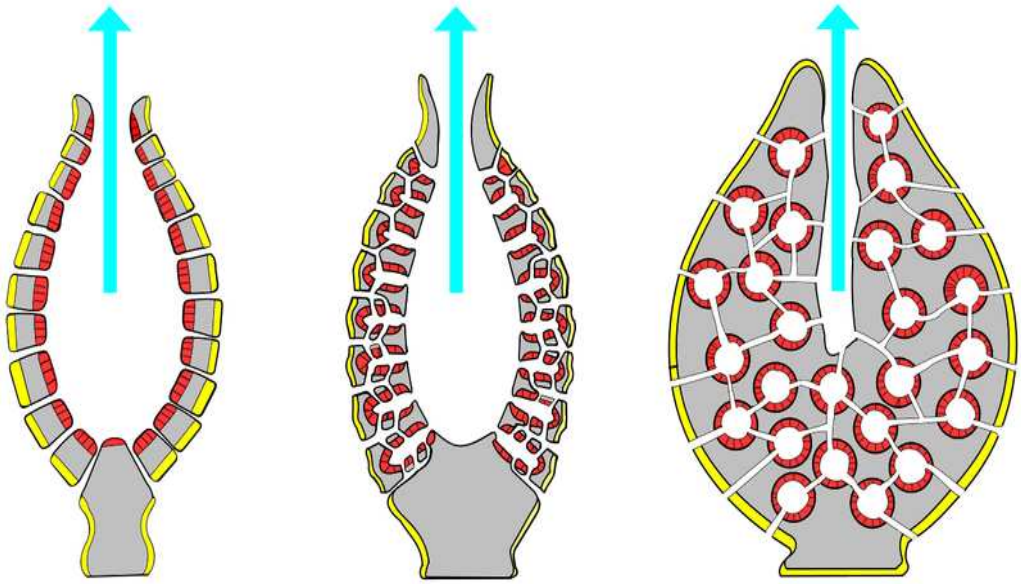

Figure 1.1. A schematic of the structure of the three major types of sponge aquiferous system. Red signifies choanocytes, grey mesohyl, and yellow outer layer of cells. Image released under the GNU license.

Phylum Porifera has traditionally been regarded as the most basal major phylum within the Metazoa, and is divided into three extant classes: the Calcarea, the Hexactinellida and the Demospongiae. New phylogenetic evidence suggests that the phylum might be paraphyletic, though no formal reclassification has yet been established (Hooper et al. 2002; Halanych 2004).

Class Demospongiae includes the vast majority of sponges, accounting for over 85% of described species (Hooper and Van Soest 2002a). The body is insulated from the outside by a single cell perforated epithelium, called the choanoderm within the aquiferous system, and the pinacoderm on the external surface of the sponge (Bergquist 1978). Between these layers is a less well defined area called the mesohyl. The organization, extent, and structure of this region varies, though it in most cases contains free or fiber bound skeletal spicules and provides a means for motile cells to get around the sponge. In contrast to the calcareous spicules of class Calcarea, the Demospongiae carry siliceous spicules. Compared to other animal taxa sponges are highly amorphous, and individual shape, size, and other properties vary considerably. Spicules, of which there are often several types, are typically more constant in their properties, and have for this reason been of critical importance in establishing the current taxonomic classification.

The existence of associations between sponges and microorganisms has been known for a long time, though the complexity of these interactions has often made them difficult to study (Imhoff and Stöhr 2003). In recent years research has increased markedly, aided by new molecular tools such as 16S gene libraries and flourescent *in situ* hybridization (FISH) (Hoffmann et al. 2006; Taylor et al. 2007). Such studies have shown that sponge mesohyl can contain highly specific microbial communities differing from the surrounding area (Hoffmann et al. 2005; Hentschel et al. 2006). Symbionts may be bacteria, archaea or eukaryotes. They can act as food sources, provide symbiotic functions, be commensalists of the sponge environment, or be parasites on the host (Taylor et al. 2007). For some sponges, the so-called high-microbial-abundance sponges, up to 40% of total sponge tissue volume has been reported to be composed of microbial cells (Taylor et al. 2007), though other sponges may contain comparatively little microbial volume (Hentschel et al. 2006). As a food source, the sponge can benefit from the metabolic capabilities of highly diverse microorganisms through phagocytosis of ecto- and endosymbionts, supplementing direct filter-feeding.

Deep-sea hydrothermal vent areas present unique opportunities for symbiotic arrangements between microorganisms and host animals. Most vents form in areas of active seafloor spreading such as at the Mid-Ocean ridges of the world's oceans. Since the discovery of the first vent habitat along the Galápagos Rift in 1977, they have been the subject of intense study. These high-energy habitats have been called "oases of the sea" owing to the huge increase in biomass around the chimneys and fissures where heated mineral-rich water exits the seafloor. In contrast, the surrounding seabed some distance away is typically life and resource poor (van Dover et al. 2006).

Biomass increase around the vents is ultimately dependent on chemolithoautotrophic prokaryotes possessing the metabolic pathways necessary both to turn inorganic compounds into energy, and to use this energy to fix inorganic carbon such as CO_2 into organic carbon. Capitalizing on these processes, many species of several animal phyla carry a substantial number of prokaryote symbionts. Most notably are the pogonophore giant annelids such as the up to several meters long *Riftia pachyptila* Jones, 1981 (formerly placed in the now obsolete phylum Pogonophora) (Cavanaugh et al. 1981; Halanych 2004), and mytilid bivalves of the genus *Bathymodiolus* (Belkin et al. 1986). Other vent organisms graze on mats of free-living bacteria and form the nutritional basis for larger vent fauna, creating a vent-specific habitat (van Dover et al. 2006). Vent-associated fauna in the surrounding area benefit in a more indirect fashion from the gradient of increased enrichment around the vent systems (Vacelet 2006b).

While the hydrothermal hot vents associated with Mid-Ocean ridges are best known, studies have shown other examples of environments where inorganic compounds provide the basis for specialized ecosystems. Vent-like complex chemosynthetic systems have been discovered at seep areas, localized regions of subsurface hydrocarbon (mainly methane) and sulfur seepage that have been shown to be a widespread feature along continental margins (Sibuet and Olu 1998). Depending on their precise properties, they are variously described as cold seeps, methane seeps, pockmarks, mud volcanoes, or if highly saline, brine pools (Smith et al. 2000; van Dover et al. 2006; Levin and Mendoza 2007). Interestingly, other areas of transient but intense nutrient access and decomposition such as whale skeletons (whale falls), wood falls and kelp falls have been shown to contain closely related fauna as well (Distel et al. 2000; Smith and Baco 2003).

In studies of chemoautotrophic processes from the inorganic compounds in the vent or seep discharge, reduced sulfur compounds have attracted much attention; however methanotrophic symbionts have also been investigated. Examples are seen in gill symbionts carried by *Bathymodiolus thermophilus* Kenk and Wilson, 1985 from the Galápagos Rift hydrothermal vent that are sulfur oxidizers (Belkin et al. 1986), while the symbionts of closely related mytilid *B. childressi* Gustafson et al. 1998 from a cold seep in the Gulf of Mexico prove to be methanotrophic (Childress et al. 1986; Smith et al. 2000). Methanotrophic symbionts have also been found in pogonophore vent and seep annelids (Gebruk et al. 2003).

Sponges at or in the vicinity of deep-sea vents are mainly either hexactinellids or members of the deep-sea demosponge family Cladorhizidae (Vacelet 2006b). As the family name implies, these sponges are usually erect, and in many cases branching. On normal deep-sea seabed, the Cladorhizidae are typically found as small, single individuals owing to the poor nutrient availability of the deep-sea. Close to vent sites however, numerous cladorhizids are common, occurring with a high diversity, many of them still undescribed (Vacelet 2006b).

While the cladorhizids have been found in the vicinity of hydrothermal vents at small distances to active smokers, and at cold seep areas, they are typically not documented among the true vent fauna directly benefiting from vent effluent, a surprising fact given the great number of symbiotic interaction between sponges and other microorganisms (Vacelet et al. 1996; Vacelet 2006b). An exception is the symbiotic relationship between a sponge and methanotrophic bacteria that was discovered in a cladorhizid from a mud volcano in the Barbados Trench (Vacelet et al. 1995; Vacelet et al. 1996). This species, later named *Cladorhiza methanophila* Vacelet and Boury-Esnault, 2002 (Vacelet and Boury-Esnault 2002) was found to carry two distinct methanotrophic symbionts within the sponge tissue.

In addition to being the demosponge family most typical in deep-sea vent and near-vent areas, the cladorhizids are of great interest as they are quite atypical sponges. Another highly unusual mode of nutrient acquisition was discovered within the Cladorhizidae at about the same time as the discovery of methanotrophic symbionts in *C. methanophila*: the capture and subsequent digestion of small crustaceans (Vacelet and Boury-Esnault 1995).

G. O. Sars, when erecting the genus *Cladorhiza* (1872), remarked on the lack of an aquiferous system in *C. abyssicola* Sars, 1872, and noted the adhesiveness of the sponge surface. His hypothesis that the observed microscopic spicule hooks catch prey and thus “probably fulfil

an essential condition for its nourishment" (Sars 1872) was wrongfully discounted, as filter-feeding was later deemed ubiquitous for the sponges. When pores or oscula could not be found, it was typically assumed that the specimens were damaged or had missing parts (Ridley and Dendy 1887; Lundbeck 1905).

This remained the consensus until 1995, when Vacelet and Boury-Esnault made a detailed study demonstrating the process of carnivory in the cladorhizid species *Asbestopluma hypogea* Vacelet and Boury-Esnault, 1996 (Vacelet and Boury-Esnault 1995; Vacelet and Boury-Esnault 1996). Carnivory was soon also described within the genera *Cladorhiza* (Vacelet et al. 1995) and *Chondrocladia* (Kübler and Barthel 1999). The genera *Abyssocladia* and *Neocladia* have recently been revived (Vacelet 2006a; Vacelet 2008), and the genus *Lollipocladia* erected (Vacelet 2008), so that the Cladorhizidae currently consists of six genera.

With the exception of *Chondrocladia*, where a modified aquiferous system is used to inflate prey-capturing globules, choanocytes, choanocyte chambers and channels are absent. This secondary loss or modification is a novel alternative to the strategy of nutrient acquisition and associated structures that is one of the major defining characters of phylum Porifera. Carnivory might be the result of adaptation to the oligotrophic waters of the deep-sea, where filter-feeding would be less advantageous given the low nutrient content of the water and unpredictable food supply (Vacelet and Boury-Esnault 1995; Vacelet and Duport 2004). For most species, carnivory is inferred through morphological features and presence of crustacean debris, though a few studies have actually described the process directly.

The method for capturing prey seems to vary somewhat among the genera where it has currently been described but in all cases depend on trapping prey which come in contact with the sponge. Given the passive method of feeding, the prey items are potentially all small animals of a suitable size, but are generally small crustaceans, especially copepods as they carry numerous appendages that might become entangled in the sponge and contain a high degree of energy-rich lipids (Vacelet and Duport 2004; Watling 2007).

The first description of prey capture and manner of digestion was Vacelet and Boury-Esnault's study of *A. hypogea* in 1995. This *Asbestopluma* species is erect and pinnate in shape (Vacelet and Duport 2004), may be perched on a stalk, and carries up to 10 mm long filamentous protrusions. These protrusions feature hook-like palmate anisochelate

microscleres that passively trap the setae and other appendages of small crustaceans. Typically, a prey becomes stuck to one or more filaments and frequently gets more entangled while struggling to break free of the sponge. Cells soon migrate to the general area, and are able to completely envelop the prey in about one day, creating a temporary digestive cavity where the prey is subsequently digested over the course of 8-10 days (Vacelet and Boury-Esnault 1995; Vacelet and Duport 2004). In *Chondrocladia*, this system complemented by spheres inflated by the remnant aquiferous system, deflating on contact and thus surrounding the prey (Kübler and Barthel 1999). For *Cladorhiza*, a sticky, mucous surface probably aids the protrusions, less orderly placed than in Asbestopluma, in prey capture (Watling 2007). Based on morphology, lack of an aquiferous system and microsclere spicule arrangement, *Abyssocladia*, *Lollipocladia* and *Neocladia* are assumed to be carnivorous as well, though the process has not been described in greater detail in these genera (Vacelet 2006a; Vacelet 2008).

Today, it is believed that carnivory is the general feeding mode in all of the approximately 100 described species within the Cladorhizidae (Vacelet 2007), in addition to certain members of other families such as the Guitarridae, Esperiopsidae and Mycalidae (Watling 2007; Vacelet 2008). The question of whether Cladorhizidae is mono- or paraphyletic has been raised as well. Molecular data, which are almost completely lacking for these groups, are necessary to resolve the issue of phylogeny where morphological methods are insufficient (Vacelet 2007).

1.2 Objectives

Cladorhizidae is the family of demosponges most frequently found in close proximity to vent systems. A carnivorous method of feeding has made the cladorhizids adapted to the otherwise nutrient-poor deep sea. Vent systems are often found at great depth, and the cladorhizids would thus be pre-adapted to colonize these local areas of higher nutrient access, where they occur in greater number and diversity (Vacelet 2006b).

Sponges, including one reported cladorhizid, are known to be able to carry microorganism symbionts. As several vent species are reported to carry symbiotic bacteria, vent-associated cladorhizids are good candidates for harboring such symbionts. As the fauna of vent and near-vent systems and other habitats such as cold seeps and mud volcanoes in many cases have been found to be closely related (e.g. Smith et al. 2000), specimens from these types of habitat are also of great interest.

Vent-associated cladorhizids have been shown to contain a high proportion of undescribed species (Vacelet 2006b), making specimens from vent areas interesting from a taxonomic perspective. Doubts have been raised concerning the current embryo and spicule-based classification of the Cladorhizidae and related taxa with similar morphology and carnivorous feeding mode (e.g. Vacelet 2007). Few molecular studies have yet tried to provide an alternative method to establish the phylogenetic relationships within these groups (Vacelet 2006b).

The R/V "G.O. Sars" cruises from 2006-2008 have obtained numerous cladorhizids at or near the newly discovered Mid-Arctic Ridge vent areas, at the Nyegga cold seeps off the coast of Norway, and at the Håkon Mosby mud volcano. Using the R/V "G.O. Sars" material in this study, I have pursued several of the issues above, which may be summed up in the following objectives:

- To carry out a taxonomic inventory of the 2006-2008 R/V "G.O. Sars" cladorhizid material based on spicule and morphological characters.

- To perform an exploratory study to ascertain the feasibility of, and provide some preliminary results in using COI and ITS molecular data to provide insight into the phylogenetic relationships of the cladorhizid species, and their placement within the Demospongiae.

- To test the possibility of using molecular sequencing to determine the presence of methanotrophic symbionts through amplification of *pmoA*, a gene coding for a part of a universal enzyme for methanotrophic metabolism.

2. Materials and methods

2.1 Survey area

The samples for this study were collected from four areas: (1) the vent fields of Troll Wall, Gallionella Garden and Soria Moria on the southern end of the Mohns Ridge, (2) the vent field of Loki's Castle on the southern end of the Knipovich Ridge, (3) the active cold seep area at Nyegga on the Norwegian margin, (4) and the Håkon Mosby mud volcano (Figure 2.1).

The Mohns and Knipovich Ridges, together with the Kolbeinsey, Molloy and Gakkel Ridges, collectively form the Arctic Mid-Ocean Ridge system. It marks the boundary between the Eurasian and North American continental plates, and extends the Mid-Atlantic Ridge northwards from the northern shelf of Iceland towards the pole through the Norwegian-Greenland Sea (Figure 2.1) (Okino et al. 2002; Pedersen et al. in press). Here, seafloor spreading is slower than at the Mid-Atlantic Ridge and the ridge system is classified as an ultra-slow spreading system (<20 mm spreading per year), decreasing from 18-20 mm per year at the Kolbeinsey Ridge directly north of Iceland to 6-12.7 mm per year at the northernmost Gakkel Ridge (Okino et al. 2002; Pedersen et al. in press). Based on extrapolation from faster moving ridge systems, it was believed that ultra-slow spreading systems would contain few or no vents. However, more than 20 active or ancient vent sites have been found either on the seafloor or detected as seawater anomalies along the Arctic Mid-Ocean Ridge system to date (Pedersen et al. in press). The fact that the ridge system borders on and enters the Arctic Ocean makes it particularly interesting. The Arctic Ocean is an area known for its high species endemism and is therefore of great importance in furthering our knowledge of vent fauna world-wide (van Dover et al. 2006).

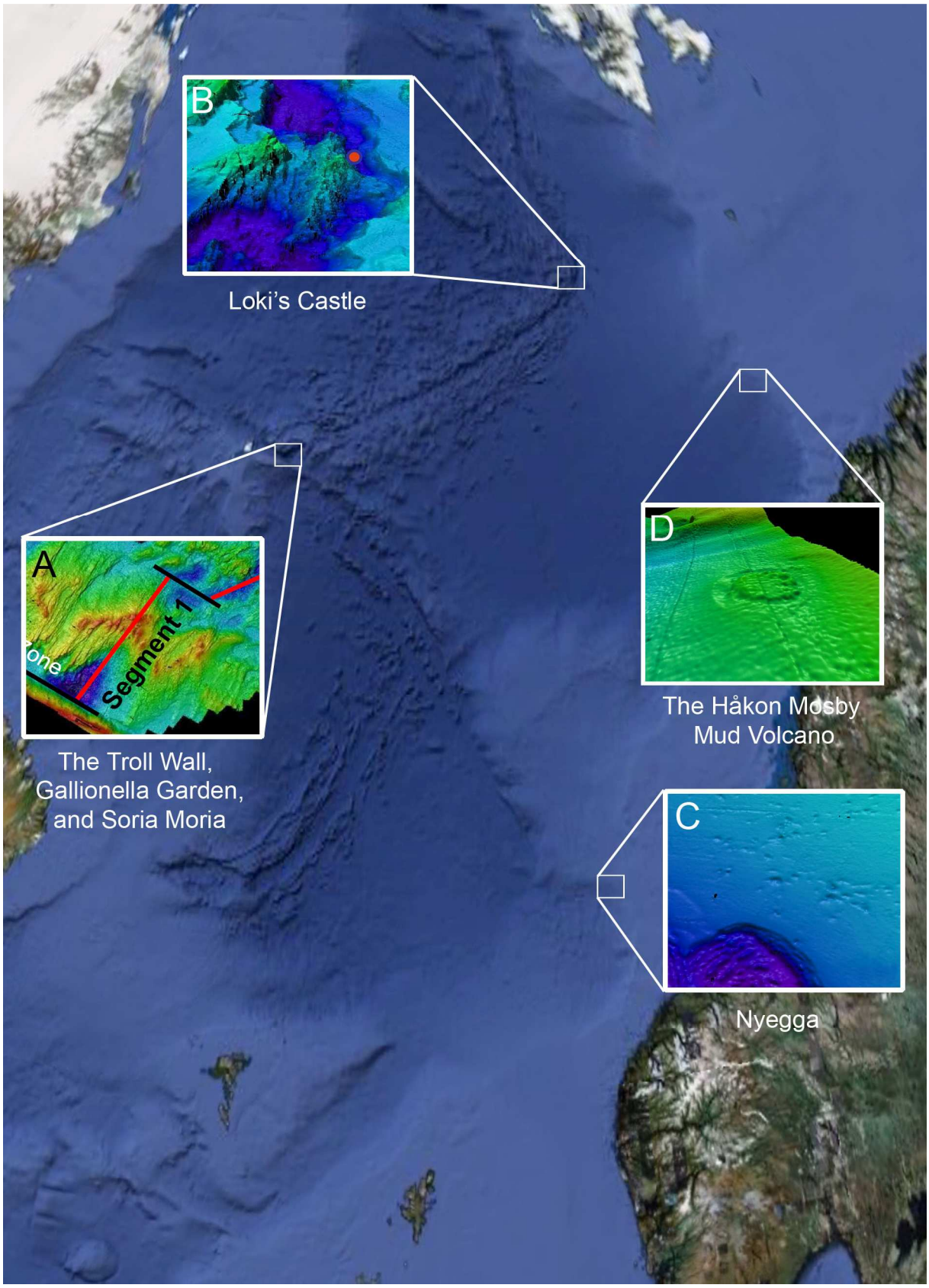

Figure 2.1. Overview of the sample locations showing the approximate location of (A) the Troll Wall, Gallionella Garden and Soria Moria, (B) Loki's Castle, (C) Nyegga, and (D) the Håkon Mosby Mud Volcano. Main map from Google Earth, detailed maps courtesy of Haflidason (C) and Pedersen[1] (A, B, D).

[1] Haflidason, H. Dept. of Geology; Pedersen, R. B. Centre for Geobiology: University of Bergen.

The Troll Wall (Trollveggen) is a vent area on the eastern margin of the southern part of the rift valley of the Mohns Ridge (71°18'N and 5°47'W), where the rift descends into the Jan Mayen fracture zone (Pedersen et al. in press). It is the site of both low and high temperature vents (Figure 2.2). The vent field, at a depth of approximately 550 m, contains at least 10 major vent sites composed of multiple 5-10 m white smoker chimneys with measured temperatures exceeding 260 °C, and is surrounded by larger areas of more diffuse lower temperature flow (7 °C as opposed to an ambient seawater temperature of -0.7 °C) with large iron oxide-hydroxide deposits, site of a bacterial carpet of iron oxidizing *Gallionella ferruginea* Ehrenberg, 1836, and hence named Gallionella Garden (Thorseth et al. 2007; Pedersen et al. in press). Discovered at the same time, Soria Moria is a smaller vent field situated on the other side of the ridge (71°16'N and 5°49'W), 5 km to the southwest, containing numerous white smokers. At 71°N, these vent fields were at the time of discovery the northernmost vent fields known (Pedersen et al. 2005; Schander et al. in press). While very few vent-endemic species have been found in this area, some species may be vent-associated (Schander et al. in press).

The Loki's Castle (Lokeslottet) vent field (73°30'N and 8°E) was discovered during the 2008 H_2DEEP-08 expedition with R/V "G.O. Sars" and is the northernmost vent field currently found, though chemical and physical anomalies indicating possible vent fields have been reported even farther north along the ridge (Pedersen et al. 2009; Pedersen et al. in press). It is located on an axial volcanic ridge about 700 m from the rift bottom, on the bend where the Mohns Ridge meets the Knipovich Ridge (Figure 2.1) at a depth of 2,400 m. It contains five active up to 11 m high black smoker chimneys with a temperature reaching 317 °C, located on a mound of sulfide minerals approximately 200 m across and 20-30 m tall (Pedersen et al. in press) (Figure 2.3). The fauna recovered from this site, as yet unpublished, contains a greater number of true vent-endemic taxa than the Mohns Ridge area (Rapp pers. comm.[2]).

[2] Rapp, H. T. Dept. of Biology/Centre for Geobiology: University of Bergen

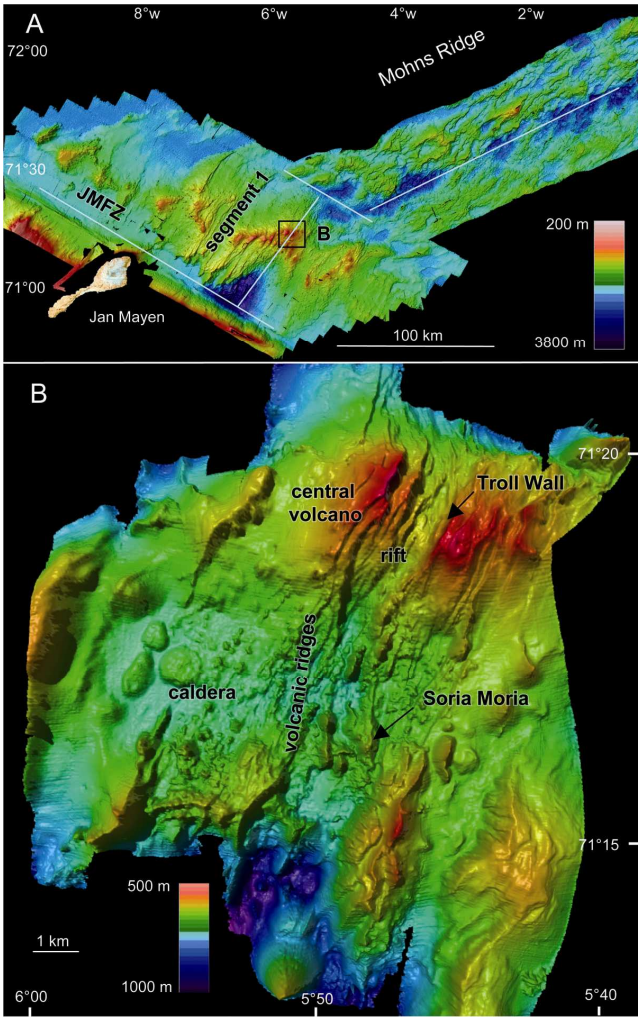

Figure 2.2. (A) The southern slope of the Mohns Ridge with a detailed view of the (B) Troll Wall and Soria Moria vent fields. Maps courtesy of Pedersen.

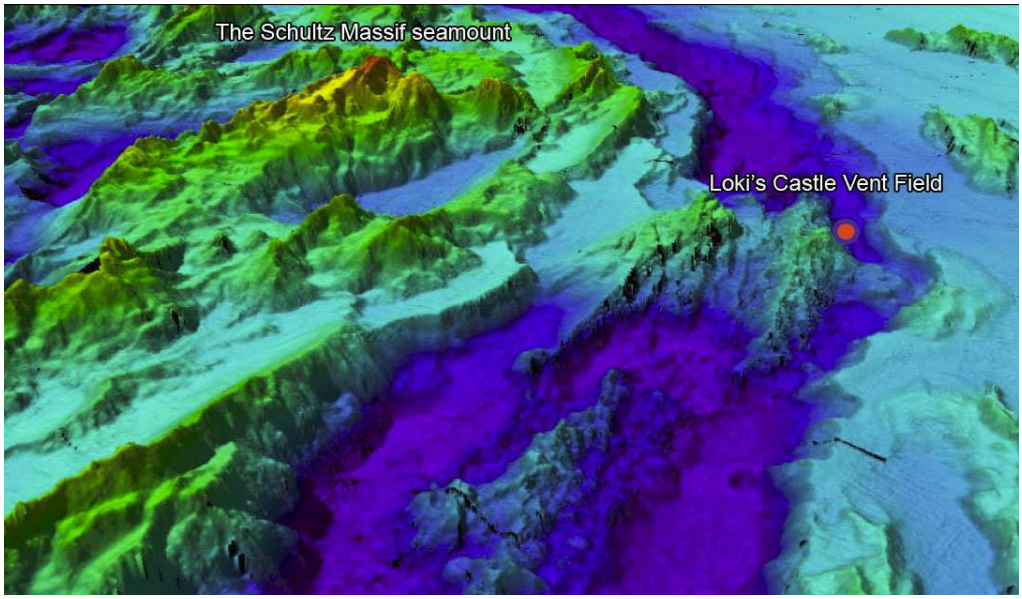

Figure 2.3. Loki's Castle and the Schultz Massif seamount. Map courtesy of Pedersen.

The Schultz Massif is a seamount due west and close to the Loki's Castle vent field, where sampling was carried out during the BIODEEP-07, H_2DEEP-08 and -09 cruises (Figure 2.3). At the top of the seamount, depth is 500-600 m. The slope down to Loki's Castle is composed of several terraces and plains and descends down into the rift valley and back to the mid-rift volcanic ridge containing the Loki's Castle vent field. While close to Loki's Castle, it is distant enough that it is not in the immediate vent enrichment area, and fauna composition is similar to the normal distribution in the wider Norwegian Sea area (Barthel and Tendal 1993). Still, the geology of the area does not preclude vent or seep areas closer to the seamount, and thus the present degree of vent and seep enrichment is unknown (Rapp pers. comm.).

The Nyegga area (approximately 64°40'N and 5°17'E) is previously known to be a site of seepage from underlying rock-bound hydrocarbon deposits, evidenced by a number of pockmarks formed by the eruption of methane deposits (Hovland et al. 2005) (Figure 2.4). The depth varies from 550-1,100 m with a general slope of 1° down to the abyssal plain at 3,000 m. Ambient temperatures are at –0.7 °C. A pockmark-specific fauna has been reported, including bacterial mats, annelids and pycnogonids similar to a faunal composition earlier reported from the Håkon Mosby mud volcano (Gebruk et al. 2003; Hovland et al. 2005). Sponge samples in this study were recovered from the pockmarks G11, Dodo and Tobic (Figure 2.4).

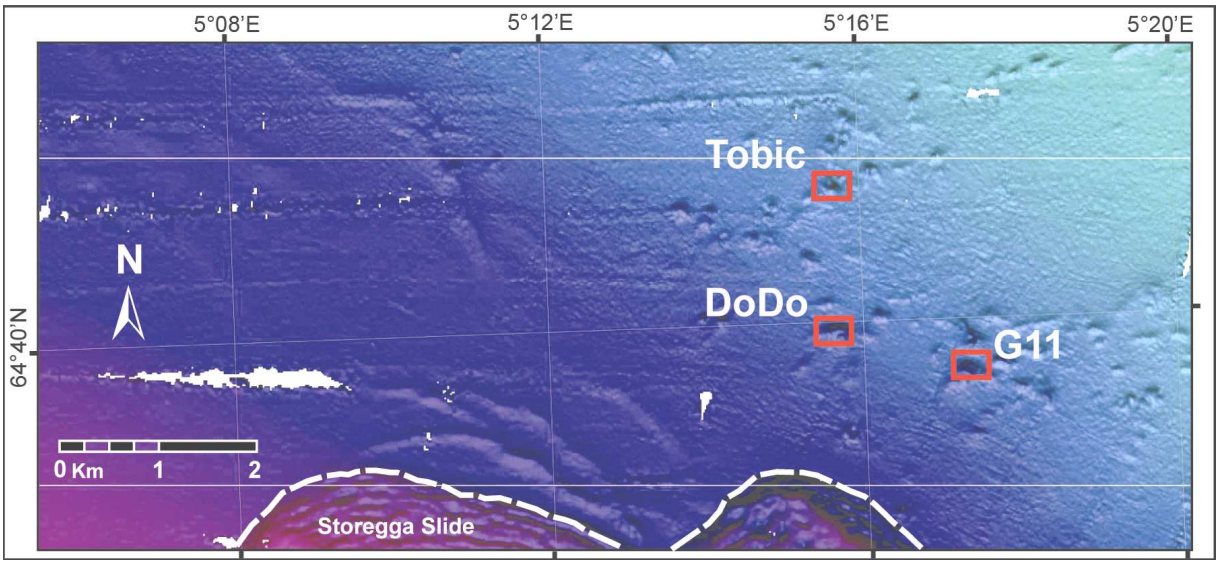

Figure 2.4. The Nyegga area showing the three pockmarks from where samples were recovered. Map courtesy of Haflidason.

The Håkon Mosby mud volcano (72°00'N and 14°44'E), located in the Barents Sea, is situated on top of 6,000 m sediment pile at a depth of 1,250 m and is more than 1,000 m in

diameter (Figure 2.5). There is a large influx of methane in the crater of the volcano, and it is dominated by bacterial mats and pogonophorans containing methanotrophic symbionts. In addition to seep-specific fauna, there is also a general increase in biomass, compared to the surrounding area, related to the general enrichment of the area (Gebruk et al. 2003).

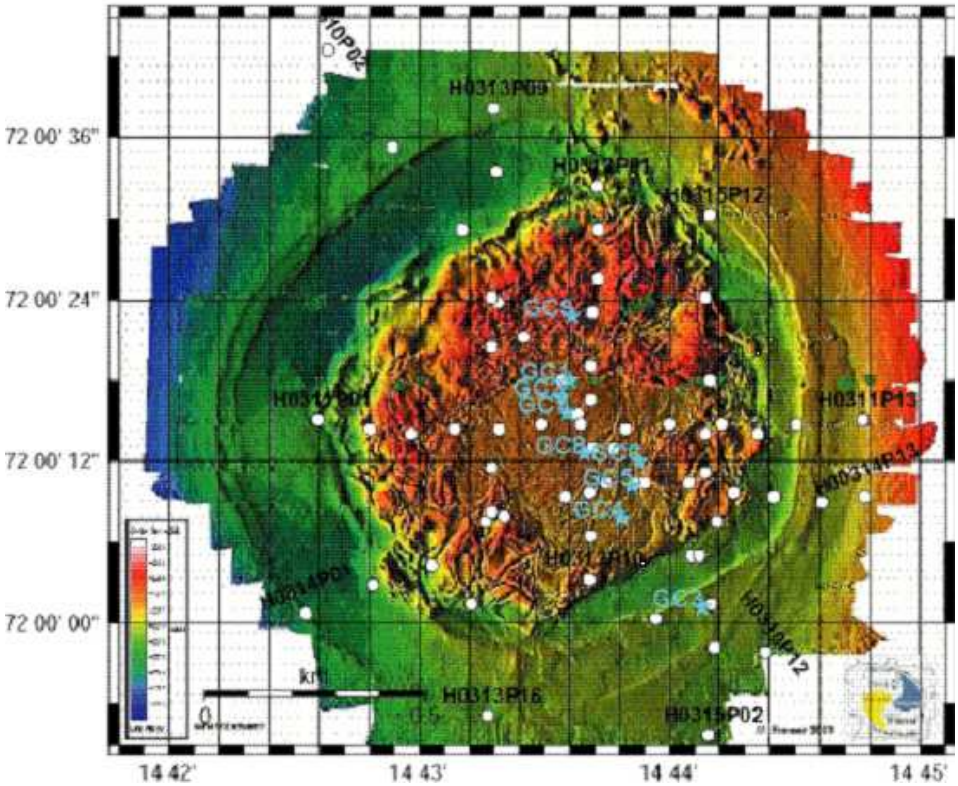

Figure 2.5. The Håkon Mosby Mud Volcano (Kaul et al. 2006).

2.2 Sampling

The 2006 and 2007 cruises undertook further surveys of the Troll Wall/Gallionella Garden and Soria Moria vent fields, discovered in 2005, and the 2007 cruise made an unsuccessful attempt to discover the source of detected anomalies at the then undiscovered field at Loki's Castle. The 2008 cruise discovered the Loki's Castle vent field on the southern part of the Knipovich Ridge. The following cruise made surveys of methane seeps along the Norwegian margin. Lastly, the 2009 cruise included a revisit to the Loki's Castle area as well as a short survey of the Håkon Mosby mud volcano.

The Troll Wall/Gallionella Garden specimens were collected during the 2006 BIODEEP cruise. One specimen was taken on the Schultz Massif slope during the 2007 BIODEEP cruise. Specimens on the Schultz Massif slope and near Loki's Castle were collected during the 2008

H_2DEEP cruise, Nyegga seep specimens were collected at the subsequent R/V "G.O. Sars" cruise (GS08-155) right after H_2DEEP-08. Further specimens were collected at the Håkon Mosby mud volcano and near Loki's Castle during the 2009 H_2DEEP cruise (Appendix A).

2.2.1 ROV sampling

Remotely operated vehicle (ROV) samples provided most of the specimens examined in this thesis. The medium-work class ROV Bathysaurus XL, tethered to a deck-based container-housed control unit, from ARGUS Remote Systems (Haflidason et al. 2008), was used on all cruises, providing video footage of samples, and enabling collection of specimens through a horizontal box corer and a suction sampler with collection container. The 2006 and 2007 ROV samples were collected at three different dives at or close to the Troll Wall and Gallionella Garden. The 2008 samples were collected at the Schultz Massif or on the irregular slope between the Schultz Massif and Loki's Castle, as well as at the Tobic, Dodo and G11 Nyegga pockmarks.

2.2.2 Box corer

A 25 x 25 cm box corer with an inner removable box was used in the collection of one specimen close to the methane seep at pockmark G11 during the Nyegga part of the 2008 cruise (Haflidason et al. 2008).

2.2.3 Agassiz trawl

An Agassiz trawl was used to collect samples during the 2009 cruise both at the Håkon Mosby mud volcano and between the Schultz Massif and Loki's Castle.

2.2.4 Sorting, preservation, and naming

The contents of the ROV container and box corer were sorted into animal phyla, then further into taxonomic groups depending on taxon. Cladorhizid specimens used in this study were identified as such aboard R/V "G.O. Sars", and thoroughly cleaned for any visible contaminants before preservation in 96% ethanol. A number of specimens were fixated in formaldehyde; for some of these specimens a smaller part was preserved in ethanol for genetic analysis. Samples were labeled with station name, location, date and equipment used. Pictures of the fresh specimens were taken in many cases.

All cladorhizid samples were named and numbered to discriminate between them for the purposes of this thesis based on cruise year. Appendix A contains a full list of sampling stations and samples used in this study.

2.3 Morphological methods

Species identification was carried out through measuring the type and size of specimen spicules, supplemented by noting sponge general shape, size and form. For species descriptions, pictures of the habitus and detailed pictures of structures of interest, light microscopy slide pictures (Appendix C), as well as SEM-images of the spicules themselves, were taken. A short description of the different spicule types can be found in Appendix B.

Establishing species identity was done through comparing spicule measurements and habitus with a list of relevant species in the literature, including described species from the North-Atlantic north of Gibraltar up to and including species in the Arctic and Bering Seas. Some spicule preparations from older Bergen Museum specimens, and a number of slides made by Lundbeck for the Danish Ingolf-Expedition report (Lundbeck 1905), were available for comparison.

2.3.1 Spicule microscopy slide preparations

Sponges are highly amorphous, and differ in size, shape, and structure as a response to local conditions. For this reason, characterization of the size and type of spicules found in a specimen is crucial for identification, although intraspecific variation with regards to these properties can be found as well. Due to the spicules normally minute size, these measurements are carried out using microscopy slide preparations, created by dissolving a small piece of sponge tissue in a suspension, and applying some of this suspension on a microscopy slide for examination.

The method used to create permanent microscopy slide preparations for this thesis follows the standard procedure described in the *Thesaurus of Sponge Morphology* (Boury-Esnault and Rützler 1997), though sodium hypochlorite (NaClO, bleach) was used to dissolve tissue instead of nitric acid (HNO_3) in many cases, as a commonly used alternative to nitric acid (Rapp 2006). Euparal or Eukitt was used as a mounting medium.

For each preparation, a small tissue fragment was cut from the specimen and put in a test tube containing a solution of NaClO or HNO_3 and allowed to dissolve overnight. Excess tissue solvent was then removed by pipette and distilled water added, allowing at least two hours until the spicules resettled at the bottom of the tube. This procedure was repeated twice. Next, the water solution was removed and 75% ethanol added, again allowing at least two hours for settling, and this procedure was then repeated twice using a concentration of 96% ethanol. For

some of the NaClO preparations, 1.5 ml Eppendorf tubes were used instead of test tubes so that waiting times could be reduced through centrifugation of the samples to resettle spicules (5,000 rpm for 60s) (Cárdenas pers. comm.[3]). A standard microscope slide was then put on a hot plate (150 °C), and spicules from the ethanol suspension were applied using a pipette to the slide to let the ethanol evaporate. 3-5 drops of mounting medium was applied to the slide and covered with a cover slide. The slide was then allowed to harden at 55-60 °C in the case of Euparal, or at room temperature in the case of Eukitt, for 48 hours.

A Leitz Dialux 20 microscope with a measuring reticule eyepiece was used to measure individual spicules, using a calibration slide to establish accurate conversion factors between the reticule at different objective magnifications and actual spicule size. For each individual specimen, a minimum of 30 spicules of each type of spicule found was measured when possible, though in cases where spicules of a certain type were very scarce this was not attained. The part of the sponge where tissue was removed was noted in case significant differences between spicule type and sizes were found in preparations from the same specimens or specimens of the same suspected species.

2.3.2 Specimen habitus description

While spicules are necessary to identify specimens to species level, the habitus can also give a general idea of species identity. The Cladorhizidae are typically erect, often branched, sponges, with a number of small protrusions. The type (if any) of branching and the structure of any smaller protrusions therefore provide good supporting characters to the size and shape of specimen spicules.

In situ stills from the ROV video feed provide important information on the habitus of the still living sponge. Pictures were in many cases taken aboard the R/V “G.O. Sars” of freshly obtained specimens. Additionally, pictures were taken of all preserved specimens prior to tissue removal for spicule preparations or DNA extraction using a Canon PowerShot G9 digital camera. Pictures of structural details were taken using a Leica MZ16A stereo microscope connected to a Nikon Digital Sight DS-U1 camera and ACT-2U v1.31 software (Nikon Corp.). Sharper images were obtained by taking several pictures at slightly different focus depths, and then combined into one picture using Auto-Montage Pro v5.02 (Synoptics Ltd.).

[3] Cárdenas, P. Dept. of Biology: University of Bergen

2.3.3 Spicule SEM preparations

For pictures of spicules based on scanning electron microscopy (SEM) preparations, nitric acid (HNO_3) was used exclusively in order to dissolve tissue more completely and thus obtain cleaner pictures. A small, round glass slide was taped using double-sided or carbon tape to a SEM stub to provide a substrate for the spicules. Part of the tape was left uncovered by the slide to improve conductivity. A couple of drops of spicule solution were then put on the glass slide and allowed to air dry overnight before being coated in a thin layer of gold-palladium. Spicules pictures were taken using a combination of a Zeiss Supra 55 VP and a JEOL JSM-7400F scanning electron microscope.

2.3.4 Image processing

Adobe Photoshop CS3 was used to cut away the background, adjust brightness and contrast, and standardize color conditions between specimens from different pictures.

2.4 Molecular methods

2.4.1 Sequenced genes

Genetic sequencing was carried out for two purposes: to obtain sponge sequences in order to investigate phylogenetic relationships within the Cladorhizidae and the Poecilosclerida, and detect the presence of methanotrophic symbionts in the specimen samples. To investigate phylogeny, partitions of two genes were sequenced: part of the gene coding for subunit I of the mitochondrial cytochrome oxidase complex (COI, also referred to as CO1 or COX1), and the rDNA internal transcribed spacers (ITS1 and ITS2), with 5.8S and fragments of 18S, 28S rDNA.

The COI gene is a popular choice in the molecular phylogeny of higher animal taxa (Erpenbeck et al. 2005). It has received special attention as the focus of a Metazoa-wide attempt to provide a standardized short sequence for species level identification as well, so-called DNA barcoding (Hebert et al. 2003). The most common primer pair for the subunit is the LCO1490 and HCO2198 metazoan primer pair (Folmer et al. 1994) which was also used in this thesis. The Folmer subunit partition is in the Demospongiae a 710 bp long sequence (of which ~650 bp is typically retrievable using direct sequencing) of the ~1550 bp long COI gene (Figure 2.6) (Folmer et al. 1994; Erpenbeck et al. 2005; Schander and Willassen 2005). While the resolution of the Folmer partition is usually sufficient to resolve even closely related species in nearly all animal groups (Hebert et al. 2003), it has been shown that in the Porifera and the Cnidaria, the subunit resolution is sometimes not adequate to resolve species

level relationships owing to the partition being more conservative because of DNA repair systems unique to these groups (Erpenbeck et al. 2005; Schander and Willassen 2005). Nevertheless, the Folmer partition was still chosen first, to ascertain its applicability in resolving the internal Cladorhizidae relationships, second, in order to establish a tentative phylogeny of the Cladorhizidae within the Poecilosclerida using GenBank sequences for comparison, and to broaden the usefulness of recovered sequences for further studies.

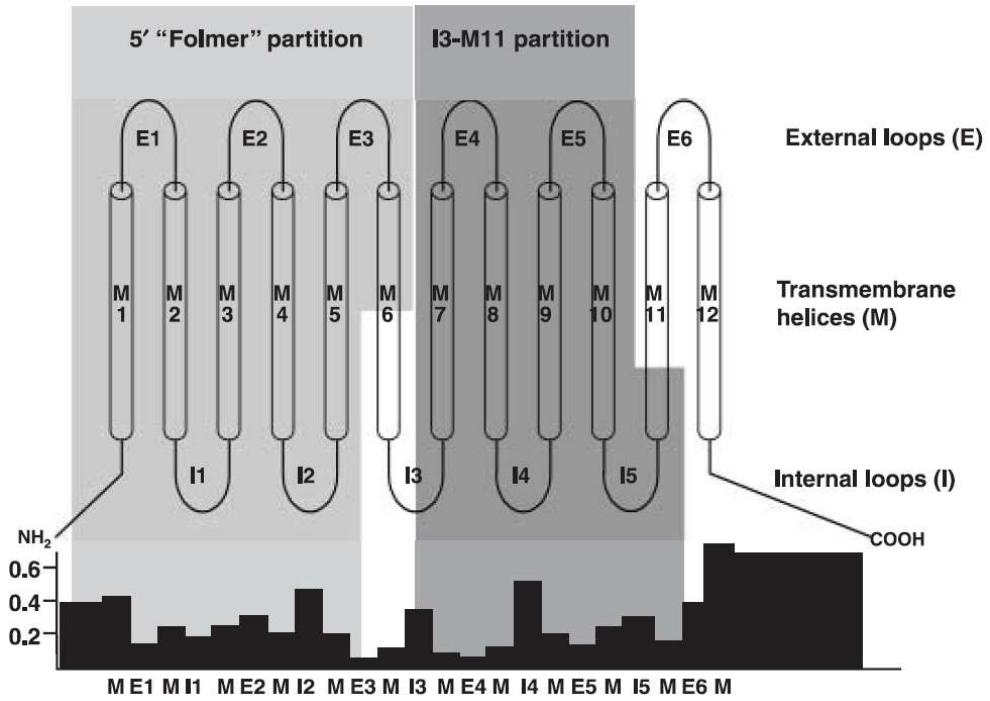

Figure 2.6. The COI protein (520 amino acids), with transmembrane helices, showing the Folmer and l3 M11 partitions (Erpenbeck et al. 2005).

The eukaryote nuclear ITS1 and ITS2 sequences are usually well suited for species and population level analysis (Hillis and Dixon 1991), and were chosen to provide resolution between specimens where the Folmer COI partition might prove too conservative. The ITS regions are non-coding and very variable regions, part of the rDNA array found in prokaryotes, eukaryotes and eukaryote mitochondria. While the two ITS regions themselves are variable, they are surrounded by three highly conserved ribosomal DNA regions, which are themselves useful for the phylogeny of more distant groups. In eukaryote nuclear DNA, these regions are the 18S, 5.8S and 28S subunits (Figure 2.7). The conserved nature of these sites makes them well suited as universal primer sites across a large range of taxa (Hillis and Dixon 1991). Demosponge specific 18SFow and 28SRev primers (Lôbo-Hajdu et al. 2004) were chosen as amplification primers. The 18SFow primer covers the -63 to -40 bp of 18S relative to the start of ITS1 while the 28SRev covers the +58 to +70 bp of 28S after the end of

ITS2 (Lôbo-Hajdu et al. 2004) (Figure 2.7).While eukaryotes may have up to several hundred tandem repeats of the entire array across the nuclear genome, intraspecific homogeneity is generally high, owing to concerted evolution (Hillis and Dixon 1991).

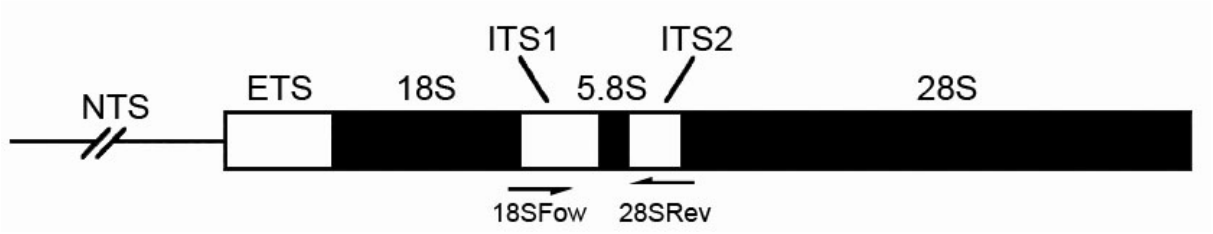

Figure 2.7. The eukaryote rDNA array, showing the non-transcribed spacer (NTS), external transcribed spacer (ETS), the 18S small subunit, the 5.8S subunit and the 28S large subunit as well as the two internal transcribed spacers (ITS1 and ITS2). Lôbo-Hadju et al. 2004 128SFow and 28SRev primer sites added. Figure adapted from Hillis and Dixon 1991.

The strategy used to ascertain whether the samples contained methanotrophic symbionts was to attempt to sequence a gene unique to methanotrophic bacteria: the presence or absence of product would serve as an indicator of whether this type of organism was present. Methanotrophic bacteria are able to utilize methane and other one-carbon compounds both as a carbon and energy source. To this end, enzymes known as methane monooxygenases (MMOs) are employed to catalyze the oxidation of methane to methanol, which is then further metabolized (Hanson and Hanson 1996). There are three groups of methanotrophic bacteria: type I alpha-proteobacteria, type II gamma-proteobacteria, and type X beta-proteobacteria. Within these groups, two forms of MMO have been discovered, a soluble form, and a ubiquitous particulate form (pMMO) common to all known methanotrophs (Hanson and Hanson 1996). For this reason, a gene coding for the expression of the alpha part of the enzyme pMMO, *pmoA*, was used as this would allow detection of any and all methanotrophs in the samples (Hoffmann et al. 2002). To sequence this gene, the 189A (Holmes et al. 1995) and mb661 (Costello and Lidtrom 1999) primer pair was used, producing a 470 bp *pmoA* fragment (Figure 2.8).

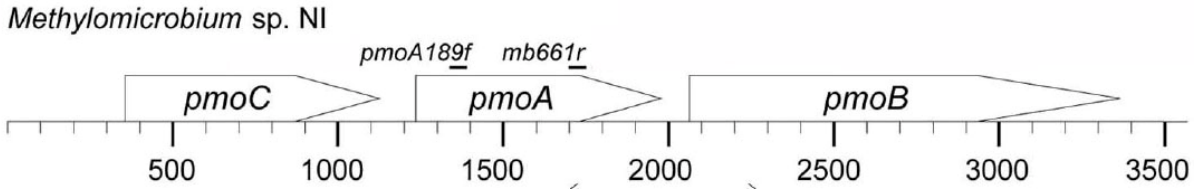

Figure 2.8. Three of the genes coding for particulate monooxygenase in an unknown *Methylomicrobium* including the pmoA primer sites for the 189A and mb661 primer pair (Tavormina et al. 2008).

2.4.2 Samples

One 2006- and sixteen 2008-specimens were chosen for sequencing representing six morphologically identified cladorhizid species (Table 2.1). For sample details, refer to Appendix A. Though a specimen from the 2009 H_2DEEP cruise was identified as *C. tenuisigma*, lack of time made it unfeasible to include this species in the analysis.

Table 2.1. Amplification and sequencing specimen list.

Species	Sample(s)
A. cupressiformis	GS06-04, GS08-05
A. furcata	GS08-04a, GS08-04b, GS08-04c, GS08-06a, GS08-06b
A. infundibulum	GS08-09
A. lycopodium	GS08-02, GS08-08, GS08-10, GS08-11a
A. sp. 1 aff. *lycopodium*	GS08-03
C. corticocancellata	GS08-07
C. gelida	GS08-01a, GS08-01b, GS08-01c

2.4.3 DNA extraction

Tissue was collected from the specimens by cutting off tips of branches or scraping off part of the cortex depending on the morphology of the sponge. No effort was initially made to distinguish sponge tissue from any other organisms living in or on the sponge. When early sequencing showed presence of DNA contamination, a second extraction was made during which a stereo microscope was used to check for other organisms on the tissue sample. Small particles, and in a couple of cases some partially digested copepods, were removed from the specimens before they were air-dried to remove ethanol.

The Viogene Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene BioTek) was used for extraction of DNA according to the tissue protocol in the kit instruction manual: For each extract the collected tissue was cut into smaller parts before insertion into a 1.5 ml Eppendorf tube. 20 µl Proteinase K was added to the samples and vortexed for 20 sec to start lysing the tissue while preserving DNA. The solution was incubated at 60 °C for several hours and vortexed regularly. While a longer incubation time is an alternative to vortexing during tissue lysis, the compactness of the tissue made vortexing necessary for complete dissolution. The solution was incubated at 70 °C for 20 min to catalyze usage of remaining proteinase K. 200 µl EX buffer was added, the solution was vortexed, and incubated at 70 °C for 10 min. Following similar sponge DNA extraction in earlier studies (Cárdenas et al. 2007), a single

centrifugation step was added and the mixture pipetted into a new 1.5 ml Eppendorf tube in order to remove spicules from the solution which might otherwise clog the filter of the column. 210 µl ethanol was then added and the sample vortexed. The mixture was pipetted into a mini column and collection tube, and centrifuged at 8,000 rpm for 2 min before switching the column to a new collection tube. The column was then washed twice with 500 µl WS buffer containing ethanol, centrifuging each time at 8,000 rpm for 2 min and the flow-through was discarded after each run. To remove excess ethanol an additional centrifugation step at 14,000 rpm for 2 min was done, after which the column was quickly moved to a new 1.5 ml Eppendorf tube so as to avoid ethanol evaporating back from the collection tube into the column. 200 µl preheated (70 °C) AE elution buffer was added to the column, which was allowed to stand for 5 min before a final 13,000 rpm centrifugation was run to elute DNA into the 1.5 ml Eppendorf tube.

2.4.4 PCR gene amplification

PCR was performed on a PTC220 DNA Engine Dyad (Bio-Rad Laboratories, formerly MJ Research). The total volume of PCR reaction mixture for the COI, ITS, and *pmoA* genes were 25 µl, containing 2.5 µl 2 mM Takara 10x buffer (TaKaRa Bio Inc.), 2 µl 2.5 mM dNTP mix (TaKaRa Bio Inc.), 1 µl 10 µM of each primer in the primer pair (Table 2.2), 0.15 µl 5 units/µl TaKaRa Ex Taq Hot Start (TaKaRa Bio Inc.), 2 µl template, and purified water. For certain samples, 50 µl mixtures were subsequently prepared in order to yield enough product for gel extraction of individual bands. In these cases, the reagent amounts were doubled, using the same amount of template adding 2 µl extra purified water. For amplification of vector DNA from bacterial clones using the M13 primer pair, a similar 25 µl reaction was used, applying cells from selected clones directly from the petri dish into the reaction mixture at the same time as the clones were transferred onto a new dish using a toothpick.

Table 2.2. Primers used for sequencing.

Gene	Primer	Sequence (5'->3')	Source
COI	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
ITS	18SFow	TCATTTAGAGGAAGTAAAAGTCG	Lôbo-Hadju et al. 2004
ITS	2004 28SRev	GTTAGTTTCTTTTCCTCCGCTT	Lôbo-Hadju et al. 2004
pmoA	A189	GGNGACTGGGACTTCTGG	Holmes et al. 1995
pmoA	mb661	CCGGMGCAACGTCYTTACC	Costello and Lidstrom 1999
vector	M13Fow	CTGGCCGTCGTTTTAC	Invitrogen
vector	M13Rev	CAGGAAACAGCTATGAC	Invitrogen

Reaction mixtures using 0.15 µl Amplitaq polymerase (Life Technologies, formerly Applied Biosystems Inc.) were also made using a standard MgCl amount of 2.5 µl. These mixtures showed no visible results however, and the use of this Taq polymerase was abandoned.

For COI amplification, the HCO2198 and LCO1490 general metazoan primer pair was used (Folmer et al. 1994). The two-step program used contained an initial denaturation at 94 °C for 5 min followed by five cycles of 94 °C for 45 sec, 45 °C for 30 sec, and 72 °C for 1 min, then followed by 30 cycles of 94 °C for 45 sec, 50 °C for 30 sec, and 72 °C for 1 min, then a final step of 72 °C for 10 min. For samples where band strength was low, the number of cycles in the second phase was increased to 35 to obtain a better yield in subsequent amplification.

For ITS amplification, 18SFow and 28SRev sponge-specific primers were used (Lôbo-Hajdu et al. 2004). The program contained an initial denaturation at 95 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 1 min, followed by a final cycle of 72 °C for 7 min.

In examining for the presence of methanotrophy, the primers A189 (Holmes et al. 1995) and mb661 (Costello and Lidtrom 1999) were used to check the existence of the *pmoA* gene. An amplification gradient was set up to find optimal annealing temperature using a program adapted from a previous study (Jensen et al. 2008). As results were inconclusive, annealing temperature was set at 55 °C as in Jensen et al. The program contained an initial 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, then finally a single cycle of 72 °C for 7 min. A second identical PCR was run on the initial amplification product as the initial run provided very little in terms of visible product.

The Invitrogen pCR4-TOPO vector M13F and M13R primers were used to amplify vector-bound DNA during cloning. The program contained an initial 95 °C denaturation step followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, then finally a single step of 72 °C for 10 min.

2.4.5 Gel electrophoresis

Gels were cast with 1% agarose mixed with 0.5 M TBE-buffer and 3-5 µl ethidium bromide or GelRed (Biotium Inc.), and run at 90V for 30-90 min. Gel electrophoresis was employed on DNA extracts directly in order to give a general idea of the amount of DNA in each mixture, using 8 µl extract and 2 µl 5x loading buffer; and to evaluate the success and amount

of amplified DNA, using 4 µl extract and 1 µl 5x loading buffer. The ΦX174 DNA-HaeIII marker (Promega Corp.) was used to evaluate size and quantity of DNA.

2.4.6 Gel extraction

Where gel samples yielded multiple bands, gel extraction was employed to isolate individual bands, using either the E.Z.N.A gel extraction kit (Omega Bio-Tek Inc.) or the Viogene DNA/RNA Extraction kit (Viogene BioTek). Sample bands were cut out of the gel using a clean scalpel blade on a UV table and put in 1.5 ml Eppendorf tubes. In the case of the E.Z.N.A kit, each resulting gel slice was weighed. An equal number of ml of buffer CP to the weight in grams to the slice (E.Z.N.A) or 0.5 ml GEX buffer (Viogene) was added to the tube. The sample was incubated at 60 °C for up to 10 min or until the gel dissolved, while vortexing every 2 min. The sample was then briefly centrifuged to collect the liquid to the bottom of the tube. The sample was transferred to the provided spin-column mounted in a 2 ml collection tube and centrifuged for 60 sec at 10,000 rpm. The flow-through was discarded and either 0.3 ml binding buffer (E.Z.N.A) or 0.5 ml WF buffer (Viogene) was added before a second identical centrifugation, discarding the flow-through. 0.7 ml SPW buffer (E.Z.N.A) or WS buffer (Viogene) was diluted with ethanol, and was added to the column. The sample was centrifuged for 60 sec at 10,000 rpm, after waiting 3 min in the case of the E.Z.N.A kit, and discarding the flow-through. The SPW/WS buffer step was repeated, and the liquid discarded again. The empty column was centrifuged again at 12,000 rpm for 60 sec to remove residual ethanol and quickly placed in a new 1.5 ml Eppendorf tube. Finally, 30 µl elution buffer was added to the column, and the sample was centrifuged for 60 sec to collect DNA into the 1.5 ml tube.

2.4.7 TOPO cloning

As direct amplification and subsequent purifying in most cases gave low quality, ambiguous sequences, a number of samples were cloned in order to separate sponge sequences from other amplified DNA. The Invitrogen TOPO TA Cloning Kit for Sequencing (Life Technologies, formerly Invitrogen Corp.) was used using chemically competent *Escherichia coli* bacteria. Reagent solutions were provided in the kit. The procedure followed the guidelines in the kit manual:

An initial standard PCR 25 µl amplification was done for each sample to be cloned in order to obtain fresh product as the necessary adenine-ends of Taq-amplified DNA are known to fall off after about 24 hours. The reaction was then evaluated using gel electrophoresis to decide

the amount of template to use in the initial TOPO cloning reaction. Where samples during previous amplification had displayed more than one band, 50 µl reactions were used so that gel extraction could be performed on the desired amplified DNA band before cloning. Otherwise, PCR product was used directly in the cloning reaction.

For each sample, a combination consisting of 1 µl salt solution, 0.5-4 µl PCR product, 1 µl TOPO vector, and purified water to a total of 6 µl was gently mixed and incubated for 30 min at room temperature, then placed on ice. Vials containing chemically competent *E. coli* were thawed on ice from -80 °C, and 2 µl TOPO cloning reaction added and gently mixed. The vials were incubated for 15-20 min on ice, then heat-shocked using a 42 °C water bath for 30 seconds before immediately being transferred back to the ice in order for the vector to enter the cells. 250 µl room temperature S.O.C medium was added to each vial, and the vials were then incubated by being shaken horizontally at 200 rpm at 37 °C for 1 hour. Each medium solution was spread on two selective medium agar plates containing ampicillin pre-coated with 40 µl X-gal, one using 50 µl, the other 100 µlsolution. The plates were incubated at 37 °C overnight. As ampicillin-resistance is conferred to the cells via the vector, only cells with vector inserts (transformants) would grow on the medium.

After overnight incubation, transformants containing vector inserts were identified as white colonies while vectors containing no insert were stained blue. 10 white clones for each sample were respread to a new selective plate and also added to the M13 PCR reaction described above. The respread plates were incubated at 37 °C overnight for potential later use while the PCR products were assessed using gel electrophoresis. From these 10 clones, 5-10 PCR products were chosen for enzyme purification and sequencing.

2.4.8 Purification

PCR products with promising single bands were purified using exonuclease I (EXO) and shrimp alkaline phosphatase (SAP) enzymes to remove excess primers and nucleotides from PCR product (USB Affymetrix). 0.05 µl EXO and 0.5 µl SAP were mixed with 1.45 µl purified water and 8 µl PCR product on ice, and run at 37 °C for 30 min, then 80 °C for 15 min. For gel-extracted product, no further purification was necessary.

2.4.9 Sequencing

The amount of DNA in PCR products was estimated comparing sample band strength to concentration and strength of the gel marker. This gave an approximate product concentration,

deciding the amount of template used in preparing the sequencing PCR-reaction. For the sequencing reaction, 1 µl BigDye v 3.1 (Life Technologies, formerly Applied Biosystems Inc.), 1 µl 5x buffer, 0.5 µl primer, 0.5-4 µl template and purified water to a total of 10 µl was used and run, using a program containing a single step of 96 °C for 5 min, 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. After the reaction, 10 µl purified water was added to the product before it was delivered on ice to the Sars International Centre for Marine Molecular Biology for sequencing.

2.4.10 Assembly

Initial assessment of sequence chromatograms was done using FinchTV 1.4 (Geospiza). The chromatogram data was visually examined to evaluate sequence quality, and BLAST searches were employed to identify which sequences could be reliably identified as poriferan through comparison with GenBank sequences, using the Sponge Genetree Server (Erpenbeck et al. 2008) to check the identity and putative phylogeny of related sponge species already in GenBank. Contig assembly was primarily done using SeqMan Pro, part of the Lasergene 8.0 suite of programs (DNASTAR), while using Contig Express 11 for comparison (Life Technologies).

2.4.11 Alignment

Sequence data were aligned using ClustalX 2.0.10 (Larkin et al. 2007), with default alignment parameters for COI. For the Lôbo-Hadju et al. primer pair, 18S, ITS1, 5.8S, ITS2 and 28S parts of the sequence were identified and aligned separately. As large inserts are generally relatively common in the ITS partitions (Meland, pers. comm.[4]), the gap extension penalty for the ITS fragments was reduced to 2 while keeping default parameters intact for the non-ITS fragments. The COI sequences were exported directly, the Lôbo-Hadju et al. fragments were joined together, and both alignments were exported in nexus format to Phylogenetic Analysis Using Parsimony (PAUP) 4.0b10 (Sinauer Associates).

In order to evaluate the phylogenetic relationships of *Cladorhiza* and *Asbestopluma* within the Demospongiae, poecilosclerid COI and ITS sequences were retrieved from GenBank and included in the alignment together with the COI and ITS sequences gained from this study. A list of downloaded sequences may be found in Appendix D. Where several sequences of the same species were available through GenBank, sequences were manually compared to each other. In all cases, none or only a couple of bases were different; thus only one sequence was

[4] Meland, K. Dept. of Biology: University of Bergen

chosen as representative per species. Previous studies suggest that Suberitidae or Tethyidae are sister groups to Poecilosclerida (Borchiellini et al. 2004; Nichols 2005; Lavrov et al. 2008). Accordingly *Suberites aurantiacus* (Duchassaing & Michelotti, 1864), *Tethya actinia* de Laubenfels, 1950 and *Sidonops neptuni* (Sollas, 1886) were chosen as outgroups for COI, and *Su. ficus* (Johnston, 1842) and *Tethya* sp. for ITS.

2.4.12 Phylogenetic analysis

An unweighted maximum parsimony (MP) search was performed in PAUP using 1,000 repetitions adding sequences in random order. The majority rule consensus tree was saved. A bootstrap analysis was run on the data using 2,000 boot replicates each using 10 MP repetitions, which would ensure a precision—that is the probability that a certain number of pseudoreplicates gives the same result as an infinite number of pseudoreplicates—of ±1% at 95% bootstrap support (BS) (Hedges 1992; Hillis and Bull 1993). Bootstrap values were appended to the MP consensus tree. For maximum likelihood (ML) analysis, ModelTest 3.7 (Posada and Crandall 1998) was used to establish a fitting model, and a ML search was performed in PAUP using 200 repetitions. MrModeltest 2.2 (Nylander 2004) was used to establish a model for the Bayesian analysis. A Bayesian search was carried out using 1,000,000 generations with 2 simultaneous runs, each employing 3 hot and 1 cold chain, sampling a tree every 1,000 generations. The trace files were examined in Tracer 1.4.1 (Rambaut and Drummond 2007), and the generations before reaching stable tree values were removed (burn-in). The remaining trees were combined to make a posterior probabilities tree, and the posterior probability values were applied to the ML/Bayesian tree. Trees were visualized and exported to vector graphic using FigTree 1.2.2 (Rambaut 2006).

3. Results

3.1 Taxonomic index

Systematics is based on Systema Porifera (Hooper and van Soest 2002c).

Class Demospongiae Sollas, 1885
Order Poecilosclerida Topsent, 1928
Suborder Mycalina Hadju, van Soest & Hooper, 1994

Family Cladorhizidae Dendy, 1922

Genus *Asbestopluma* Topsent, 1901
- *Asbestopluma cupressiformis* (Carter, 1874)
- *Asbestopluma furcata* Lundbeck, 1905
- *Asbestopluma infundibulum* (Levinsen, 1887)
- *Asbestopluma lycopodium* (Levinsen, 1887)
- *Asbestopluma* sp. 1 aff. *lycopodium*
- *Asbestopluma* sp. 2 aff. *lycopodium*

Genus *Cladorhiza* Sars, 1872
- *Cladorhiza corticocancellata* Carter, 1876
- *Cladorhiza gelida* Lundbeck, 1905
- *Cladorhiza tenuisigma* Lundbeck, 1905

3.2 Species list

ASBESTOPLUMA Topsent, 1901

Synonyms

Cometella (Schmidt 1870) (nomen oblitum)
[*Asbestopluma*] Ray Lankester, 1882:478 (nomen nudum)
Asbestopluma Topsent, 1901:23

Type species

Cladorhiza pennatula (Schmidt, 1875:119) (by subsequent designation; Topsent, 1901:23).

Description

Cladorhizidae lacking an aquiferous system, carrying palmate anisochelae (adapted from Hooper & van Soest, 2002).

Remarks

The genus name *Asbestopluma* was first used by Norman to refer to several specimens collected in the outer Hardangerfjord near Leirvik. Ray Lankester wrote a short report of the trip in *Nature*, where the genus was briefly mentioned without further description (Ray Lankester 1882). Vosmaer therefore put *Asbestopluma* into a list of *incertae sedis* genera (Vosmaer 1887). Topsent later re-examined some of Norman's specimens and found labels connecting *Asbestopluma* with the then named species *Cladorhiza pennatula* (Topsent 1901).

3.2.1 *Asbestopluma cupressiformis* (Carter, 1874)

Figure 3.1; Figure 3.10; Figure C.1

Synonyms and citations

Esperia cupressiformis in part Carter, 1874:215
Esperia bihamatifera in part (Armauer Hansen 1885:15)
Esperella cupressiformis var. *robusta* (Levinsen 1887:364)
Cladorhiza cupressiformis (Fristedt 1887:457)
Esperella fristedtii in part (Lambe 1900a:157)
Asbestopluma cupressiformis (Lundbeck 1905:58)
Asbestopluma cupressiformis (Hentschel 1929:934)
Asbestopluma cuppressiformis (Burton 1934:27) [sic]
Asbestopluma cupressiformis (Koltun 1959:77)
Asbestopluma cupressiformis (Koltun 1964:151, 163)

Type locality

Between Scotland and Faroe Islands at 60°25'N 9°40'W at 702 m (Carter 1874).

Material examined

GS06-01, GS06-04a, GS06-04b, GS06-04c, GS06-04d, GS08-05e, GS09-06. Lundbeck: *A. cupressiformis* preps. 1, 3.

Description

An *Asbestopluma* species with a single, erect stem growing from a small cylindrical stem. The main stem broadens out from the start of the main body, giving the samples the shape of a flattened club, but may also be slender. The surface of the body is irregular, with knobs and grooves along the surface. The color is white to light brown.

The complete specimens are 22, 30, 31, 40, 45 and 91 mm long, and overall similar in shape and texture except GS08-05e, which is just a stem with a couple of small, thin filaments. The width is between 1 mm at the base up to 7 mm at the apical end. One of the specimens has a large, round swelling at the top end.

Spicules

The megascleres are styli, sometimes subtylostyli. Grouping these is difficult as many overlapping forms exist, but they generally become shorter and more curved in the basal part of the sponge. There are two forms of microscleres: palmate anisochelae and forcipes. The forcipes were uncommon and unevenly distributed in some specimens, and mostly found in the top part. In a couple of specimens, small tylostyli, smaller palmate anisochelae, and in one case some tylostrongyla are found. According to Lundbeck (1905), these are associated with embryos within the sponge tissue.

Table 3.1. Spicules of *Asbestopluma cupressiformis*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS06-01							
Styli/subtylostyli	238	535	±95	656	10.6	±1.7	30
Palmate anisochelae	21.0	23.2	±1.0	25.6			30
GS06-04							
Styli/subtylostyli	456	628	±76	713	9.3	±1.2	30
Palmate anisochelae	21.0	23.2	±1.0	25.6			30
Forcipes	37.3	40.6	±2.4	44.3			30
GS08-05							
Styli/subtylostyli	551	703	±58	798	10.4	±1.5	30
Palmate anisochelae	18.6	22.0	±1.3	23.3			30
Forcipes	35.0	40.5	±2.5	44.3			15
Tylostyli	83.9	112.3	±6.4	125.8	5.1	±0.0	5
Small palmate anisochelae	9.3	12.7	±1.6	16.3			20
Tylostrongyla	14.0	18.3	±4.7	32.6			18
GS09-06							
Styli/subtylostyli	344	571	±173	804	10.2	±1.0	60
Palmate anisochelae	20.2	23.2	±1.2	24.2			30
Forcipes	36.4	41.3	±2.3	44.4			30
Tylostyli	88.9	125.2	±15.0	155.5	4.2	±0.5	30
Small palmate anisochelae	10.1	12.1	±1.0	14.1			30
Total							
Styli/subtylostyli	238	600	±136	804	10.2	±1.4	153
Palmate anisochelae	16.3	21.9	±2.3	25.6			110
Forcipes	35.0	30.9	±2.4	44.4			75
Tylostyli	83.9	123.4	±15.5	155.5	4.3	±0.7	35
Small palmate anisochelae	9.3	12.3	±1.3	16.3			50
Tylostrongyla	14.0	18.3	±4.5	32.6			18

Previously reported distribution

Between Scotland and the Faroe Islands (Carter 1874), the Kara Sea (Levinsen 1887), west of the Taymur peninsula (Fristedt 1887), Davis Strait (Lambe 1900b), between Faroe and Iceland, at East-Greenland, the south coast of Jameson Land (Lundbeck 1905), and in the Barents and Laptev Seas (Koltun 1959). Reported depth range is 18-2,387 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Gallionella Garden	71°18'N 05°46'W	557 m	BIODEEP-06
Troll Wall	71°18'N 05°46'W	557 m	BIODEEP-06
Schultz Massif	73°50'N 07°35'E	688 m	H_2DEEP-08
Btw. Loki and Schultz	73°35'N 07°45'E	2,387 m	H_2DEEP-09

Remarks

The spicule complement of this species is the same as *A. lycopodium*, though the size is different. Though megascleres have been described as subtylostyli in earlier descriptions (Lundbeck 1905; Koltun 1959) the swellings at the blunt end are in the specimens here examined barely visible, and almost style-like. The upright stem of the specimens is quite similar in general shape to *A. lycopodium*, but the surface lacks the protrusions of that species, instead being unevenly wrinkled.

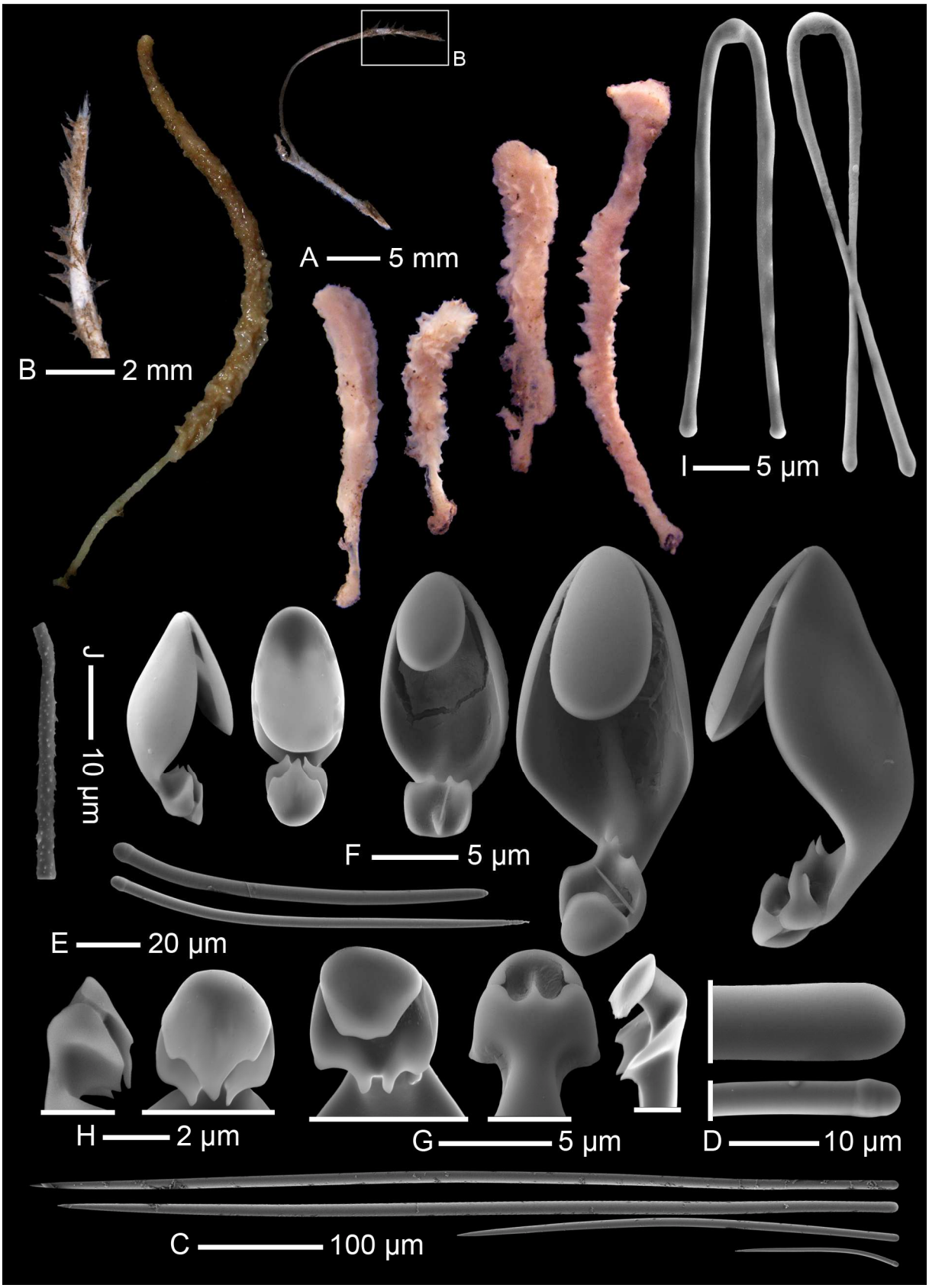

Figure 3.1. *Asbestopluma cupressiformis*. (A) Six specimens, (B) detail of the top, (C) styli/subtylostyli (D) with detail, (E) tylostyli, (F) palmate anisochelae from main sponge and embryos (G, H) with head end detail, (I) forcipes, (J) and tylostrongyla.

3.2.2 *Asbestopluma furcata* Lundbeck, 1905

Figure 3.2; Figure 3.10; Figure C.2; Figure C.3

Synonyms and citations

Esperia bihamatifera in part (Armauer Hansen 1885:15)
Asbestopluma furcata Lundbeck, 1905:54
Asbestopluma furcata (Hentschel 1929:933)
Asbestopluma furcata (Koltun 1964:152, 163)
Asbestopluma furcata (Barthel and Tendal 1993:84)

Type locality

Southeast of Iceland at 62°23'N 12°05'W at 982 m (Lundbeck 1905).

Material examined

GS06-03, GS08-04a, GS08-04b, GS08-04c, GS08-04d, GS08-06a, GS08-06b, GS09-02a, GS09-02b, GS09-02c. Lundbeck: *A. furcata* preps. 1-2.

Description

A long and slender, erect *Asbestopluma* species with a repeatedly dichotomously branched stem. The lower part of the main stem is brown, changing abruptly to grayish white in the branches. Upper branches carry fine, lateral processes 1-2 mm in length arranged in one plane opposite each other on either side of the stem. The surface is otherwise comparatively smooth.

The largest specimen is ca. 180 mm tall, branched up to 5 times. The width is 2-3 mm at the root decreasing to less than 1 mm in the branches. A root-like holdfast branches out from the base. The branches of this and a second specimen is considerably thicker than those of the smaller specimens and carry slight swellings at their terminal ends, not evident in the smaller samples. In a couple of cases, different branches in contact with each other have fused together at the point of contact. At 35-75 mm, the other specimens are considerably smaller. The width of the smaller specimens is approximately 0.5 mm, tapering off in the branches. One specimen showed no bifurcation; the others branch up to 2 times.

Spicules

There are three kinds of megascleres: Styli, subtylostyli and tylostrongyla. The tylostrongyla are only found in the lower stem; here subtylostyli are lacking however. Styli are long and straight when encountered together with subtylostyli in the upper branches, but are shorter and bent to a various degree in samples taken in the lower parts of the main stem. There are three kinds of microscleres: large palmate anisochelae, small palmate anisochelae, and

sigmata. The large palmate anisochelae are very uncommon in the lower stem. As the specimens GS09-02d and GS09-02e are fragments certain spicules were few or lacking.

Table 3.2. Spicules of *Asbestopluma furcata*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
S06-03a							
Styli	551	630	±31	684	13.1	±1.7	30
Subtylostyli	295	350	±24	390	9.3	±1.2	30
Tylostrongyla	58.6	74.9	±5.1	90.9			30
Large palmate anisochelae	41.9	49.4	±2.8	53.6			30
Small palmate anisochelae	9.3	9.4	±0.4	11.7			30
Sigmata	16.3	17.3	±1.5	21.0			30
S08-02a							
Styli	618	720	±50	865	14.6	±2.1	30
Subtylostyli	295	344	±22	399	9.4	±1.0	30
Tylostrongyla	58.6	70.0	±5.4	80.8			30
Large palmate anisochelae	41.9	47.9	±2.8	53.6			30
Small palmate anisochelae	7.0	9.2	±0.4	9.3			30
Sigmata	14.0	17.6	±1.6	21.0			30
S08-02b							
Styli	608	752	±62	855	17.4	±2.1	30
Subtylostyli	342	400	±20	447	10.2	±1.2	30
Tylostrongyla	56.6	69.6	±5.6	78.8			30
Large palmate anisochelae	41.9	46.4	±2.4	51.2			30
Small palmate anisochelae	7.0	9.2	±0.6	9.3			30
Sigmata	14.0	16.5	±1.1	18.6			30
S08-05b							
Styli	352	552	±99	713	15.0	±3.2	51
Subtylostyli	295	356	±20	390	8.3	±1.5	30
Tylostrongyla	55.9	67.3	±4.8	76.9			30
Big palmate anisochelae	41.9	46.8	±2.1	51.2			30
Small palmate anisochelae	7.0	8.8	±1.0	9.3			30
Sigmata	14.0	15.7	±1.6	21.0			30
S08-05c							
Styli	352	570	±97	741	15.1	±3.0	66
Subtylostyli	295	362	±27	475	8.3	±1.4	46
Tylostrongyla	55.9	69.4	±6.1	81.6			45
Large palmate anisochelae	41.9	47.0	±2.0	51.3			43
Small palmate anisochelae	7.0	8.9	±0.9	9.3			45
Sigmata	14.0	16.1	±1.6	21.0			50
S08-05d							
Styli	582	695	±43	779	13.2	±1.7	30
Subtylostyli	371	410	±18	456	8.9	±1.2	30
Tylostrongyla	56.6	73.7	±5.7	84.8			30
Large palmate anisochelae	42.4	46.8	±2.2	52.5			30
Small palmate anisochelae	7.0	9.0	±0.9	10.1			30
Sigmata	14.1	16.3	±1.2	18.6			30

Table 3.2 (cont.). Spicules of *Asbestopluma furcata*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
S08-06							
Styli	333	501	±112	703	12.3	±2.6	47
Subtylostyli	312	355	±17	377	8.3	±1.1	30
Tylostrongyla	37.3	65.1	±10.9	79.2			30
Large palmate anisochelae	42.4	49.3	±3.6	56.6			30
Small palmate anisochelae	7.0	8.5	±1.0	10.1			30
Sigmata	14.0	16.2	±1.9	21.0			30
GS09-02a							
Styli	500	685	±60	771	15.3	±2.4	30
Subtylostyli	279	331	±20	369	9.8	±1.0	30
Tylostrongyla	52.5	70.3	±7.5	82.8			30
Large palmate anisochelae	46.5	53.0	±3.6	62.6			30
Small palmate anisochelae	8.1	9.2	±1.0	10.1			30
Sigmata	12.1	15.3	±1.1	16.2			30
GS09-02b							
Styli	599	763	±73	968	17.8	±3.0	30
Subtylostyli	295	339	±22	385	10.0	±1.0	30
Tylostrongyla	56.6	68.3	±5.8	80.8			30
Large palmate anisochelae	42.4	49.7	±3.8	56.6			30
Small palmate anisochelae	8.1	9.2	±1.2	12.1			30
Sigmata	14.1	15.6	±0.9	16.2			30
GS09-02c							
Styli	426	586	±74	697	14.7	±2.5	30
Subtylostyli	287	344	±28	394	10.2	±0.9	30
Tylostrongyla	56.6	71.6	±6.8	90.9			30
Large palmate anisochelae	42.4	47.5	±2.4	52.5			30
Small palmate anisochelae	8.1	9.0	±1.0	10.1			30
Sigmata	14.1	15.2	±1.0	16.2			30
GS09-02d							
Styli	631	739	±48	845	19.0	±2.4	30
Subtylostyli	312	265	±29	426	9.9	±1.0	30
Tylostrongyla	58.6	69.2	±11.1	80.8			4
Large palmate anisochelae	42.4	49.6	±3.4	54.5			30
Small palmate anisochelae	8.1	8.9	±1.0	10.1			30
Sigmata	14.1	15.6	±1.1	18.2			30
GS09-02e							
Styli	336	514	±98	746	17.8	±2.5	30
Tylostrongyla	64.6	76.8	±8.5	101.0			30
Large palmate anisochelae	44.4	49.2	±4.6	54.5			6
Small palmate anisochelae	8.1	8.9	±1.0	10.1			30
Sigmata	14.1	15.5	±1.0	16.2			30
Total							
Styli	333	626	±123	968	15.3	±3.2	434
Subtylostyli	279	360	±32	475	9.3	±1.3	346
Tylostrongyla	37.3	70.7	±7.5	101.0			349
Large palmate anisochelae	41.9	48.5	±3.4	62.6			349
Small palmate anisochelae	7.0	9.0	±0.9	12.1			375
Sigmata	12.1	16.1	±1.5	21.0			376

Previously reported distribution

The North Atlantic Ocean (Armauer Hansen 1885), southeast and northeast of Iceland, off the coast of Norway (Lundbeck 1905), central Greenland Sea (Koltun 1964), and west of Spitsbergen (Barthel and Tendal 1993). Reported depth range is 557-2,387 m.

***G.O. Sars* distribution**

Location	Coordinates	Depth	Cruise
Troll Wall	71°18'N 05°46'W	557 m	BIODEEP-06
Schultz Massif	73°48'N 07°49'E	1,764 m	H_2DEEP-08
Schultz Massif	73°50'N 07°35'E	1,764 m	H_2DEEP-08
Btw. Loki and Schultz	73°35'N 07°45'E	2,387 m	H_2DEEP-09

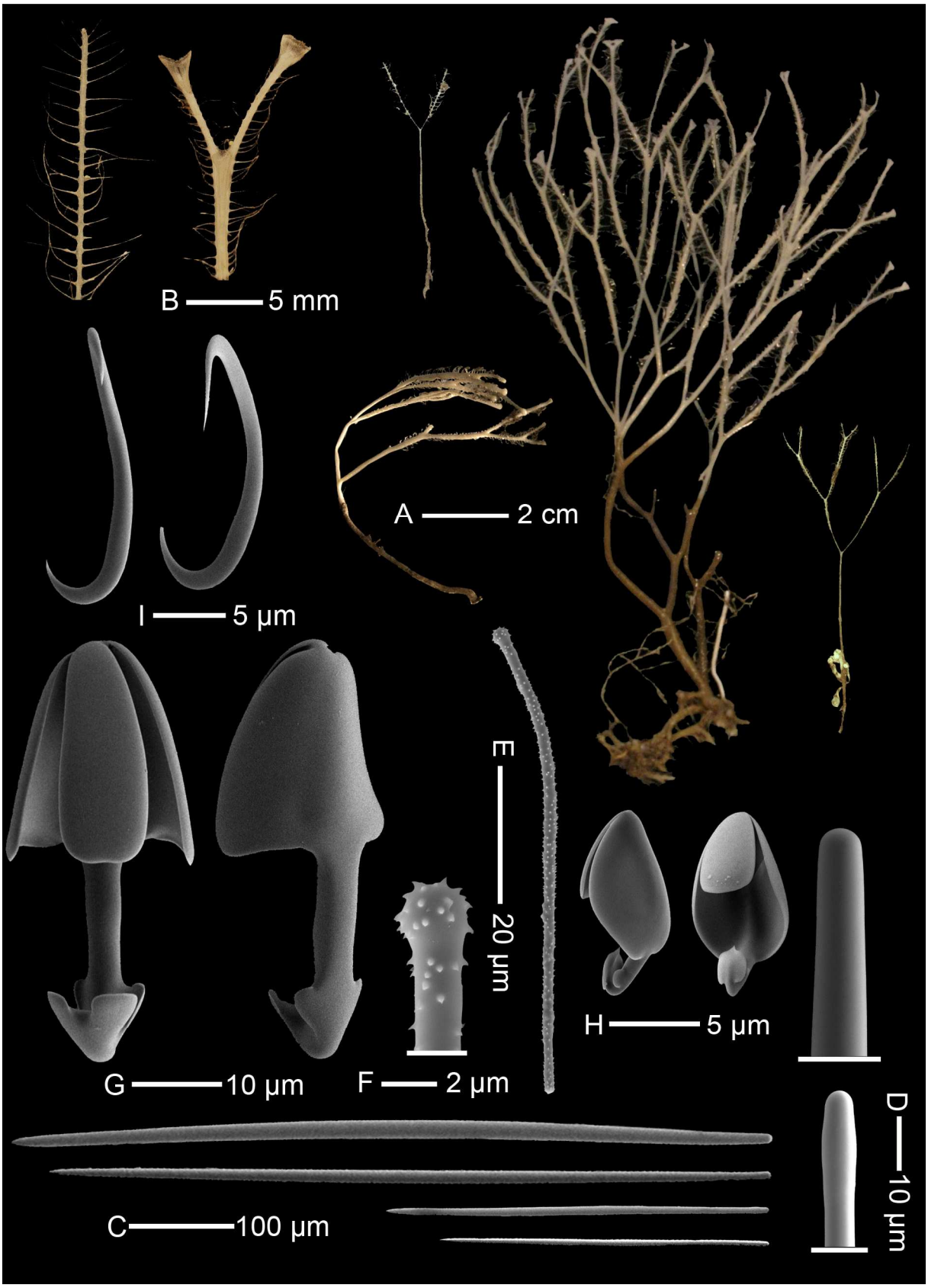

Figure 3.2. *Asbestopluma furcata*. (A) Four specimens, (B) a detailed view of two branch-ends, (C) styli and subtylostyli (D) with blunt ends, (E) tylostrongyla (F) with detail, (G) large palmate anisochelae, (H) small palmate anisochelae (I) and sigmata.

3.2.3 *Asbestopluma infundibulum* (Levinsen 1887)

Figure 3.3; Figure 3.10; Figure C.4

Synonyms and citations

Esperia cupressiformis in part (Carter 1874:215)
Esperia bihamatifera in part (Armauer Hansen 1885:15)
Esperella infundibulum (Levinsen 1887:366)
Asbestopluma infundibulum (Lundbeck 1905:68)
Asbestopluma infundibulum (Hentschel 1929:875, 935)
Asbestopluma infundibulum (Koltun 1959:77)
Asbestopluma infundibulum var. *orientalis* (Koltun 1972:202)

Type locality

The Kara Sea at 91-128 m (Levinsen 1887).

Material examined

GS08-09a, GS08-09b. Lundbeck: *A. infundibulum* prep. 4.

Description

A small, 15-35 mm tall sponge formed like a calyx on a short, thin stem. The lower part of the stem is covered in a sheath, giving it a brownish color. The stem abruptly changes to a white color before widening to form the head. The calyx is shaped like a hollow bulb or cup with a terminal opening up to 5 mm across and might be flattened to varying degree. Spicules protrude from both the stem and the calyx, giving it a slightly hirsute appearance.

Four specimens were recovered from the same site. Three of them are 15-20 mm tall, and the largest around 35 mm.

Spicules

Megascleres are styli and shorter subtylostyli. Generally the swelling at the blunt end becomes more pronounced as the style becomes shorter. While the megascleres tend to congregate around two average sizes, there is partly overlap both in length and degree of swelling. There is also a form of easily distinguishable flagelliform style/subtylostyle tapering off into a very long and fine point. Microscleres are palmate anisochelae.

Table 3.3. Spicules of *Asbestopluma infundibulum*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS08-09a							
Styli/subtylostyli	171	333	±100	627	6.6	±1.0	60
Short subtylostyli	133	168	±28	257	4.9	±0.7	30
Flagelliform subtylostyli	371	454	±41	561	4.6	±0.7	30
Palmate anisochelae	18.6	20.0	±1.2	21.0			30
GS08-09b							
Styli/subtylostyli	361	551	±76	675	8.2	±1.2	30
Short subtylostyli	162	359	±70	466	6.8	±0.9	30
Flagelliform subtylostyli	371	471	±45	561	4.7	±0.6	30
Palmate anisochelae	16.3	20.8	±1.2	23.3			30
Total							
Styli/subtylostyli	171	412	±135	675	7.2	±1.3	90
Short subtylostyli	133	263	±110	466	5.8	±1.3	60
Flagelliform subtylostyli	371	463	±43	561	4.6	±0.7	60
Palmate anisochelae	16.3	20.4	±1.3	23.3			60

Previously reported distribution

The North Atlantic (Armauer Hansen 1885), the Kara Sea (Levinsen 1887; Koltun 1959), off the Faroe Islands (Carter 1874; Lundbeck 1905), north of Iceland, south of Jan Mayen (Lundbeck 1905), in the Barents Sea (Hentschel 1929), the Laptev and Greenland Seas (Koltun 1959). Reported depth range is 91-734 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Pockmark G11	64°40’N 05°17’E	734 m	H2DEEP-08

Remarks

Earlier descriptions of this species include forcipes to the list of microscleres (length: 75-104 μm) (Levinsen 1887; Lundbeck 1905; Hentschel 1929), and also mention smaller palmate anisochelae connected to embryos in the tissue. Despite carefully completely scanning a number of slides, no forcipes were found; however Lundbeck mentions the scarcity of these spicules, and Hentschel was able to find only two. As the number of forcipes seems to vary between specimens in other species such as *A. lycopodium* and *A. cupressiformis*, the most likely explanation is that the *A. infundibulum* specimens in this case contained few or no forcipes. Forcipes have been reported being associated with spermatic cysts in another species from the same genus. A probable cause for the lack of forcipes in the samples may therefore be the lack of spermatic cysts within specimen tissue.

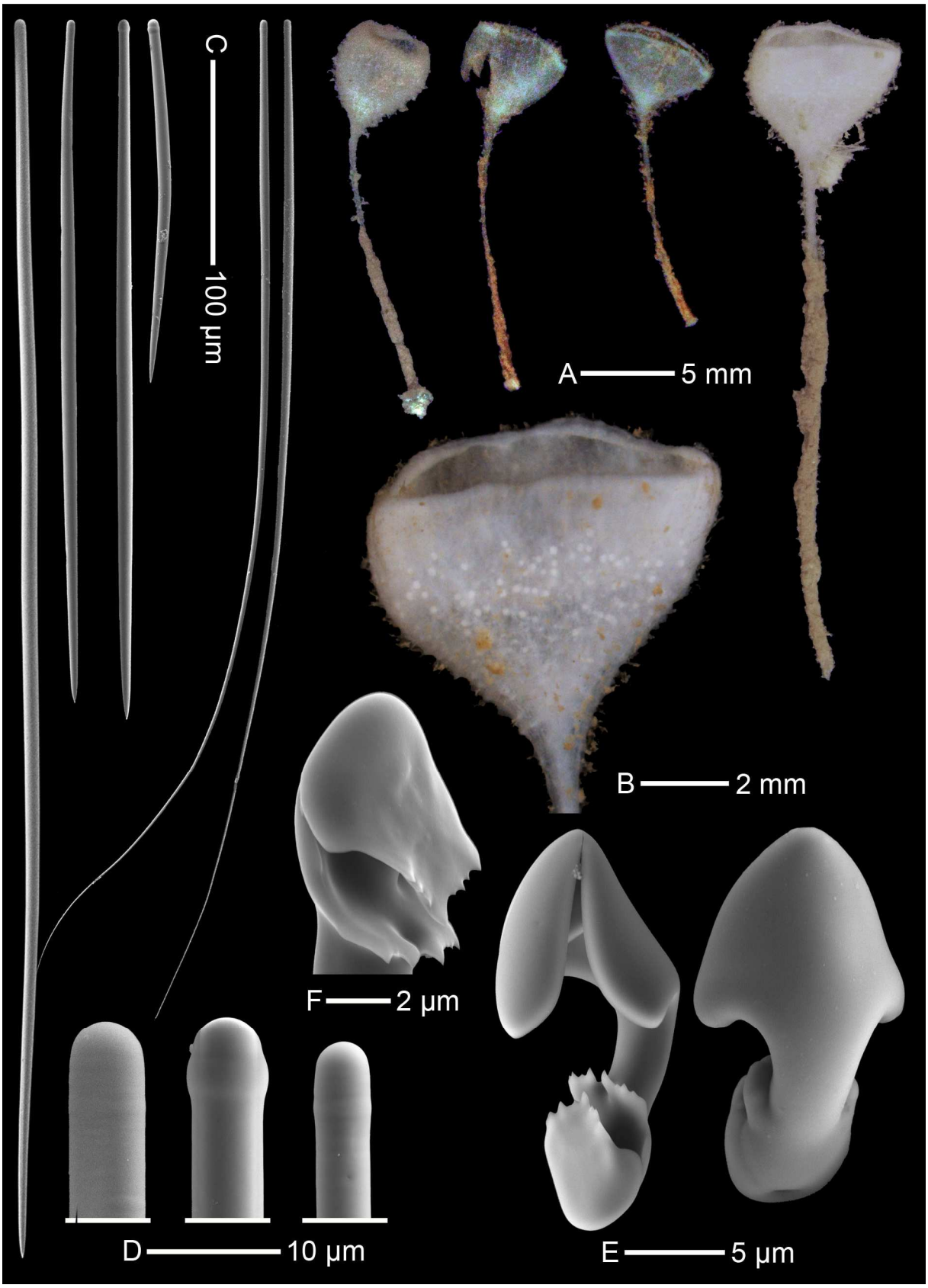

Figure 3.3. Asbestopluma infundibulum. (A) Four specimens, (B) detail of the calyx of largest specimen showing probable embryos, (C) styli, subtylostyli and flagelliform subtylostyli, (D) with detail, (E) palmate anisochelae, (F) with detail.

3.2.4 *Asbestopluma lycopodium* (Levinsen, 1887)

Figure 3.4; Figure 3.10; Figure C.5

Synonyms and citations

Esperia bihamatifera in part (Armauer Hansen 1885:15)
Esperella cupressiformis var. *lycopodium* Levinsen, 1887:365
Esperella fristedtii in part (Lambe 1900a:157)
Asbestopluma lycopodium (Lundbeck 1905:62)
Asbestopluma lycopodium (Hentschel 1929:934)
Asbestopluma lycopodium (Burton 1934:9, 33)
Asbestopluma lycopodium (Koltun 1959:75)
Asbestopluma lycopodium (Koltun 1964:151, 163)
Asbestopluma lycopodium (van Soest et al. 2007:130)

Type locality

The Kara Sea at 93-148 m (Levinsen 1887).

Material examined

GS06-05a, GS06-05b, GS07-01, GS08-02, GS08-08, GS08-10, GS08-11a, GS08-11b, GS09-07. Lundbeck: *A. lycopodium* prep. 4.

Description

A single, erect, cylindrical main stem connects to the substrate with a short basal stem ending in a conical holdfast. The stem gradually widens from a little over 1 mm in diameter toward halfway up the sponge, where it can reach almost 3 mm, and then tapers off towards the top. In some specimens, a rough sheath covers a short (up to 20 mm) part of the basal stem. Closely spaced 1-3 mm long protrusions radiate equally in all directions from the sponge, reaching their maximal length about halfway up the stem. These protrusions may be reduced to knobs. The holdfast and basal stem are either the same color as the rest of the body or brown. In the last case, the color abruptly changes below the protrusion-bearing part of the stem, which is grayish white.

Collected specimens are 25-60 mm long including holdfast. Protrusion length varies between specimens as well as along the stem within single specimens.

Spicules

The megascleres are slender styli. In most specimens the size is less than 1416 μm, but in a couple of samples some styli were longer, being measured up to 2974 μm. In the basal part of the sponge, styli are generally shorter, and often curved. Styli forms overlap, with many

intermediate forms. There are two forms of microscleres: palmate anisochelae and forcipes. The forcipes, though observed in all specimens, are relatively rare in some specimens. In the very closely related species *A. occidentalis*, forcipes are found in conjunction with spermatic cysts (Riesgo et al. 2007). It is reasonable to assume a similar relationship for *A. lycopodium*, in which case the relative abundance of spermatic cysts probably has a great effect on the number of forcipes found in each sample. In a couple of specimens, smaller styli were found. According to Lundbeck (1905), these are associated with embryos within the tissue.

Table 3.4. Spicules of *Asbestopluma lycopodium*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS06-05a							
Styli	485	768	±233	1330	9.8	±1.8	30
Short styli	100.2	114.9	±5.5	125.8	3.5	±1.6	30
Palmate anisochelae	14.0	14.1	±0.4	16.3			30
Forcipes	46.6	48.9	±2.4	53.6			12
GS06-05b							
Styli	428	786	±278	1368	11.0	±1.6	30
Short styli	95.5	112.8	±8.2	123.5	3.4	±1.6	30
Palmate anisochelae	14.0	14.0	±0.0	14.0			30
Forcipes	46.6	49.5	±2.2	51.3			4
GS07-01							
Styli	741	1326	±580	2974	11.4	±2.1	55
Palmate anisochelae	16.3	16.2	±0.0	16.3			30
Forcipes	41.9	51.0	±3.3	55.9			30
GS08-02							
Styli	314	618	±223	1197	7.3	±2.2	38
Palmate anisochelae	14.0	14.3	±0.8	16.3			30
Forcipes	25.6	35.9	±8.0	46.6			10
GS08-08							
Styli	238	550	±271	1311	8.3	±2.2	60
Palmate anisochelae	11.7	13.7	±0.8	14.0			30
Forcipes	41.9	46.1	±2.6	51.3			30
GS08-10							
Styli	808	1213	±478	2489	10.2	±2.5	30
Palmate anisochelae	14.0	15.1	±1.2	16.3			30
Forcipes	37.3	44.0	±4.6	48.9			8
GS08-11a							
Styli	656	959	±252	1416	12.3	±2.5	32
Palmate anisochelae	11.7	14.0	±0.9	16.3			30
Forcipes	37.3	48.4	±5.1	53.6			9

Table 3.5 (cont.). Spicules of *Asbestopluma lycopodium*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS08-11b							
Styli	238	558	±242	1444	11	±1.9	90
Palmate anisochelae	14.0	15.1	±1.2	16.3			30
Forcipes	51.3	55.9	±2.0	58.3			30
GS09-07							
Styli	812	1240	±407	2148	10.4	±0.8	30
Palmate anisochelae	16.2	17.0	±1.0	18.2			30
Forcipes	42.4	47.5	±2.9	52.5			30
Total							
Styli	238	837	±462	2974	10.1	±2.5	395
Short styli	95.5	113.9	±125.8	125.8	3.4	±1.2	60
Palmate anisochelae	11.7	14.8	±1.4	18.2			270
Forcipes	25.6	48.8	±5.9	58.3			163

Previous reported distribution

In the area around the Faroe Islands and Shetland, off the coast of Norway, south of Jan Mayen (Lundbeck 1905), Vegasund and the Greenland Sea (Burton 1934; Koltun 1964), the Barents, Kara, Laptev and East Siberian Seas, the Vilkitsky Strait, and off the coast of Franz Josef Land (Koltun 1959; Koltun 1964). Reported depth range is 41-2,387 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Troll Wall	71°18'N 5°46'W	557 m	BIODEEP-06
Schultz Massif slope	73°50'N 7°38'E	1,262 m	BIODEEP-07
Schultz Massif seamount	73°50'N 7°35'E	688 m	H_2DEEP-08
Pockmark G11	64°40'N 5°17'E	734 m	H_2DEEP-08
Pockmark Dodo	64°40'N 5°16'E	730 m	H_2DEEP-08
Btw. Loki and Schultz	73°35'N 7°44'E	2,387 m	H_2DEEP-09

Remarks

Some variation between specimens was found: Several specimens that are included as *A. lycopodium* in this thesis contain styli up to 40% longer than in earlier descriptions. The palmate anisochelae of specimen GS09-07 are also slightly larger than for the other species. Given other shared characteristic traits I have still chosen to include them as *A. lycopodium*.

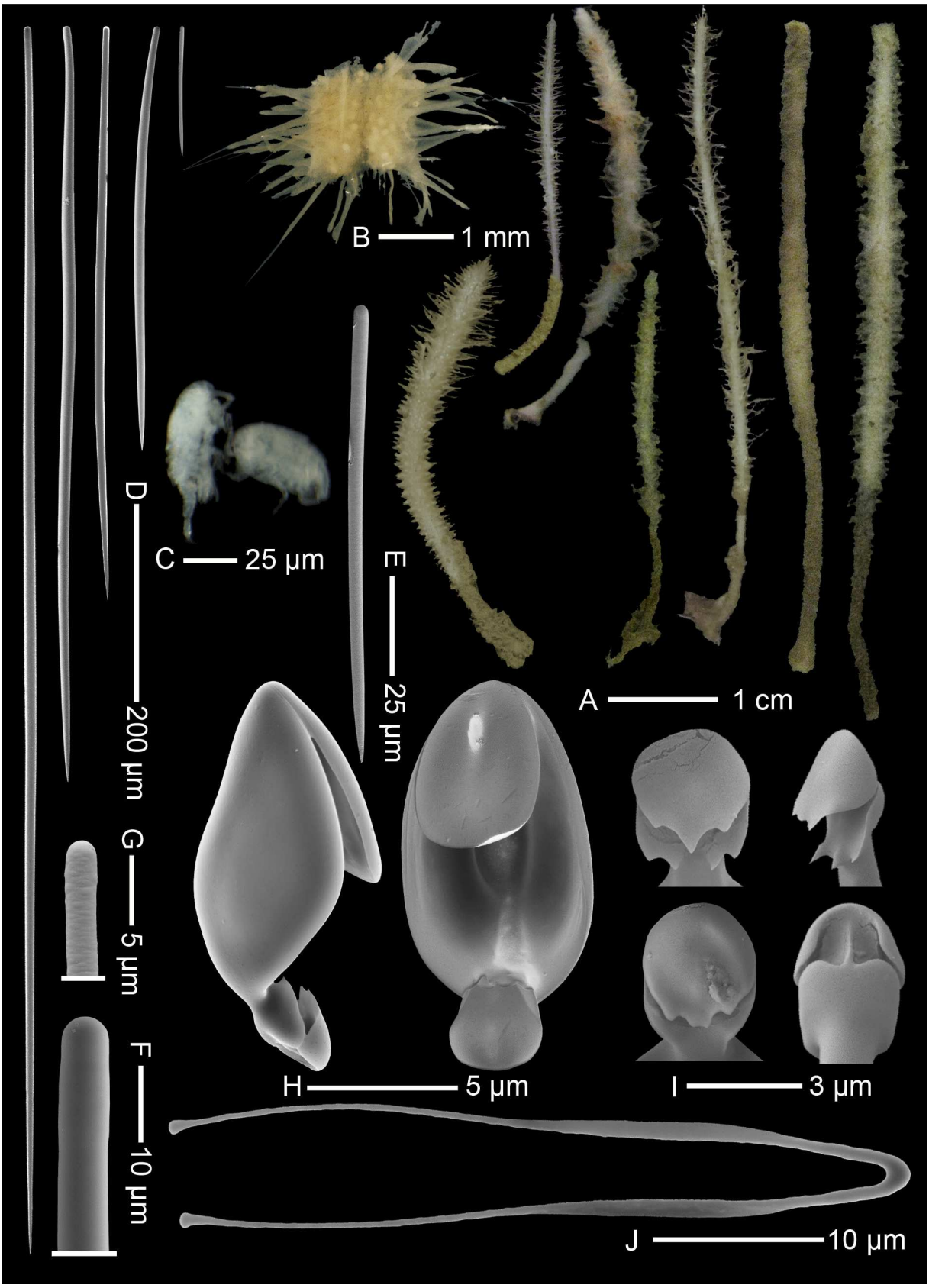

Figure 3.4. Asbestopluma lycopodium. (A) Seven specimens, (B) a more detailed transect of the main stem showing visible embryos, (C) a couple of partially digested copepods, (D) styli (F) with detail, (D) smaller, embryo associated style (E, G) with detail, (H) palmate anisochelae (I) with detail, (J) and forcipes.

3.2.5 *Asbestopluma* sp. 1 aff. *lycopodium*

Figure 3.5; Figure C.6

Material examined

GS08-03.

Description

This specimen is a small, erect individual similar whose description is similar to that of *A. lycopodium* above. It is approximately 50 mm long. Protrusions are short or otherwise reduced compared to the most common *A. lycopodium* form, but are within observed morphological variation for this species.

Spicules

The megascleres are styli and easily distinguishable subtylostyli in the basal part of the specimen. Microscleres are palmate anisochelae and forcipes, of which only a few were found. Forcipes have the same general form as for *A. lycopodium*.

Table 3.6. Spicules of *Asbestopluma* sp. 1 aff. *lycopodium.*

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS08-03							
Styli	542	864	±206	1406	8.5	±2.1	30
Subtylostyli	247	430	±144	732	5.4	±1.3	30
Palmate anisochelae	14.0	15.4	±1.2	16.3			30
Forcipes	32.6	35.0	±3.3	39.6			4

***G.O. Sars* distribution**

Location	Coordinates	Depth	Cruise
Loki's Castle sea mount	73°50'N 7°35'E	688 m	H_2DEEP-08

Remarks

This specimen is very similar to *A. lycopodium* except that it carries subtylostyli in the basal part of the sponge. The general shape of the sponge together with the presence of subtylostyli is like that of *A. occidentalis* (Lambe 1893) previously reported around Vancouver Island and in the Kurile-Kamchatka Trench (Lambe 1893; Koltun 1972; Riesgo et al. 2007). The subtylostyli of *A. occidentalis*, not mentioned by Lambe, are shorter, at 121.7-369.5 μm (Riesgo et al. 2007), and the palmate anisochelae of that species are, at 10-13 μm (Lambe

1893; Riesgo et al. 2007) (but see Koltun 1972: 10-22 µm), also slightly smaller.
A. occidentalis and *A. lycopodium* are morphologically very closely related, and this specimen is very similar to both. It should be noted that Koltun at one time considered *A. lycopodium* and *A. occidentalis* similar enough for them to be regarded as the same species, but later described them separately (Koltun 1955; Koltun 1959; Koltun 1972). ITS sequences were recovered from both this specimen and *A. lycopodium* specimens for the molecular part of this thesis, with 88% identity of ITS2 (complete ITS1 sequence unavailable for this species).

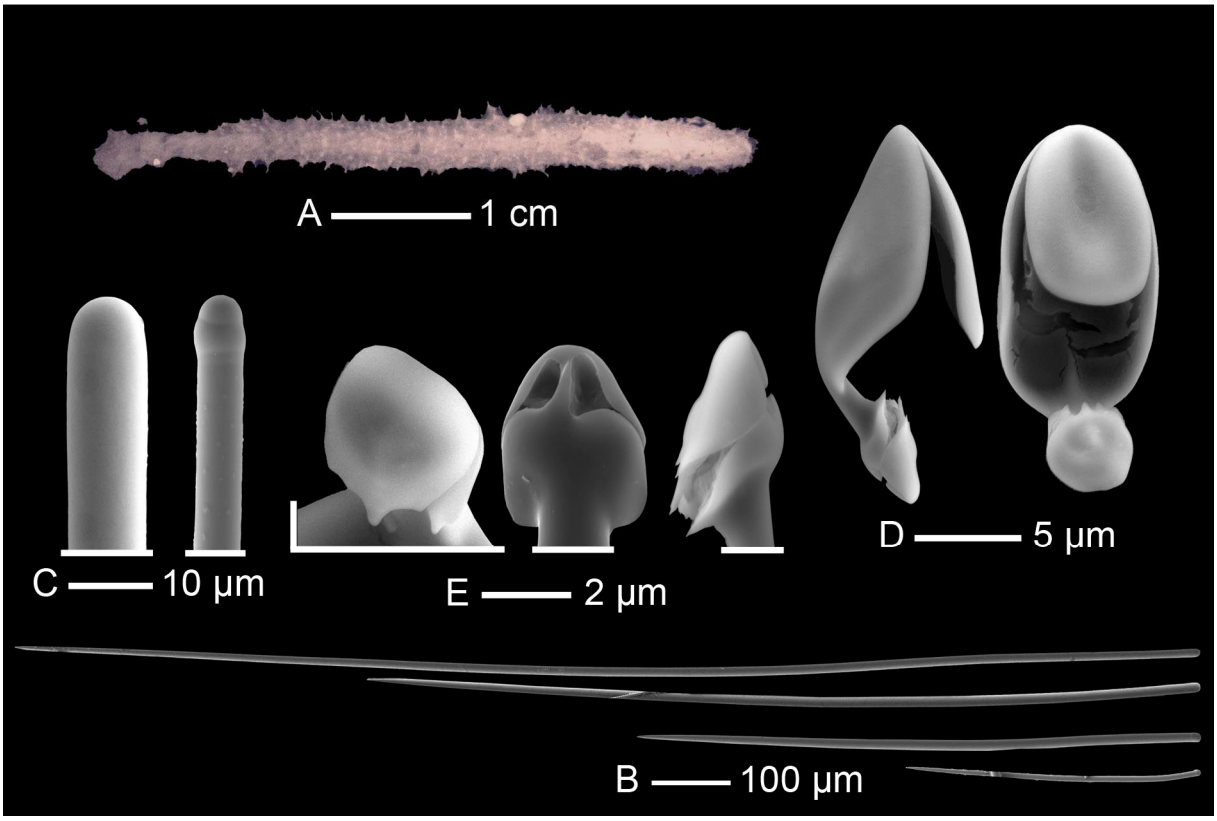

Figure 3.5. *Asbestopluma* sp. 1 aff. *lycopodium*. (A) Morphology, (B) styli and subtylostyli (C) with detail, (D) palmate anisochelae (E) with detail. No forcipes were found on the SEM preparations.

3.2.6 *Asbestopluma* sp. 2 aff. *lycopodium*

Figure 3.6; Figure C.7

Type locality

Gallionella Garden (71°17.98'N 5°46.92'W at 616 m).

Material examined

GS06-02, GS06-06a, GS06-06b.

Description

A tiny stem fastened to the substrate with a small, conical holdfast suspends a head containing a central harder core surrounded by softer tissue. From the core, 0.5-1 mm long protrusions project in all directions. The color is white to slightly yellow.

The specimens examined are 2.5, 3, and 8 mm long. This minute size might reflect their age rather than the size range of the species.

Spicules

Megascleres are longer styli/subtylostyli, separate, shorter subtylostyli, and tiny subtylostyli. Microscleres are palmate anisochelae. The smaller subtylostyli are very characteristic, having a short but robust fusiform shape with a very pronounced swelling at the blunt end.

Table 3.7. Spicules of *Asbestopluma* sp. 2 aff. *lycopodium*

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS06-02							
Styli	371	591	±114	941	7.8	±1.1	30
Subtylostyli	219	382	±102	618	7.6	±1.9	30
Palmate anisochelae	11.7	11.7	±0.0	11.7			30
Short subtylostyli	65.2	85.0	±8.5	102.5			30
GS06-06b							
Styli	390	590	±174	998	8.7	±1.6	30
Subtylostyli	143	314	±107	513	5.5	±1.3	23
Palmate anisochelae	11.7	11.7	±0.4	14.0			30
Short subtylostyli	79.2	138.8	±32.5	181.7			7
Total							
Styli	371	590	±146	998	8.2	±1.5	60
Subtylostyli	143	352	±108	618			53
Palmate anisochelae	11.7	11.7	±0.3	14.0			60
Short subtylostyli	65.2	95.2	±26.3	181.7			37

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Gallionella Garden	71°18'N 05°47'W	616 m	BIODEEP-06
Troll Wall	71°18'N 05°46'W	557 m	BIODEEP-06

Remarks

There is no doubt that this is a close relative to *A. lycopodium*, given the similar shape of the palmate anisochelae. The palmate anisochelae are slightly smaller than in *A. lycopodium* however, and more similar in size to those of *A. occidentalis* (10-13 µm) (Lambe 1893; Riesgo et al. 2007). Like *A. occidentalis* this species carries short subtylostyli rather than styli as *A. lycopodium* does. No molecular data is available for any of the specimens identified to this variety, but as *A.* sp. 1 aff. *lycopodium* is likely another species as evidenced by ITS2 identity (see species description), and as these specimens differ from the description of *A. lycopodium* to an equal, though not identical, degree, there is enough indication that these specimens could be tentatively considered as a separate species.

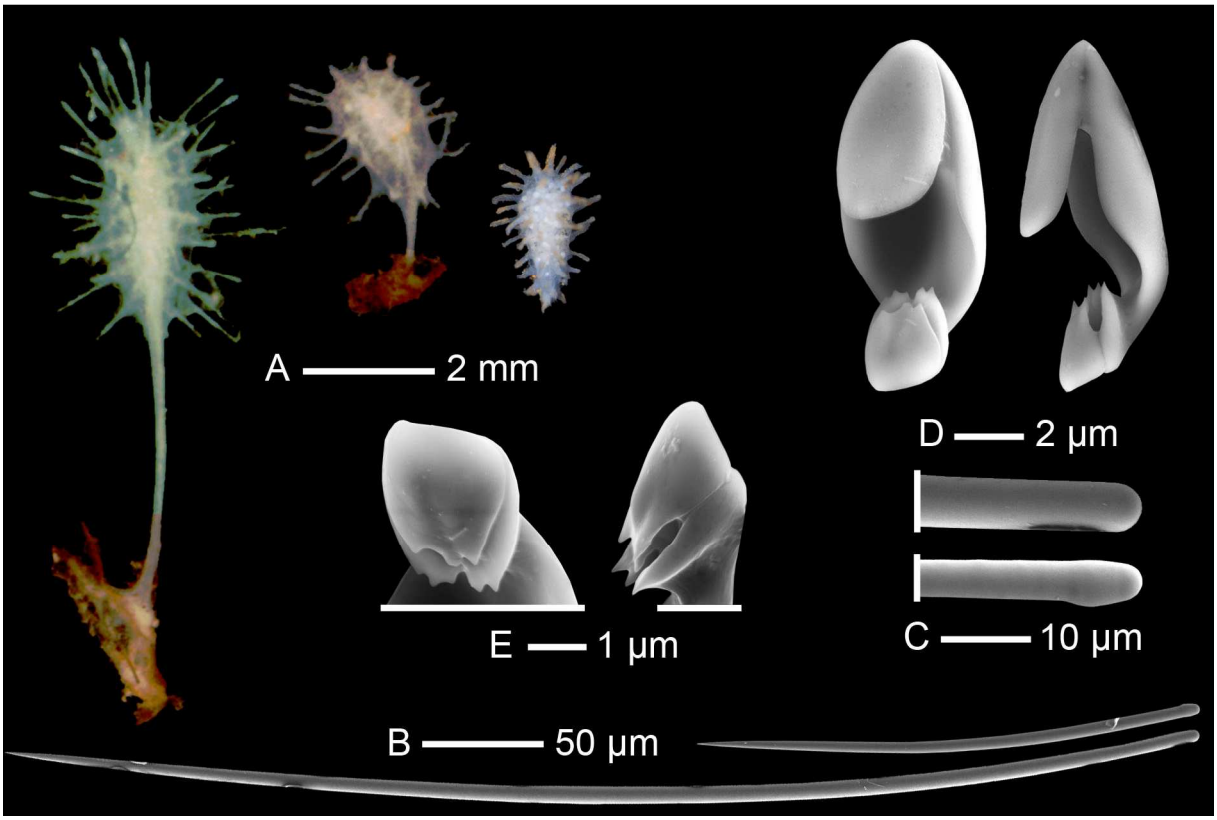

Figure 3.6. *Asbestopluma* sp. 2 aff. *lycopodium*. (A) Two specimens and fragments of a third, (B) styli and subtylostyli (C) with detail, (D) and palmate anisochelae (E) with detail. No short subtylostyli were found on the SEM preparations, but check Figure C.7 for light microcopy pictures..

CLADORHIZA Sars, 1872

Synonyms

Cladorhiza Sars, 1872
[*Trochoderma*] (Ridley and Dendy 1886) (preoccupied)
Axoniderma (Ridley and Dendy 1887)
Exaxinata (de Laubenfels 1936)

Type species

Cladorhiza abyssicola Sars, 1872

Description

Hooper & van Soest, 2002: Cladorhizidae lacking an aquiferous system, carrying anchorate unguiferate anisochelae.

3.2.7 *Cladorhiza corticocancellata* Carter, 1876

Figure 3.7; Figure 3.10; Figure C.8

Synonyms and citations

Cladorhiza abyssicola var. *corticocancellata* Carter, 1876:320
Cladorhiza abyssicola in part (Armauer Hansen 1885:16)
Cladorhiza abyssicola var. *corticocancellata* (Arnesen 1903:12)
Cladorhiza corticocancellata (Lundbeck 1905:93)
Cladorhiza corticocancellata (Burton 1934:9, 24)
Cladorhiza corticocancellata (Koltun 1964:163)

Type locality

Near the Faroe Islands at 60°14’N 06°17’W at 1156 m (Carter 1876).

Material examined

GS08-07, GS09-05. Lundbeck: *C. corticocancellata* prep. 3.

Description

A whitish grey, particularly robust *Cladorhiza* species with one or several stems carrying shorter branches all covered with a thick and porous cortex from which protrusions project. The branches are generally 4-10 mm wide. A thick, outer, porous cortex surrounds a darker, hard stem, an arrangement which is common in several cladorhizids but especially prominent in this species as the outer layer is quite soft, and easily falls apart from the stem, in contrast to the thinner, spiny exterior of *C. gelida* or *C. abyssicola*.

The GS08-07 specimen in particular is very fragile and easily falls apart, but the main piece remaining is still 180 mm tall. The GS09-05 fragments are somewhat smaller and less robust.

Spicules

The megascleres are straight styli. There are three different kinds of microsclere: anchorate anisochelae, large sigmata, and small sigmata. The small sigmata of this species are quite rare, even when examining the branch ends noted by Lundbeck to contain this microsclere (1905).

Table 3.8. Spicules of *Cladorhiza corticocancellata*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS08-07							
Styli	475	624	±52	703	17.6	±1.7	30
Anchorate anisochelae	28.0	31.6	±1.7	35.0			30
Large sigmata	135.1	156.0	±8.2	170.1			30
Small sigmata	46.6	60.0	±13.5	95.5			17
GS09-05							
Styli	549	659	±50	738	17.2	±2.2	30
Anchorate anisochelae	28.3	33.3	±2.0	36.4			30
Large sigmata	137.4	156.3	±12.9	181.8			30
Small sigmata	56.6	61.3	±2.7	64.6			30
Total							
Styli	475	642	±54	738	17.4	±2.0	60
Anchorate anisochelae	28.0	32.4	±2.0	36.4			60
Large sigmata	135.1	156.2	±10.7	181.8			60
Small sigmata	46.6	60.8	±8.3	95.5			47

Previous reported distribution

Near the Faroe Islands (Carter 1876; Lundbeck 1905), off the Norwegian coast (Arnesen 1903), and the Western Greenland Sea (Burton 1934). Reported depth range is 710-2,387 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Pockmark Tobic	64°41’N 5°16’E	720 m	GS08-155
Btw. Loki and Schultz	73°35’N 7°45’E	2,387 m	H_2DEEP-09

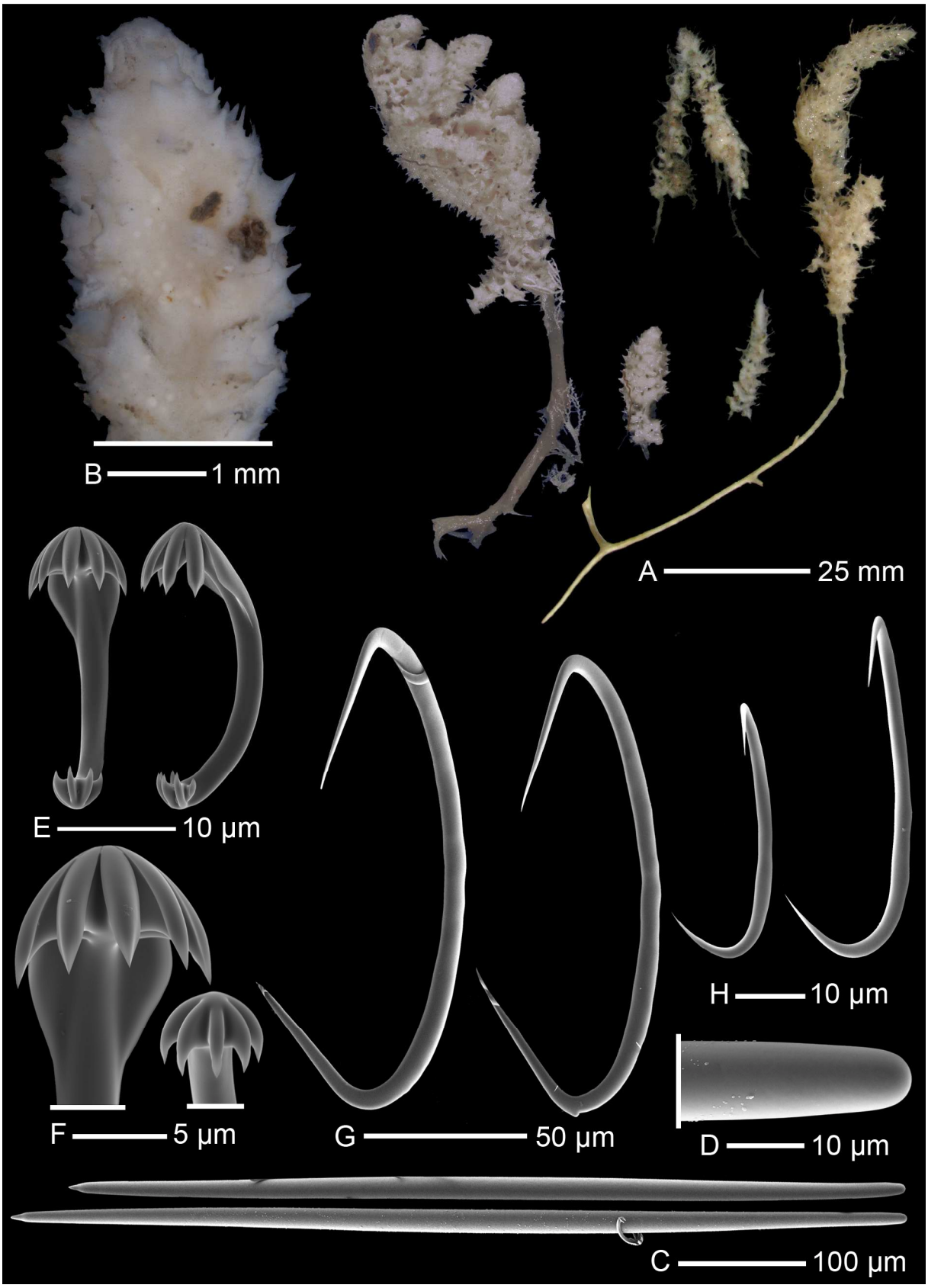

Figure 3.7. *Cladorhiza corticocancellata*. (A) Specimen fragments, (B) detail from branch end, (C) styli (D) with detail, (E) anchorate anisochelae (F) with alae detail, (G) large, (H) and small sigmata.

3.2.8 *Cladorhiza gelida* Lundbeck, 1905

Figure 3.8; Figure 3.10; Figure C.9

Synonyms and citations

Cladorhiza abyssicola in part (Armauer Hansen 1885:16)
Cladorhiza gelida Lundbeck, 1905:83
Cladorhiza gelida (Topsent 1909:6)
Cladorhiza gelida (Topsent 1913:48)
Cladorhiza gelida (Arnesen 1920:18)
Cladorhiza gelida (Hentschel 1929:935, 1001)
Cladorhiza gelida (Burton 1930:492)
Cladorhiza gelida (Koltun 1959:81)
Cladorhiza gelida (Koltun 1964:152, 163)
Cladorhiza gelida (Barthel and Tendal 1993:84, 90)
Cladorhiza gelida (van Soest et al. 2007:130)

Type locality

Between Jan Mayen and Iceland at 67°57'N 06°44'W at 2317 m (Lundbeck 1905).

Material examined

GS08-01a, GS08-01b, GS08-01c. Lundbeck: *C. gelida* prep. 1. Bergen Museum (Arnesen 1920) specimen.

Description

A *Cladorhiza* species with a slender central stem from which lateral branches project at regular 4-8 mm intervals. The color is grayish white and almost uniform, with a slight darkening of the basal part of the stem. No root or holdfast was recovered. The main stem is approximately 3 mm in diameter at the base, gradually tapering off towards the upper stem and branches. Lateral branches are around 1-2 mm in diameter. All branches except at the basal stem are closely covered with thin 1-3 mm long protrusions. The structure of the stem and branches is that of a light brown, hard and smooth central stem covered with a cortex of slightly softer tissue from which the protrusions emerge.

One large specimen is 160 mm in length, a smaller specimen, and several smaller fragments are 12-32 mm in length.

Spicules

The megascleres are straight styli. There are three types of microsclere: anchorate anisochelae, large sigmata, and small sigmata. The small sigmata are much less common than other

spicules, and in several preparations were completely lacking. This finding is consistent with earlier reports (Lundbeck 1905).

Table 3.9. Spicules of *Cladorhiza gelida*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS08-01a							
Styli	485	618	±42	684	17.6	±3.4	30
Anchorate anisochelae	25.6	28.7	±1.6	32.6			45
Large sigmata	130.5	146.9	±9.4	167.8			45
Small sigmata	41.9	51.4	±6.7	76.9			30
GS08-01b							
Styli	466	597	±66	741	16.5	±3.7	30
Anchorate anisochelae	23.3	28.0	±1.8	32.6			30
Large sigmata	116.5	140.5	±8.3	160.8			30
Small sigmata			±				0
GS08-01c							
Styli	494	600	±58	694	16.2	±3.7	30
Anchorate anisochelae	21.0	28.0	±1.8	30.3			30
Large sigmata	121.2	143.6	±9.0	158.4			30
Small sigmata			±				0

Previous reported distribution

Between Jan Mayen and Iceland, the Faroe Channel (Lundbeck 1905), the Western Greenland Sea (Arnesen 1920), off Spitsbergen (Topsent 1913; Barthel and Tendal 1993), and the Rockall Bank (van Soest et al. 2007). Reported depth range is 763-2,860 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Btw. Loki and Schultz	73°39’N 07°47’E	2,345 m	H_2DEEP-08

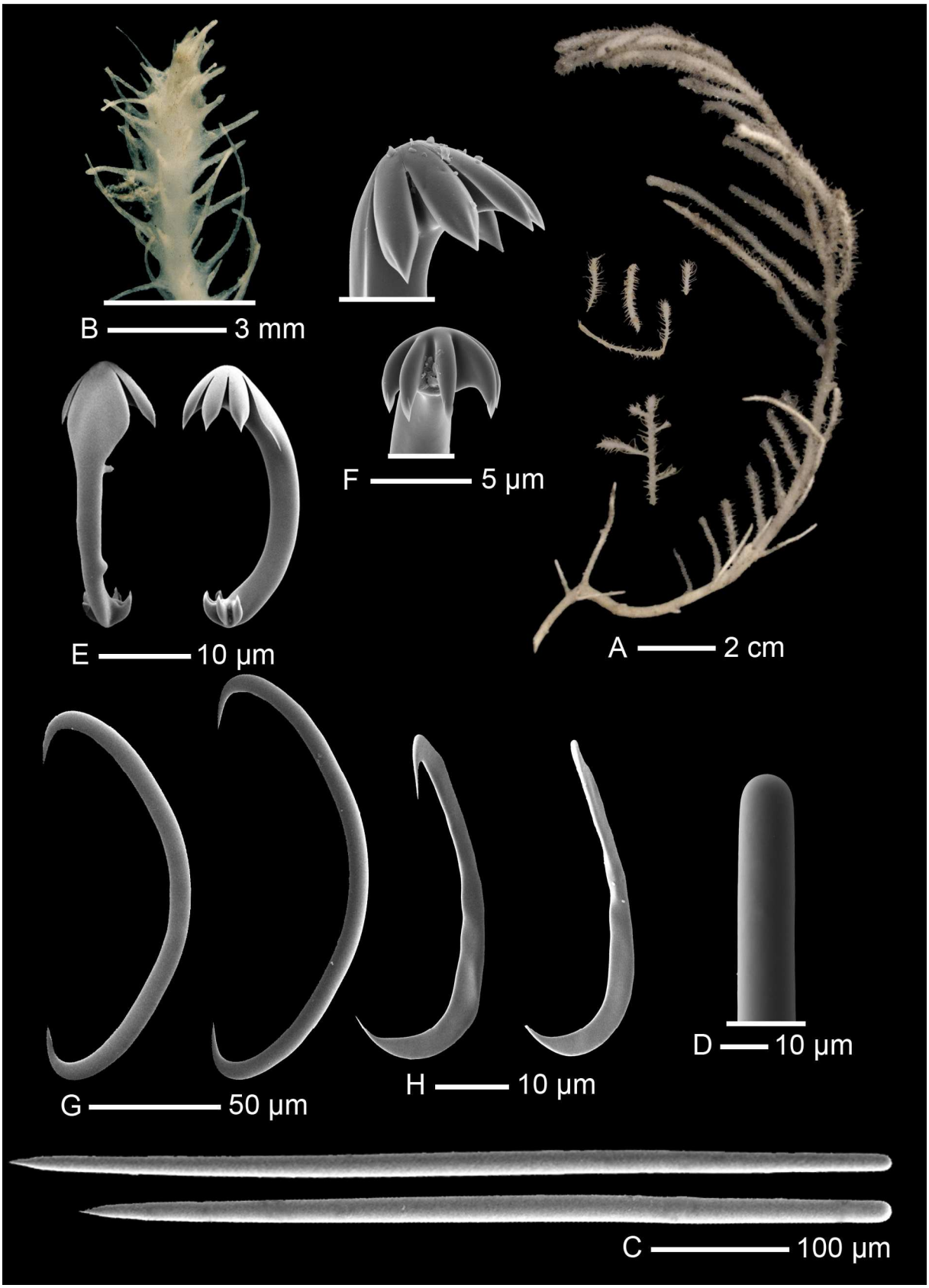

Figure 3.8. *Cladorhiza gelida*. (A) Three specimens or fragments, (B) detail from branch end, (C) styli (D) with detail, (E) anchorate anisochelae (F) with detailed view of alae, (G) large, (H) and small sigmata.

3.2.9 *Cladorhiza tenuisigma* Lundbeck, 1905

Figure 3.9; Figure 3.10; Figure C.10

Synonyms and citations

Cladorhiza abyssicola in part (Armauer Hansen 1885:16)
Cladorhiza tenuisigma Lundbeck, 1905:87
Cladorhiza tenuisigma (Topsent 1909:7)
Cladorhiza tenuisigma (Topsent 1913:49)
Cladorhiza tenuisigma (Hentschel 1929:935, 1001)
Cladorhiza tenuisigma (Koltun 1959:82)
Cladorhiza tenuisigma (Koltun 1964:163)
Cladorhiza tenuisigma (Janussen 2009:50)

Type locality

East of Iceland at 65°34'N 07°31'W at 1394 m (Lundbeck 1905).

Material examined

GS09-01.

Description

A *Cladorhiza* species with a central stem from which lateral branches project at regular intervals in all directions, each being covered in small protrusions and typically ending in a knob-like swelling. The color of the stem and branches is grayish white, with the basal part slightly darker. The stem ends in a root-like holdfast. The main stem at the holdfast is smooth and only slightly over 1 mm in diameter, but increases to almost 5 mm in the part of the stem bearing protrusions. Lateral branches are up to 4 mm in diameter. Structurally, the stem and branches consists of a slightly darker, hard and smooth central stem covered with a cortex of slightly softer tissue from which the protrusions emerge. The outer morphology, with the exception of the swellings at the branch-ends, is very similar to that of *C. gelida*.

One *C. tenuisigma* specimen was found at the Håkon Mosby mud volcano during the 2009 R/V "G.O. Sars" cruise: The specimen is around 112 mm long.

Spicules

The megascleres are straight, robust styli. There are three types of microsclere: Anchorate anisochelae, and two forms of small sigmata. A few large sigmata were found and are included in the table. These might be contamination from other sponges, though Topsent (1913) reported finding a few large sigmata as well.

Table 3.10. Spicules of *Cladorhiza tenuisigma*.

	Length (μm)				Width (μm)		
Spicule type	Min.	Mean.	σ	Max.	Mean.	σ	*N*
GS09-01							
Styli	540	739	±102	930	20.5	±3.3	30
Anchorate anisochelae	20.2	22.0	±1.5	24.2			30
Sigmata 1	36.4	39.8	±2.4	44.4			30
Sigmata 2	44.4	49.0	±3.1	54.5			30
Large sigmata	137.4	156.3	±13.6	175.7			5

Previous reported distribution

The Norwegian Sea, south of Jan Mayen and east of Iceland (Lundbeck 1905), west of Spitsbergen and off the coast of Greenland (Topsent 1909; Topsent 1913; Koltun 1964; Janussen 2009), and the Laptev Sea (Koltun 1959). Reported depth range is 631-1,834 m.

G.O. Sars distribution

Location	Coordinates	Depth	Cruise
Håkon Mosby mud volcano	72°00'N 14°39'E	1,302 m	H_2DEEP-09

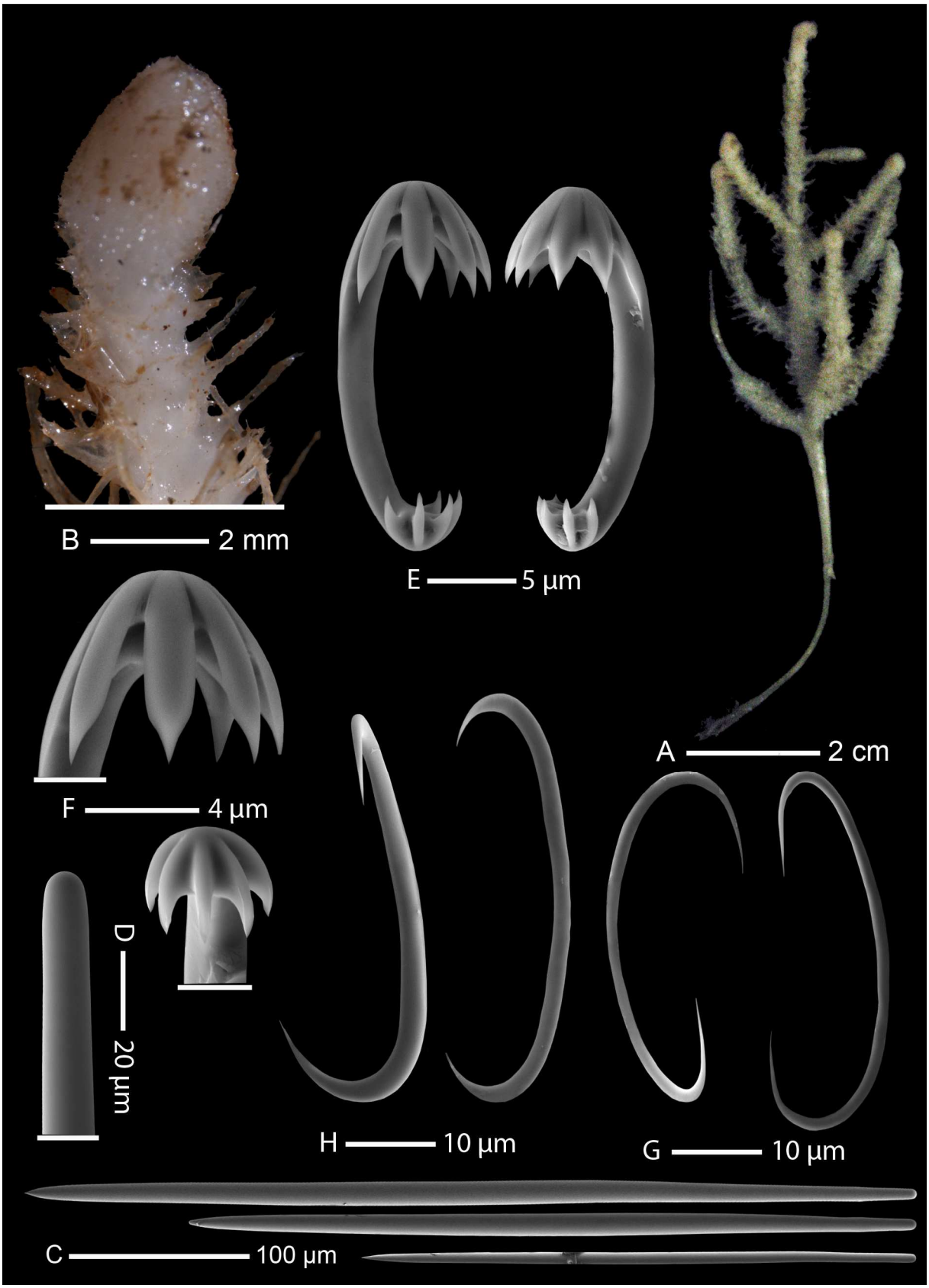

Figure 3.9. *Cladorhiza tenuisigma*. (A) Complete specimen, (B) detail from branch end, (C) styli (D) with detail, (E) anchorate anisochelae (F) with detailed view of alae, (G) smaller, (H) and larger sigmata.

3.3 Specimen habitus pictures

Figure 3.10. In-situ (where available) and habitus pictures of cladorhizid specimens.

3.4 Distribution of known Cladorhizidae in the study area

Cladorhizidae that have been described from the survey area are included in the table below (Table 3.11). While the European Register of Marine Species (ERMS) lists some species described by Koltun and Lambe from the Northern Pacific (Costello et al. 2009), these are not included here, nor are Mediterranean and Atlantic species south of the British Isles part of the list. Taxonomy and species names were checked against the World Porifera database (van Soest et al. 2008), and updated accordingly.

Table 3.11. List of known Cladorhizidae from the North Atlantic and Arctic. Species included in this thesis are marked with an asterisk.

Species	Reported depth range
Asbestopluma Topsent, 1901	
Asbestopluma comata Lundbeck, 1905	1,461 m
Asbestopluma cupressiformis (Carter, 1874)*	18-2,387 m
Asbestopluma furcata Lundbeck, 1905*	557-2,387 m
Asbestopluma hydra Lundbeck, 1905	1,847-2,394 m
Asbestopluma infundibulum (Levinsen, 1887)*	91-734 m
Asbestopluma lycopodium (Levinsen, 1887)*	41-2,387 m
Asbestopluma minuta (Lambe, 1900)	336-698 m
Asbestopluma pennatula (Schmidt, 1875)	43-1,783 m
Chondrocladia Thomson, 1873	
Chondrocladia gigantea (Hansen, 1880)	238-2,125 m
Cladorhiza Sars, 1872	
Cladorhiza abyssicola Sars, 1872	238-1,545 m
Cladorhiza arctica Koltun, 1959	2,040-2,365 m
Cladorhiza corticocancellata Carter, 1876*	710-2,387 m
Cladorhiza gelida Lundbeck, 1905*	763-2,860 m
Cladorhiza iniquidentata Lundbeck, 1905	1,783 m
Cladorhiza nobilis Fristedt, 1887	238 m
Cladorhiza oxeata Lundbeck, 1905	212-393 m
Cladorhiza tenuisigma Lundbeck, 1905*	631-1,834 m

3.5 Molecular results

3.5.1 DNA extract gel electrophoresis

Gel electrophoresis was performed directly on extracted samples to evaluate the amount of DNA recovered. Band strength was generally very weak, and showed that most extracts contained a comparatively small amount of DNA. The notable exception was both extracts from the *C. corticocancellata* specimen, where strong bands were visible.

3.5.2 COI PCR amplification and direct sequencing

Post-PCR gel electrophoresis showed that it was possible to amplify COI DNA from nearly all extracts, with representatives from all morphologically identified species. An initial set of PCR products was purified and sequenced, in which the sequence chromatograms revealed strong presence of contamination from non-sponge organisms. This was confirmed when using NCBI BLAST nucleotide search to check for similar sequences in the blastn GenBank database. A second round of all extracts was amplified where greater care was made to remove surface particles and other visible contaminants from the samples before extraction. The results from this series of extracts were almost identical to the first. Running the electrophoresis gel for a longer time period showed two closely spaced bands in the *A. infundibulum* sample in both series. Gel extraction was performed on both *A. infundibulum* extracts in order to try and sequence the bands separately; however no sequence identifiable as a sponge was retrieved using this procedure. All three *C. gelida* products showed a faint second band in both series. For the rest of the samples, only one band was visible (Figure 3.11). For most *C. gelida*, *A. furcata* and *A. lycopodium* specimens, despite a great amount of ambiguous sites, it was possible to recover sequences identifiable as poecilosclerids using BLAST. The *C. corticocancellata* and one *A. lycopodium* specimen were successfully sequenced using direct sequencing, but other specimen sequences proved too contaminated to be of further use.

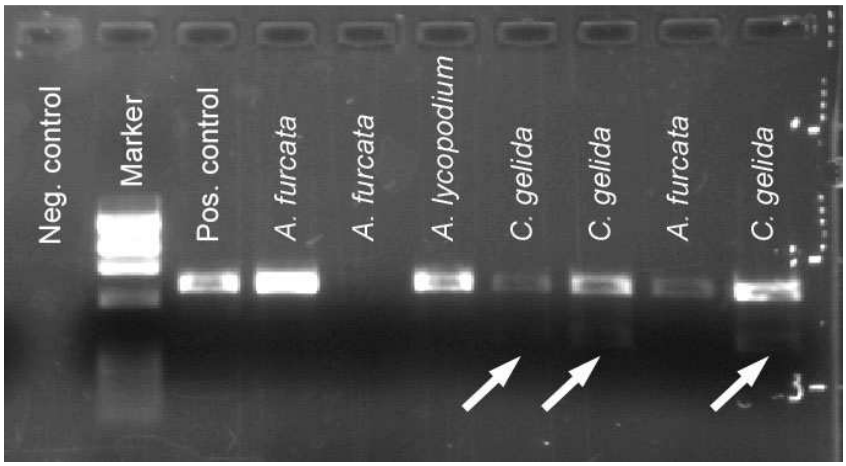

Figure 3.11. Sample COI gel showing amplified *Asbestopluma furcata*, *A. lycopodium*, and *Cladorhiza gelida*. Faint second bands (arrows) are visible for the three *C. gelida* extracts.

3.5.3 COI cloning

A representative from each morphologically identified species except *C. corticocancellata* was cloned in order to separate contaminants from sponge sequences. The cloning procedure was successful in obtaining high-quality sequences of both cladorhizid and contaminant DNA (Table 3.12; Figure 3.13). Of 10 amplified DNA clones in the *A. infundibulum* extract, all were contaminants, and no COI sequence was recovered from this species. Contaminant clones in all samples were tentatively identified by BLAST as either alpha- or gamma-proteobacteria, copepods, an amphipod, a tunicate and an annelid (Table 3.13).

Table 3.12. COI sequence specimen origin. Cloning allowed recovery of the whole 710 bp Folmer sequence.

Species	Specimen	Method	Recovered seq. length (bp)
A. furcata	GS08-06b	Cloning	710
A. lycopodium	GS08-02	Direct sequencing	648
A. cupressiformis	GS06-04a	Cloning	710
C. corticocancellata	GS08-07	Direct sequencing	677
C. gelida	GS08-01b	Cloning	709

Table 3.13. Some of the contaminating sequences from COI sequencing, with top BLAST result shown. Based on percentage coverage and identity, the top result may only give a very general indication of true sequence identity.

Species	BLAST top result	Length	Coverage	Identity
A. cupressiformis	CT573326.1 *Pseudomonas entomophila* (α-Proteob.)	704	99%	75%
A. furcata	AY116602.1 *Didemnum candidum* (Tunicata)	670	95%	77%
	AB380007.1 *Gaetanus brevispinus* (Copepoda)	710	92%	93%
A. lycopodium	NC_009434.1 *Pseudomonas stutzeri* (γ-Proteob.)	675	94%	76%
	CP000633.1 *Agrobacterium vitis* (α-Proteob.)	725	81%	78%
A. sp. 1 aff. *lycopodium*	CP000362.1 *Roseobacter denitrificans* (α-Proteob.)	693	64%	67%
	CP000489.1 *Paracoccus denitrificans* (α-Proteob.)	744	82%	72%
	CP000633.1 *Agrobacterium vitis* (α-Proteob.)	725	81%	78%
	EU856807.1 *Paracalanus aculeatus* (Copepoda)	469	98%	76%
A. infundibulum	AY830440.1 *Eurythenes gryllus* (Amphipoda)	769	99%	78%
	CT573326.1 *Pseudomonas entomophila* (α-Proteob.)	704	99%	75%
	CP001678.1 *Hirschia baltica* (α-Proteob.)	755	77%	74%
	AP009247.1 *Vesicomyosocius okutanii* (γ-Proteob.)	704	99%	79%
C. gelida	EF405598.1 *Moorebdellina biannulata* (Annelida)	537	76%	66%

3.5.4 ITS PCR amplification and direct sequencing

Gel electrophoresis showed that it was possible to recover amplified gene sequences from all samples, though in a couple of cases the band strength was very weak. The *C. gelida*, *A. infundibulum*, and two of the *A. lycopodium* samples showed single bands. The *A. furcata* and *C. corticocancellata* samples showed two distinct bands, and two *A. lycopodium* samples showed three distinct bands (Figure 3.12). The results were generally consistent across both DNA extraction series. Sequencing of the single band samples gave good results in the case of *A. infundibulum*. Of the *C. gelida* samples, only one contaminated sponge sequence was obtained, of the two *A. lycopodium* samples a couple of contaminated sequences were obtained, of the *C. corticocancellata* sample non-sponge sequence only. Gel extraction of the remaining *A. lycopodium* samples was not particularly successful as it in most cases proved difficult to get sequences from extracted gel bands. For the *A. furcata* samples, gel extraction of sample bands proved successful: In each case one band was tentatively identified by BLAST as poecilosclerid in origin, the other band as copepod. For these samples, sequences were of good quality. The LH 28SRev primer generally gave better quality sequences than the LH 18SFow primer for both single and gel extracted sequences.

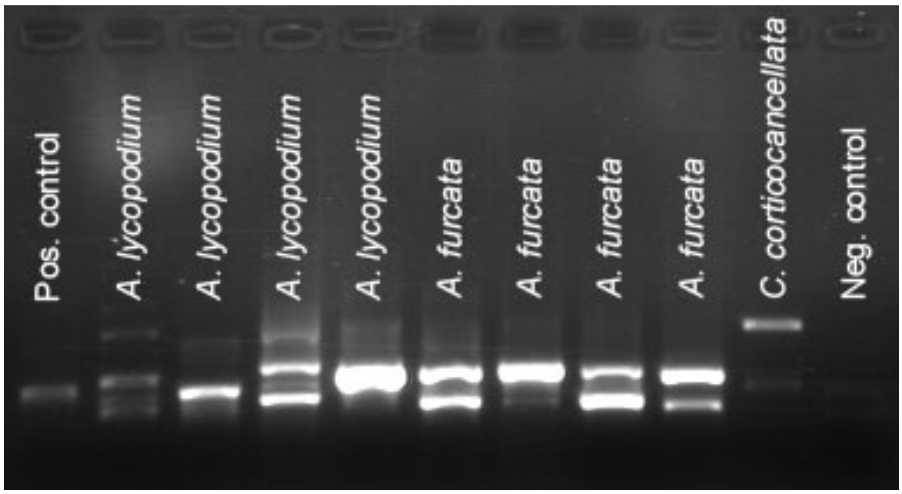

Figure 3.12. Sample ITS gel showing multiple bands after amplification.

3.5.5 ITS cloning

To resolve the issue of contaminated sequences, *C. gelida* and three different *A. lycopodium* samples were chosen for cloning. The *A. lycopodium* samples chosen were one single band extract and the lower and upper gel bands of one triple band sample; the middle of the three bands, identified as copepod during sequencing of gel extracted bands was not cloned. As only copepod sequences had been obtained from the *C. corticocancellata* samples, no cloning attempt was made on this species. As with COI, the cloning procedure successfully enabled the separation of poecilosclerid identified material and contaminant sequences (Table 3.14; Table 3.15; Figure 3.13).

Table 3.14. ITS sequence specimen origin. Total sequence length is in the case of *A. furcata* and *A.* sp. 1 aff. lycopodium somewhat more than the sum of partial lengths as sequence ends were not clean enough to be readable.

			Recovered seq. length (bp)				
Species	Specimen(s)	Method	18S	ITS1	5.8S	ITS2	28S
A. infundibulum	GS08-09	Direct sequencing	56*	235	153	206	70*
A. furcata	GS08-06a, GS08-04b, GS08-04d†	Gel extraction	26*	287	153	318	35*
A. lycopodium	GS08-10, GS08-11a†	Cloning	63*	247	153	203	70*
A. sp. 1 aff. *lycopodium*	GS08-03	Gel extraction	-	230*	153	138	41*
C. gelida	GS08-01b	Cloning	63*	306	153	287	31*

*Incomplete partition

†No intraspecies differences found in sequences examined

Table 3.15. Some of the contaminating sequences from ITS sequencing, with top BLAST result shown. Hits can be based on 18S, 5.8S and 28S similarity only, which means that coverage can be quite low. Based on percentage coverage and identity, the top results may only give a very general indication of true sequence identity.

Species	BLAST top result	Length	Coverage	Identity
A. furcata	AF315016.1 *Calanus pacificus* (Copepoda)	583	39%	85%
A. lycopodium	AF315033.1 *Cletocamptus deitersi* (Copepoda)	907	19%	94%
	DQ924559.1 *Perna perna* (Bivalvia)	1004	17%	97%
	AJ279451.1 *Galactomyces geotrichum* (Ascomycota)	418	96%	98%
	AM901707.1 Uncultured basidiomycete	690	95%	99%
A. sp. 1 aff. *lycopodium*	EU851044.1 Infectious salmon anemia virus	679	41%	84%
C. corticocancellata	AF315016.1 *Calanus pacificus* (Copepoda)	591	93%	91%
C. gelida	EU718115.1 *Scleroderma areolatum* (Basidiomycota)	437	16%	98%
	AM114420.1 *Oncaea venusta* (Copepoda)*	812	49%	84%

*This GenBank entry is 18S and ITS1 only: given ITS1 identity it is probable that sequence is a very close relative to GenBank match.

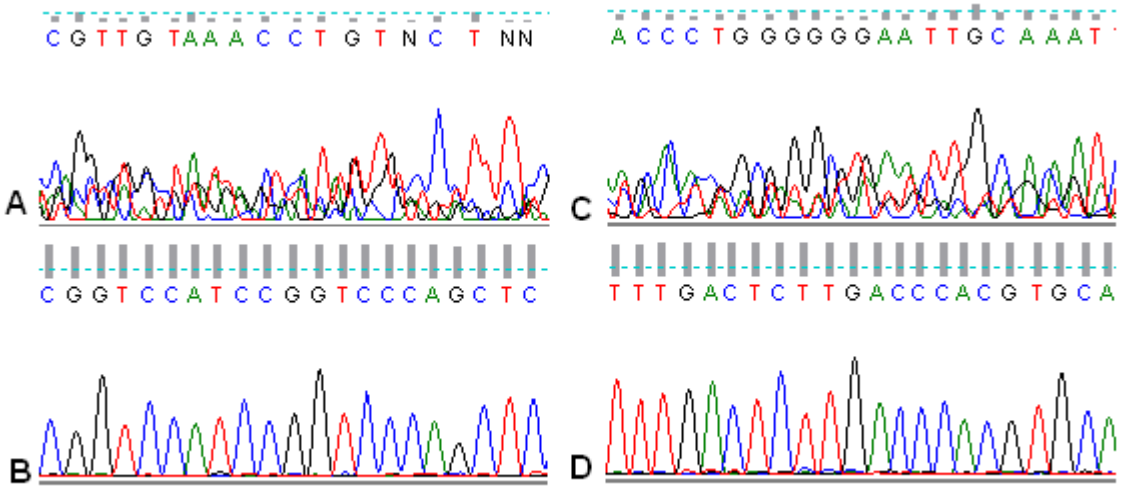

Figure 3.13. Sequence chromatograms showing (A) direct sequencing (B) and cloning of the COI, and (C) direct sequencing (D) and cloning of the rDNA and ITS fragments, from *Cladorhiza gelida*. Other specimens produced comparable results.

3.5.6 COI phylogenetic analysis

The obtained COI sequences were supplemented with GenBank poecilosclerid and outgroup sequences to determine the placement of the cladorhizids within the Poecilosclerida. As several of the imported sequences were shorter than the cladorhizid sequences, the alignment was truncated to a 582 bp sequence. For COI, 21 GenBank poecilosclerids, three outgroup

species and five cladorhizids were used to construct phylogenies using maximum parsimony (MP) maximum likelihood (ML), and Bayesian inference. To establish model of evolution and model parameters for the ML analysis, ModelTest 3.7 was used to run hierarchical likelihood ratio tests (hLRTs) and the Akaike information criterion (AIC), both suggesting the general time-reversible model with a discontinuous gamma distribution and a proportion of invariant sites (GTR+I+G) as the recommended maximum likelihood model (Table 3.16). Similarly, MrModeltest 2.2 was used to establish model and parameters for the Bayesian analysis, choosing the GTR+I+G model for the analysis. The MrBayes trace files showed that the –lnL values stabilized between generations 5,000-10,000, and the first 10,000 generations were discarded..

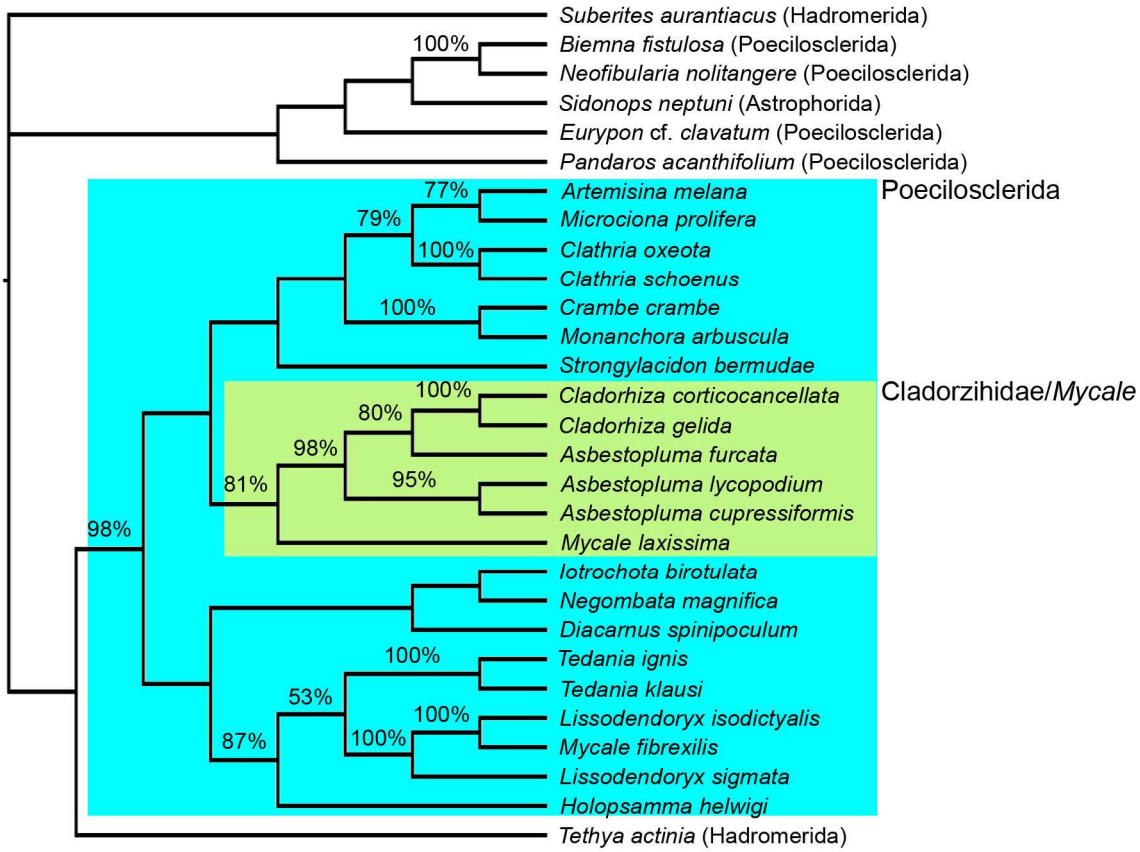

Figure 3.14. Cladogram showing phylogenetic relationships of the COI gene partition using maximum parsimony analysis with 2,000 boot replicates. Node percentages indicate bootstrap support.

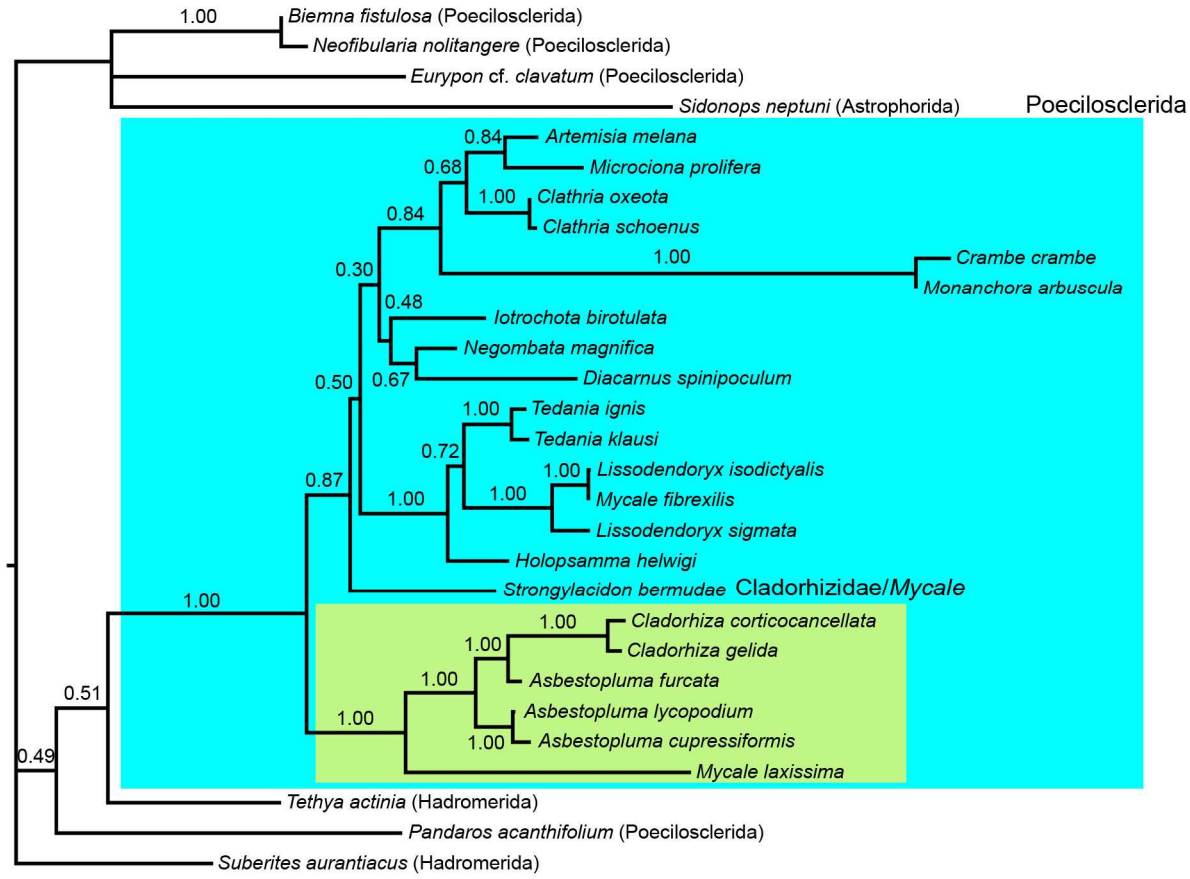

Figure 3.15. Phylogram showing phylogenetic relationships of the COI gene using maximum likelihood analysis with the GTR+I+G model and Bayesian inference using the GTR+I+G model, sampling 1,000 trees out of 1,000,000 generations discarding the first 10,000. Node values indicate posterior probability values.

Table 3.16. COI maximum likelihood model parameters.

Model: GTR+I+G		Base frequencies		Substitution rate matrix	
-lnL:	5288.0518	A	0.3009	A-C	2.5073
AIC:	10596.1035	C	0.1030	A-G	4.3852
Proportion of invariant sites:	0.2965	G	0.2312	A-T	1.3920
Gamma distribution shape:	0.4185	T	0.3648	C-G	2.7634
				C-T	7.7382
				G-T	1.0000

Both the maximum parsimony (Figure 3.14) and maximum likelihood (Figure 3.15) phylogeny provides support that Poecilosclerida is polyphyletic. *Biemna fistulosa*, *Neofibularia nolitangere*, *Eurypon* cf. *clavatum*, and *Pandaros acantifolium* constitute a clade together with *Sidonops neptuni* and *Suberites aurantiacus* in the MP cladogram, while *P. acantifolium* is recovered separately in the ML phylogram. A monophyletic clade contains the remaining poecilosclerids with *Tethya actinia* as a sister species, with 98% bootstrap support (BS). A single clade containing the cladorhizids together with *Mycale laxissima* is

well supported in both phylogenies, with BS = 81%, and a posterior probability (PP) = 1, showing up in the middle of the poecilosclerids in the MP tree, and falling basal in the poecilosclerid clade in the ML tree. While the *Cladorhiza* species are monophyletic (BS = 100%; PP = 1), the *Asbestopluma* species are deemed paraphyletic, with *A. furcata* showing a closer affinity to the *Cladorhiza* clade than to *A. lycopodium* and *A. cupressiformis* (BS = 80%; PP = 1). The other *Mycale*-species in the analyses, *M. fibrexilis*, is firmly nested in a different clade (BS = 100%; PP = 1) making *Mycale* paraphyletic. Given that only a single base differs between this species and *Lissodendoryx isodictyalis* there is a strong possibility that this sequence is a mislabeled *Lissodendoryx* and that *M. laxissima* is the only true representative *Mycale* species in the analyses.

3.5.7 ITS phylogenetic analysis

Through BLAST searches, it was possible to identify ITS sequences in GenBank as either affiliated with Poecilosclerida or as copepod through matching the ribosomal 18S and 28S ends bordering the ITS1 and ITS2 sequences, and the 5.8S partition. A MP analysis using the 5.8S partition, in addition to the 18S and 28S fragments, was made to get an approximate location of the cladorhizid and GenBank poecilosclerid samples, to be sure that the sequences used for further study were the actual cladorhizid specimens and not contaminating sequences. *Suberites ficus* and *Tethya* sp. were chosen as outgroups. The short length and high degree of conservation of these partitions make them unsuitable for any rigorous analysis (Halanych 1991), additionally 18S and 28S sequences were of different length in different sequences since they were not truncated as for the COI alignment. The partitions can still be useful to evaluate the approximate location of species within the higher taxonomy however. The resolving power of this analysis is very low, evidenced by several polytomies and the inconsistent placement of *Eurypon* cf. *clavatum* and *Tethya* sp. within the other poecilosclerids: morphological and molecular evidence shows that these are clearly not Poecilosclerida. Still, the monophyletic cladorhizid clade seems to imply that all sequences were correctly identified by BLAST, suggesting that no contaminating sequences were inadvertently included in the analysis (Figure 3.16).

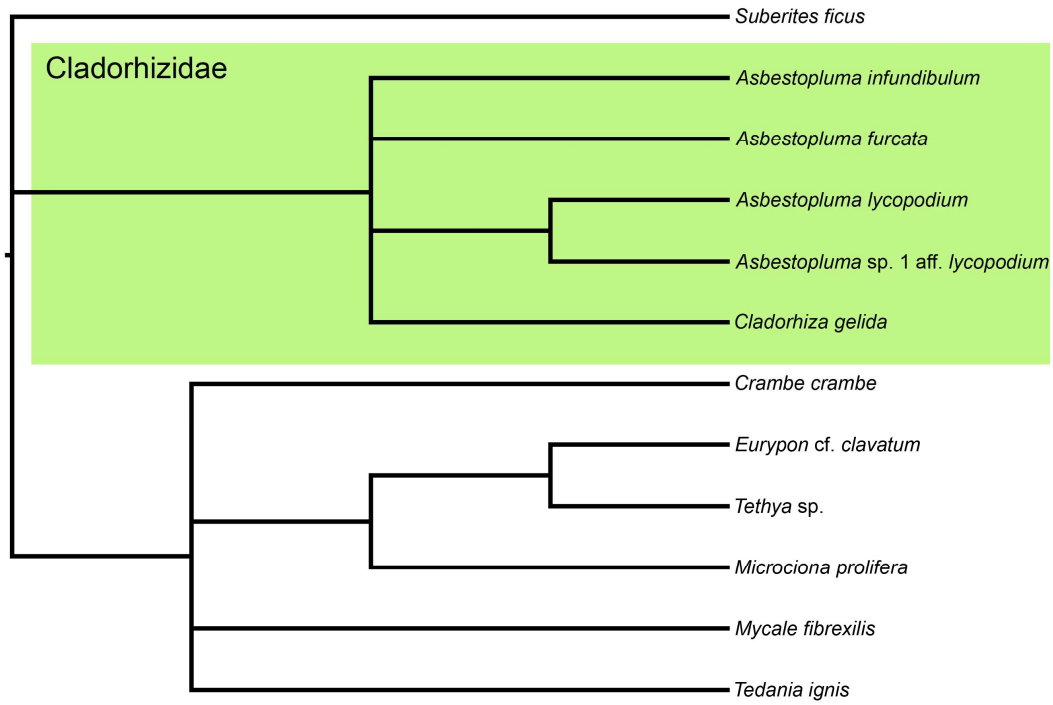

Figure 3.16. Maximum parsimony majority rule consensus tree of 5.8S and small fragments of 18S and 28S, showing the approximate location of cladorhizid and GenBank taxa.

While it was unproblematic to identify and align the 18S, 5.8S and 28S partitions, making a proper alignment of either the ITS1 or ITS2 sequences proved complicated, because of large divergences in sequence length and identity (Table 3.17). Sequences that diverge by more than 30% are of little use for phylogenetic analysis (Hillis and Dixon 1991). Thus no phylogenetic analysis was done on the ITS1 and ITS2 sequences, as they proved too divergent infer any phylogenetic relationships between the species that were successfully sequenced.

Table 3.17. Pairwise sequence inverse p-distance matrix showing similarity of ITS1 and ITS2 fragments from sequenced species as a percentage.

	A. infundibulum		*A. furcata*		*A. lycopodium*		*A.* sp. 1 aff. *lyco.*	
	ITS1	ITS2	ITS1	ITS2	ITS1	ITS2	ITS1	ITS2
A. infundibulum			51.73%	46.15%	70.82%	65.42%	32.35%*	66.33%
A. furcata	51.73%	46.15%			53.56%	45.21%	30.75%*	45.33%
A. lycopodium	70.82%	65.42%	53.56%	45.21%			31.81%*	88.41%
A. sp. 1 aff. *lyco.*	32.35%*	66.33%	30.75%*	45.33%	31.81%*	88.41%		
C. gelida	44.34%	53.90%	43.63%	39.88%	44.60%	53.28%	33.51%*	52.20%

*Incomplete *A. lycopodium* var. 1 ITS1 partition reduces sequence similarity percentage.

While the degree of divergence precluded a proper phylogenetic tree, some information can still be read from the pairwise matrix: A couple of sequence pairs showed a higher degree of

affinity than others: The *A. lycopodium* and *A.* sp. 1 aff. *lycopodium* sequences, while variant in some places, suggesting a closer relationship between these species, though an incomplete ITS1 partition for *A.* sp. 1 aff. *lycopodium* reduces identity of that sequence. The *A. infundibulum* specimen is the next closest to this group, while the *A. furcata* and *C. gelida* sequences are less similar, a pattern that broadly follows the rDNA tree (Figure 3.16), and is consistent with COI results.

3.5.8 PCR amplification and sequencing of *pmoA*

To evaluate the temperature optimum of the A189/mb661 primer pair, a gradient analysis was set up, with two amplification runs using the PCR product from the first run as template in the second (Figure 3.17). The results were inconclusive, and an annealing temperature of 55 °C was chosen, which mirrors earlier studies using the same primers (e.g. Costello and Lidtrom 1999; Jensen et al. 2008).

Only R/V "G.O. Sars" 2008-specimens were used to determine presence of methanotrophic symbionts. Samples were set up in two sets (Table 3.18).

Table 3.18. Specimens selected for amplifying pmoA sequence.

Set 1	GS08-01, GS08-06a, GS08-06b, GS08-01b, GS08-01c, GS08-02, GS08-04a, GS08-04b
Set 2	GS08-04c, GS08-04d, GS08-03, GS08-07, GS08-08, GS08-09, GS08-10, GS08-11a

Initially, both sets were amplified, then reamplified. No result except a weak positive control signal in all gels was evident, possibly due to deterioration in the primer stock. The first set was reamplified using a new primer stock. Except for the positive control, no visible product was obtained after the first amplification, but the reamplification produced multiple bands for all cladorhizid species (Figure 3.18 A).

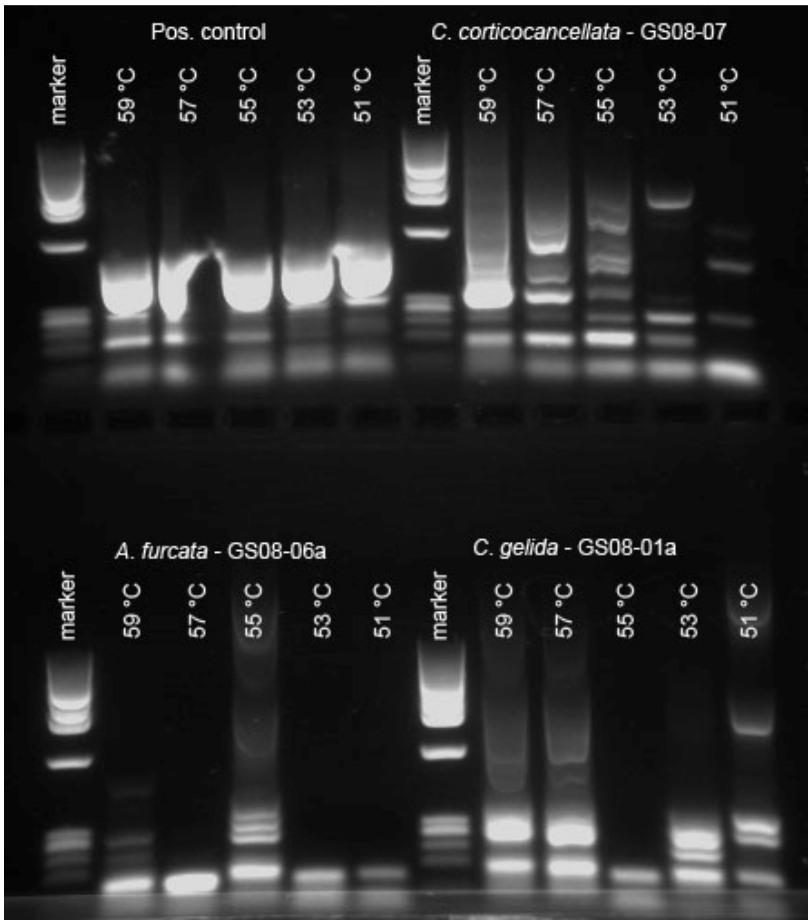

Figure 3.17. The performance of the A189/mb661 primer pair at 51, 53, 55, 57, and 59 °C using three cladorhizids and one positive control.

Table 3.19. Results of sequencing the pipette-extracted bands.

	BLAST top result	Length	Coverage	Identity
GS08-01 (*C. gelida*)	-	80	-	-
GS08-06a (*A. furcata*)	AY550729.1 Uncultured bacterium pMMO	480	42%	97%
GS08-06b (*A. furcata*)	FJ910938.1 *Zea mays* transposon	302	12%	84%
GS08-01b (*C. gelida*)	AJ295231.1 *Oncorhynchus mykiss* nitric oxide synthase	663	5%	85%
GS08-01c (*C. gelida*)	CP001393.1 *Anaerocellum thermophilum* HNH endonuclease	647	4%	92%
GS08-02 (*A. lycopodium*)	XM_002047952.1 *Drosophila virilis* mRNA	794	3%	93%
GS08-04a (*A. furcata*)	CP001510.1 *Methylobacterium extorquens*	285	67%	71%
GS08-04b (*A. furcata*)	FN394123.1 Uncultured methanotroph pMMO*	479	90%	95%
Pos. control	FN394123.1 Uncultured methanotroph pMMO	482	91%	95%
GS08-01b (*C. gelida*)	AY550729.1 Uncultured bacterium pMMO	688	29%	95%
GS08-01c (*C. gelida*)	CP000489.1 *Paracoccus denitrificans* chr. 1	377	7%	93%
GS08-02 (*A. lycopodium*)	No significant similarity found	396	-	-
GS08-04a (*A. furcata*)	CP001510.1 *Methylobacterium extorquens*	293	55%	74%

*This result is probably contamination from the neighboring positive control.

To extract single bands, a pipette was used to recover parts of individual bands for a third reamplification, which met with partial success (Figure 3.18 B). For four of the specimens, a second band was also extracted (Figure 3.18 C). The product from this third amplification was sequenced. While it was still possible to see multiple bands in most samples, they were nevertheless sequenced directly because of the time and cost involved in trying to clone each sample in the set. While it proved possible to identify some sequences as partial, though garbled, *pmoA*, in most cases the sequences proved to be a mix of several sequences (Table 3.19). As initial results were inconclusive, no further attempts were made to expand on the number of specimens for further study, and results were obtained only for the first set of 2008 specimens.

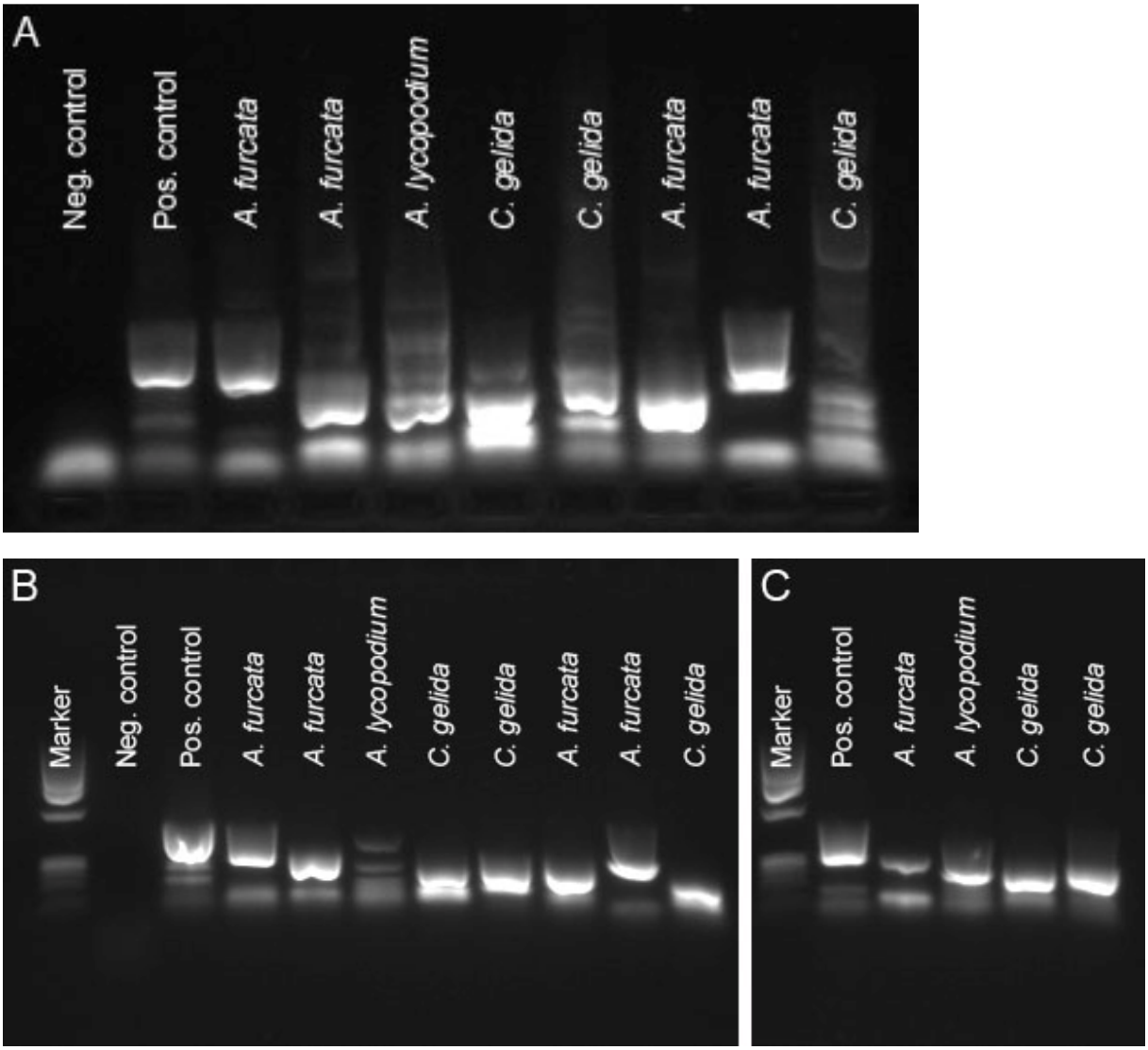

Figure 3.18. (A) PCR product of second PCR (B) and after extracting single bands. For four of the samples, (C) two bands were extracted. Specimen order is the same as in Table 3.19.

4. Discussion

4.1 Obtaining cladorhizid sequences

Sponges are generally known as a challenging group when working with molecular tools. The amount of close interaction between sponges and other organisms makes them particularly susceptible to contamination from other sources (Lavrov et al. 2008). The adhesive nature of the predatory Cladorhizidae could be an additional issue, both originally in situ and during collection, as contaminants will easily attach to the surface of specimens. Because of their slight build and branching structure, using tissue from the interior of the sponge only was not possible.

Preliminary DNA extract gel electrophoresis showed that the DNA yield of extracts was generally low, with the exception of the samples taken from the morphologically more robust *Cladorhiza corticocancellata*. While PCR amplification was successful for most samples, the small apparent amounts of extracted sponge DNA could make the amplification particularly vulnerable to even tiny amounts of contaminating DNA.

Tentative BLAST derived identity of COI contaminating sequences showed that amplification of this gene using the Folmer primer pair proved susceptible to contamination from alpha- and gamma proteobacteria (Table 3.13). This is not particularly surprising given the assumed alpha-proteobacterial origin of the mitochondrion (Gray 1999). The origin of the prokaryote DNA could be symbiotic cells, but might equally well stem from surface contaminants. Additional contamination was a mixture of copepods and other invertebrate groups. Multiple bands were generally close together, and apparent single band samples also contained contaminating sequence.

Gel electrophoresis of PCR-amplifications using the ITS primer pair revealed multiple bands, in line with the very variable length of the ITS1 and ITS2 sequences in different taxa. Contamination mostly originated from copepods, sequences of which were recovered in samples from almost all morphologically identified species, and marine fungi (Table 3.15). While this primer pair was developed for use with sponge sequences, it is clearly not specific enough to avoid amplifying contaminating sequences from other metazoans or even non-metazoan eukaryotes.

TOPO cloning proved to be a very well suited method to isolate cladorhizid DNA-fragments for sequencing from contaminated PCR product. Because of the time and cost of this procedure, cloning every specimen was unfeasible, and only a few samples were selected, representing already morphologically identified species. Thus it proved too time consuming and resource demanding in this thesis to obtain the sequences necessary to create a molecular phylogeny of all sampled cladorhizid specimens to compare to the morphological taxonomy.

4.2 Morphological taxonomy

In general, since many deep-sea sponges are described from scant material, any intra-specific variation is poorly known. Additionally, spicules may differ both in type and composition depending on the location in and the size of the specimen. Thus, there is a possibility that an exaggerated number of species has previously been described (Vacelet 2007). On the other hand, being able to complement dredging and mud bottom trawling with specimen collection using ROV, as well as focusing more sampling in the general enrichment zones around vents, has increased the number of known species, although not all of the new material has been published (Vacelet 2007).

Morphological examination of the specimens in this thesis showed that almost all specimens could readily be identified to previously described species from the North Atlantic and Arctic based on morphological characteristics. A couple of specimens were seen as affiliated with, and very similar to, but not identical to *A. lycopodium*. It proved difficult to establish whether they are actual *A. lycopodium* or if they are separate species: One the one hand, the degree of difference in spicule composition is comparable, if slightly less pronounced, than of that between *A. lycopodium* and the Pacific species *A. occidentalis*. Additionally, it was possible to extract an ITS2 sequence from *A.* sp. 1 aff. *lycopodium* with only an 88% similarity to that of *A. lycopodium* (except for a voucher specimen, all *A.* sp. 2 aff. *lycopodium* material was destroyed making spicule preparations precluding a molecular analysis). On the other hand, *A. lycopodium* spicules have been shown to vary in type and relative abundance depending on the presence of structures related to the life cycle of the sponge such as spermatic cysts and embryos. Cladorhizid spicules also vary with regards to the location in the sponge, and it would not be difficult to imagine that young specimens or specimens exposed to certain stimuli developed variations in spicule complement. The significance of the variation in the ITS2 sequence is also difficult to gauge as no studies have been made as to normal intra- and interspecific ITS variation in these sponges. It was not possible to extract COI sequence from

any variant. Both variants are also known from scant material. In conclusion, the status of these specimens must remain unknown as there are valid arguments both for and against inclusion of one or both as *A. lycopodium*.

4.3 Phylogeny

The extensive cloning procedure that proved necessary in order to obtain sequences of sufficient quality made a comprehensive comparison of all specimens impractical. A more complete range of sequences for the samples in this study would be of great use to compare genetic variation with the morphological defining characters, possibly reveal cryptic species, and elucidate the internal phylogeny of the Cladorhizidae.

The ITS1 and ITS2 partitions have in most cases been found applicable in resolving phylogenetic relationships at species and population level within the Porifera (e.g. Alvarez et al. 2007). However, the ITS1 and ITS2 partitions recovered in this analysis proved too divergent to be of use in this analysis. Still, pairwise alignment matrix values (Table 3.17) showed a slight affinity between the *A. lycopodium* and *A.* sp. 1 aff. *lycopodium* species, and between those two and the *A. infundibulum* specimen, thus providing some supporting evidence for the COI results.

Because of the many tandem repeats of the nuclear rDNA array, intragenomic polymorphism is a recognized problem with ITS sequences (Wörheide 2004). While concerted evolution tends to act as a counter to intraspecific heterogeneity in general (Hillis and Dixon 1991; Wörheide 2004), several studies have shown that polymorphism exists within a number of species, including studies focusing on different poriferan groups (e.g. Wörheide 2004; Alvarez et al. 2007). In these studies, multiple ITS versions were in some cases recoverable from the same specimen using either single strand conformation polymorphism (SSCP) or cloning procedures similar to the one used for this thesis.

Thus, the possibility that some of the sequences recovered for this thesis represent different paralogues should be considered, explaining some of the great degree of divergence in sequence identity. Two arguments may be raised against this interpretation however: First, while intragenomic variation has been found in several species, a majority of species in the mentioned studies were still found to be homogenous. Second, only a single version of the ITS/rDNA array was successfully sequenced for each species in this thesis. Intraspecific heterogeneity is thus an unlikely explanation, and the sequence differences between species

should be considered interspecific variation. It is therefore reasonable to conclude that while the ITS fragments could be applicable between very closely related species in this group, their usefulness within the Cladorhizidae at genus or even species level is low.

The ITS sequences proved too divergent for use to support a cladorhizid phylogeny, while the rDNA 5.8 partition is too short and conserved for any rigorous phylogenetic analysis (Halanych 1991), though it proved helpful in establishing the poriferan identity of the sequences (Figure 3.16). Erpenbeck et al (2005) questions the value of using only the Folmer COI partition at anything below family level in diploblastic animals. However, the Folmer COI sequences obtained in this study contained enough variation to elucidate phylogenetic relationships at the genus and even species level, as evidenced by solid bootstrap support and posterior probability values. Thus, it is possible to draw a couple of conclusions on the internal relationships of the Cladorhizidae, and to create a phylogeny of the Cladorhizidae within the poecilosclerids already available through GenBank:

First, COI supports a monophyletic Cladorhizidae clade, and groups it together with *Mycale* in a clade within the monophyletic Poecilosclerida with strong support (BS 80-100%; PP = 1). *Mycale* and the cladorhizids are the only members of suborder Mycalina included in the phylogenetic analysis. The affinity between *Mycale* and Cladorhizidae is thus in agreement with current systematics (Hooper and van Soest 2002b).

The affinities of Cladorhizidae to other Mycalina groups such as the families Esperiopsidae and Guitarridae is still unclear (Hooper and van Soest 2002b), but they are assumed to be closely related. At this point lack of sequences from these families makes it impossible to conclude anything regarding the suspected polyphyly of the Cladorhizidae with regards to these closely related families. Still, the monophyly of the Cladorhizidae compared to the rest of the current GenBank species is now established, and needs to be challenged through obtaining sequence information from species from these closely related groups.

Second, the COI *A. furcata* phylogeny suggests a closer affinity to *Cladorhiza* than to the other *Asbestopluma* species in this study. If the COI analysis is indicative of the true phylogeny, this means that the genus *Asbestopluma*, whose diagnostic feature is the presence of palmate anisochelae, is paraphyletic. Some morphological characters support this interpretation: While *Asbestopluma*-species such as *A. cupressiformis*, *A. infundibulum*, and *A. lycopodium* have a spicule complement featuring one type of palmate anisochelae (with the

exception of slightly smaller chelae of very similar form connected to embryos) and forcipes (Figure 3.1; Figure 3.3; Figure 3.4), *A. furcata* carries tylostrongyla, two types of palmate anisochelae with different size and morphology, and—like the *Cladorhiza*—sigmata (Figure 3.2). *Asbestopluma*-species described in the literature (e.g. Lundbeck 1905; Koltun 1959) fall into one or the other category: while not all species carry all spicule types mentioned, forcipes are for instance never found together with sigmata or the large type of palmate anisochela.

As with the question of the monophyly of the Cladorhizidae in relation to nearby families, expanding upon the COI results in this thesis would probably prove to be profitable in further elucidating phylogenetic relationship within the Cladorhizidae as well. Obtaining sequence data for *Asbestopluma* species with a similar spicule complement to *A. furcata*, such as for instance *A. pennatula*, would help resolve the question of paraphyly of this genus in relation to *Cladorhiza* as implied by the COI analysis.

This study has showed that COI is a well-suited gene in elucidating the phylogeny of the Cladorhizidae at the family, genus and even species level. Further studies with more sequence data are needed to gain a more complete phylogenetic picture of the cladorhizids and their relation to sister families. Adding the l3-M11 COI partition to the Folmer partition in order to obtain a complete COI sequence for each species would make the analysis more robust, as this second partition is less conserved (Erpenbeck et al. 2005), as more, closely related species, are added to the list of sequenced cladorhizid and related species.

While the l3-M11 primer pair is designed for sponges, contamination would in all probability still be an issue. Nonetheless, even though cloning or other means of separation would probably be necessary, the task of obtaining sequences to construct an expanded and even more robust phylogeny of these groups, as shown in this study, is certainly conceivable.

4.4 Methanotrophic symbiosis

Results from sequencing a fragment of the *pmoA* gene were ambiguous. While it was possible to obtain *pmoA* sequences from several specimens (Table 3.19), this was only detectable after a second PCR run, which also sequenced apparently unrelated sequences, in contrast to amplification of the positive control. The results are qualitative, though the second PCR necessary to detect traces of the gene would suggest a relatively low abundance. No ambient controls from either sediment or water samples were used, making it difficult to establish whether the sequences originate from symbionts or whether they merely represent ambient

contamination from the methane-rich environments from which the specimens were sampled. Additionally, amplification proved susceptible to contamination, making it difficult to get clean sequences. As sequences were in almost all cases a mixture of several different strands, identifying the sequences via BLAST proved difficult, evidenced by the in many cases nonsensical BLAST results.

The data do not provide sufficient basis for any strong statement regarding the question of methanotrophic symbiosis within the cladorhizids sampled. The samples successfully sequenced are mainly *C. gelida* and *A. furcata* specimens from the Loki's Castle/Schultz Massif area. Still, *pmoA* sequences were actually recovered, and it is likely that more of the sample products contained the gene than what was immediately apparent through BLAST, obscured by other amplified sequences. This implies presence of some type of methanotrophic bacteria in most samples.

Further studies probably need to be accompanied with ambient controls and structural studies to locate symbiotic cells within sponge tissue. Additionally, cloning specimen PCR product seems necessary in order to get good quality sequences. Based on an evaluation of these factors, I decided to abandon the attempt after a complete run of the first specimen set: Additional samples would have had little to add to the conclusions drawn from the first set, and even a complete cloning procedure for each sample in each set would not decisively answer the question of the sequence origin.

While the method employed proved to be insufficient the results are interesting because *pmoA* sequences were after all recovered from the samples. They also suggest what needs to be taken into account to decisively answer the methanotroph hypothesis in the North-Atlantic cladorhizids.

5. Conclusion

Based on morphological examination of the R/V “G.O. Sars” 2006-2009 material six *Asbestopluma* and three *Cladorhiza* species were identified. Almost all could be reliably identified as previously described species. Two species closely allied to *A. lycopodium* were found to be sufficiently divergent that they were described separately, based on morphological and molecular evidence.

While ITS sequences proved too divergent to be of use in phylogeny, the Folmer COI partition gave a level of resolution capable of both confidently placing the cladorhizids as a monophyletic group close to Mycalidae within the greater Poecilosclerida clade, and elucidate internal relationships within Cladorhizidae. *A. furcata* had a closer affinity to the *Cladorhiza* specimens than to the other *Asbestopluma* species. While additional molecular data are needed to make a more robust analysis, the *A. furcata* placement implies that *Asbestopluma* is paraphyletic.

Sequencing the A189/mb661 pMMO *pmoA* partition to determine presence of methanotrophic symbionts met with mixed success. Amplification produced multiple bands. As time and cost precluded proper separation of bands, band identity remained uncertain for the majority of specimens, though it was possible to identify presence of the *pmoA* partition in several samples. Band separation, in addition to examination of tissue for symbiotic cells and ambient controls, seem necessary for properly elucidating the question of whether methanotrophic symbionts are present within the Cladorhizidae.

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Appendix A: List of samples

Sample name is based on cruise, species, and specimen specimens where several species were found preserved in the same sample. In those cases where several specimens from the same sample have been examined, they are marked by small letters. Size description is comparative to other specimens of the same species.

Sample	Cruise	Species	Locality	Station	Latitude	Longtitude	Depth	Comments
GS06-01	BIODEEP-06	*A. cupressiformis*	Gallionella Garden	ROV Dive 7	71°17.99'N	5°46.08'W	616 m	Tiny fragment
GS06-02	BIODEEP-06	*A.* sp. 2 aff. *lycopodium*	Gallionella Garden	ROV Dive 12	71°17.98'N	5°46.92'W	616 m	Tiny fragment
GS06-03	BIODEEP-06	*A. furcata*	Troll Wall	ROV Dive 16	71°17.86'N	5°46.29'W	557 m	Medium specimen
GS06-04	BIODEEP-06	*A. cupressiformis*	Troll Wall	ROV Dive 16	71°17.86'N	5°46.29'W	557 m	Four small specimens
GS06-05a	BIODEEP-06	*A. lycopodium*	Troll Wall	ROV Dive 16	71°17.86'N	5°46.29'W	557 m	Small fragment
GS06-05b	BIODEEP-06	*A. lycopodium*	Troll Wall	ROV Dive 16	71°17.86'N	5°46.29'W	557 m	Small fragment
GS06-06	BIODEEP-06	*A.* sp. 2 aff. *lycopodium*	Troll Wall	ROV Dive 16	71°17.86'N	5°46.29'W	557 m	Two tiny specimens
GS07-01	BIODEEP-07	*A. lycopodium*	Schultz Massif slope	ROV Dive 9	73°50.23'N	7°38.04'E	1,262 m	A small specimen
GS08-01a	H_2DEEP-08	*C. gelida*	Btw. Loki and Schultz	08-10-ROV 1	73°39.24'N	7°47.20'E	2,345 m	Large specimen
GS08-01b	H_2DEEP-08	*C. gelida*	Btw. Loki and Schultz	08-10-ROV 1	73°39.24'N	7°47.20'E	2,345 m	Small specimen
GS08-01c	H_2DEEP-08	*C. gelida*	Btw. Loki and Schultz	08-10-ROV 1	73°39.24'N	7°47.20'E	2,345 m	Fragments
GS08-02	H_2DEEP-08	*A. lycopodium*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Medium specimen
GS08-03	H_2DEEP-08	*A.* sp. 1 aff. *lycopodium*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Small specimen
GS08-04a	H_2DEEP-08	*A. furcata*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Small branched specimen
GS08-04b	H_2DEEP-08	*A. furcata*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Small unbranched specimen
GS08-04c	H_2DEEP-08	*A. furcata*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Small unbranched specimen
GS08-04d	H_2DEEP-08	*A. furcata*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Medium branched specimen
GS08-05	H_2DEEP-08	*A. cupressiformis*	Schultz Massif top	08-41-ROV 5	73°49.78'N	7°34.87'E	688 m	Small branched specimen
GS08-06a	H_2DEEP-08	*A. furcata*	Schultz Massif slope	08-43-ROV 6	73°47.51'N	7°49.41'E	1,764 m	Big specimen
GS08-06b	H_2DEEP-08	*A. furcata*	Schultz Massif slope	08-43-ROV 6	73°47.51'N	7°49.41'E	1,764 m	Several small specimens
GS08-07	GS08-155	*C. corticocancellata*	Pockmark Tobic	GS08-155-08ROV	64°40.86'N	5°15.77'E	720 m	Big specimen
GS08-08	GS08-155	*A. lycopodium*	Pockmark G11	GS08-155-25BC	64°39.85'N	5°17.30'E	730 m	Medium specimen
GS08-09a	GS08-155	*A. infundibulum*	Pockmark G11	GS08-155-32ROV	64°39.83'N	5°17.38'E	734 m	One small specimen
GS08-09b	GS08-155	*A. infundibulum*	Pockmark G11	GS08-155-32ROV	64°39.83'N	5°17.38'E	734 m	Three small specimens
GS08-10	GS08-155	*A. lycopodium*	Pockmark G11	GS08-155-32ROV	64°39.83'N	5°17.38'E	734 m	Small specimen
GS08-11a	GS08-155	*A. lycopodium*	Pockmark Dodo	GS08-155-33d	64°40.12'N	5°15.70'E	730 m	Small specimen
GS08-11b	GS08-155	*A. lycopodium*	Pockmark Dodo	GS08-155-33d	64°40.12'N	5°15.70'E	730 m	One small, 1 medium specimen
GS09-01	H_2DEEP-09	*C. tenuisigma*	HM mud volcano rim	Agassiz trawl st. 1	72°00.39'N	14°38.88'E	1,302 m	Branched specimen

Sample	Cruise	Species	Locality	Station	Latitude	Longtitude	Depth	Comments
GS09-02a	H_2DEEP-09	*A. furcata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Several branched specimens
GS09-02b	H_2DEEP-09	*A. furcata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Branched specimen
GS09-02c	H_2DEEP-09	*A. furcata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Lower stem fragment
GS09-02d	H_2DEEP-09	*A. furcata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Stem fragment
GS09-02e	H_2DEEP-09	*A. furcata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Lower stem fragment
GS09-05	H_2DEEP-09	*C. corticocancellata*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	One specimen and 3 fragments
GS09-06	H_2DEEP-09	*A. cupressiformis*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Medium specimen
GS09-07	H_2DEEP-09	*A. lycopodium*	Btw. Loki and Schultz	Agassiz trawl st. 2	73°35.19'N	7°44.08'E	2,387 m	Small fragment

Appendix B: Spicule nomenclature

Demosponge spicules fall into two main size categories: megascleres and microscleres. The spicules found in the thesis samples are listed below; descriptions are adapted from the *Thesaurus of Sponge Morphology* (Boury-Esnault and Rützler 1997). The megascleres of the Cladorhizidae are all needle-like and monaxonal, while microscleres are more varied in shape.

Style (pl. styli): A megasclere with one pointed and one blunt end (Figure B.1 A, E).

Subtylostyle (pl. subtylostyli): A style containing a slight swelling or knob at or near the blunt end of the spicule (Figure B.1 B).

Tylostyle (pl. tylostyli): A style containing a clear swelling or know at the blunt end of the spicule (Figure B.1 C).

Tylostrongyle (pl. tylostrongyla): A monaxonal megasclere with two blunt ends, containing slight swellings or knobs at or near one or both ends (Figure B.1 D).

Anisochela (pl. anisochelae): A microsclere with a curved shaft and two unequal ends carrying recurved wing- or claw-like protrusions (alae). Anchorate chelae have three or more free alae shaped like anchor claws (Figure B.1 F). In palmate chelae, the lateral alae coalesce with the shaft over their entire length while the central ala remains free (Figure B.1 G).

Sigma (pl. sigmata): A C or S shaped microsclere (Figure B.1 H).

Forceps (pl. forcipes): A U- or V-shaped microsclere (Figure B.1 I).

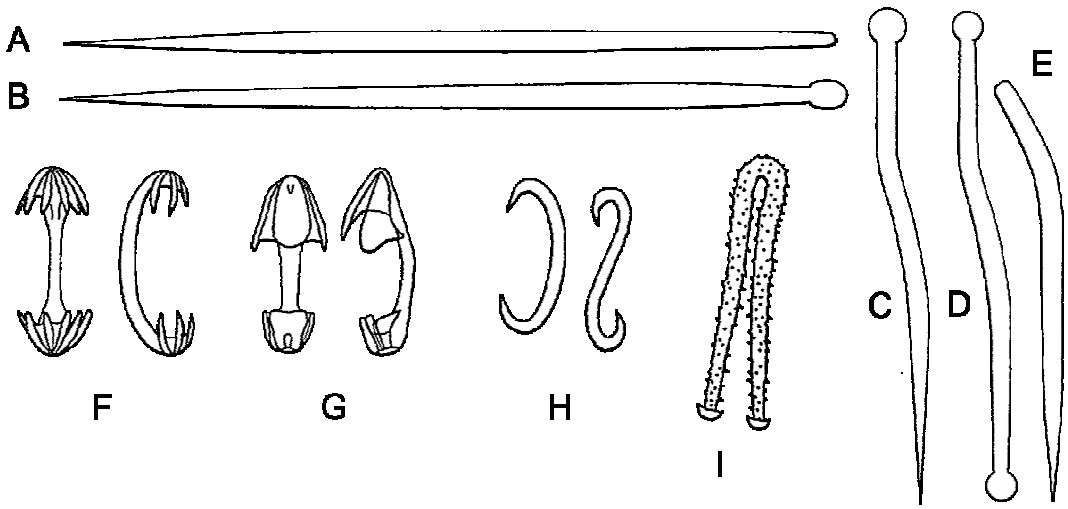

Figure B.1. Spicule type illustrations adapted from the *Thesaurus of Sponge Morphology*.

Appendix C: Species light microscopy slide pictures

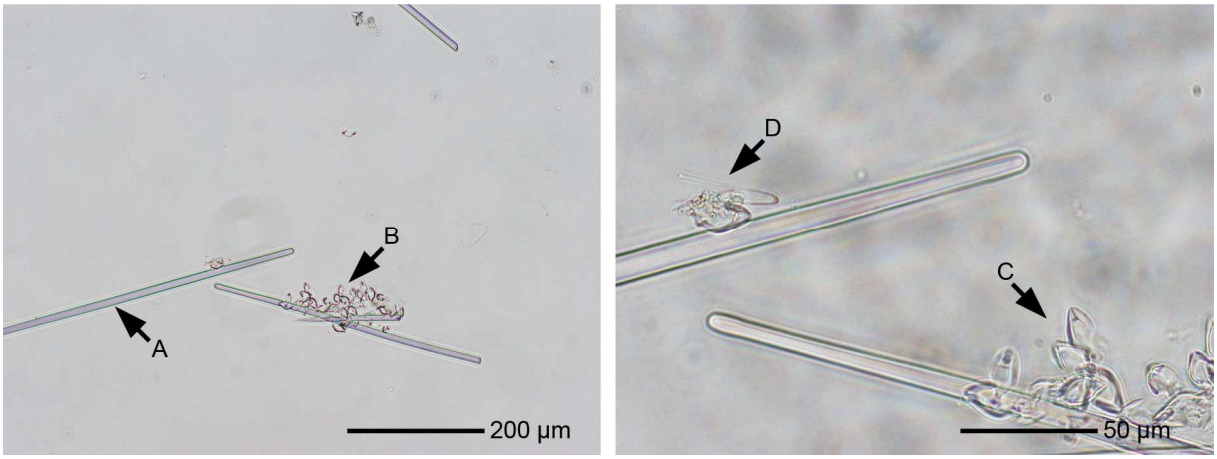

Figure C.1. Microscopy pictures of *Asbestopluma cupressiformis* at 100 and 400X. (A) Style, (B, C) palmate anisochelae, (D) forceps.

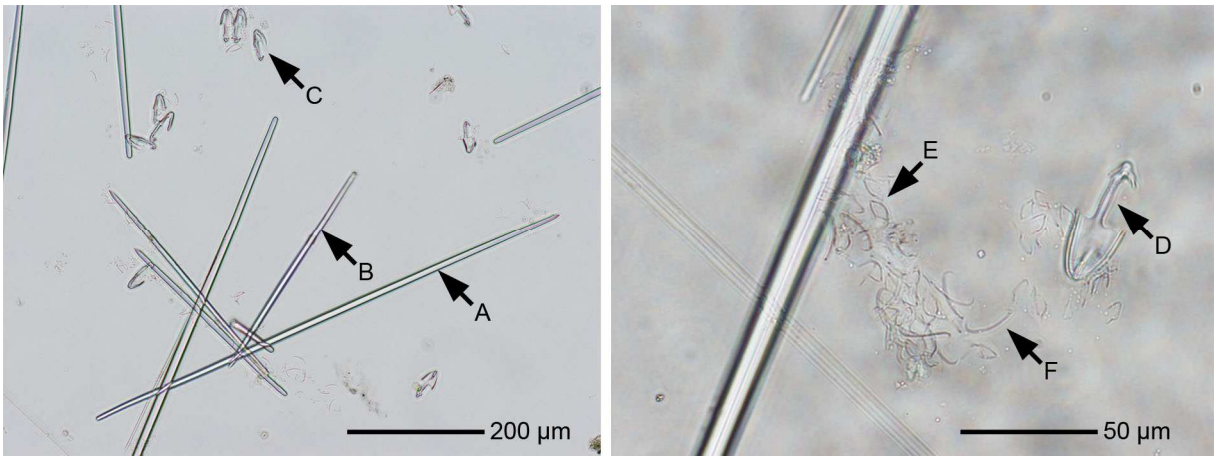

Figure C.2. Microscopy pictures of *Asbestopluma furcata* branch at 100 and 400X. (A) Style, (B) subtylostyle, (C, D) large palmate anisochelae, (E) small palmate anisochelae, (F) sigmata.

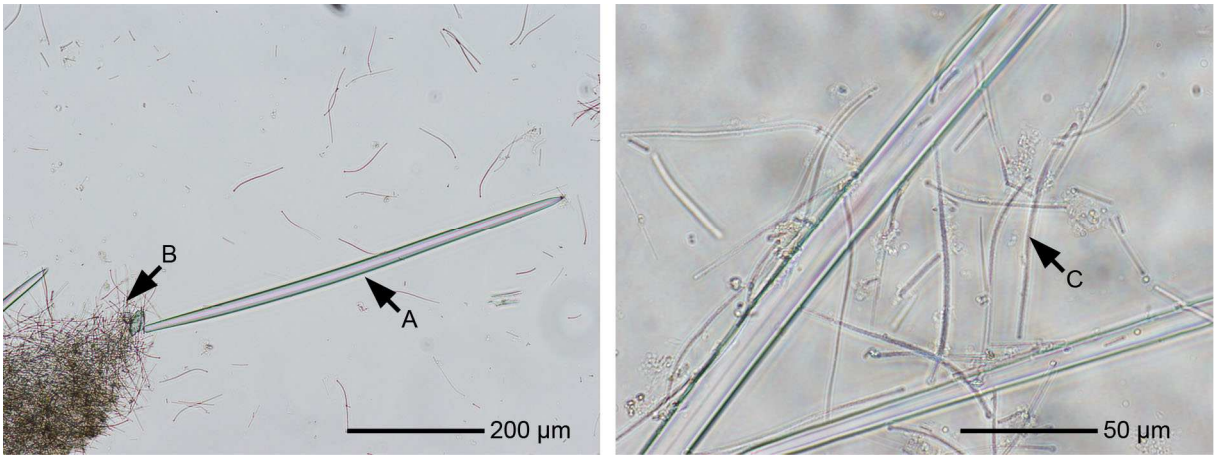

Figure C.3. Microscopy pictures of *Asbestopluma furcata* lower stem at 100 and 400X. (A) Style, (B, C) tylostrongyla.

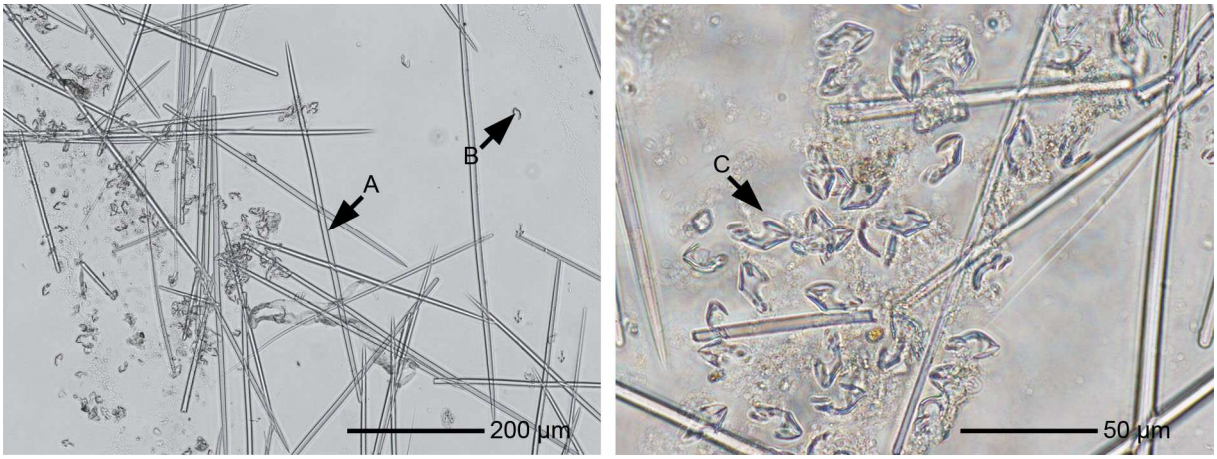

Figure C.4. Microscopy pictures of *Asbestopluma infundibulum* at 100 and 400X. (A) Style, (B, C) palmate anisochelae.

Figure C.5. Microscopy pictures of *Asbestopluma lycopodium* at 100 and 400X. (A) Styli, (B, C) palmate anisochelae, (D) forceps.

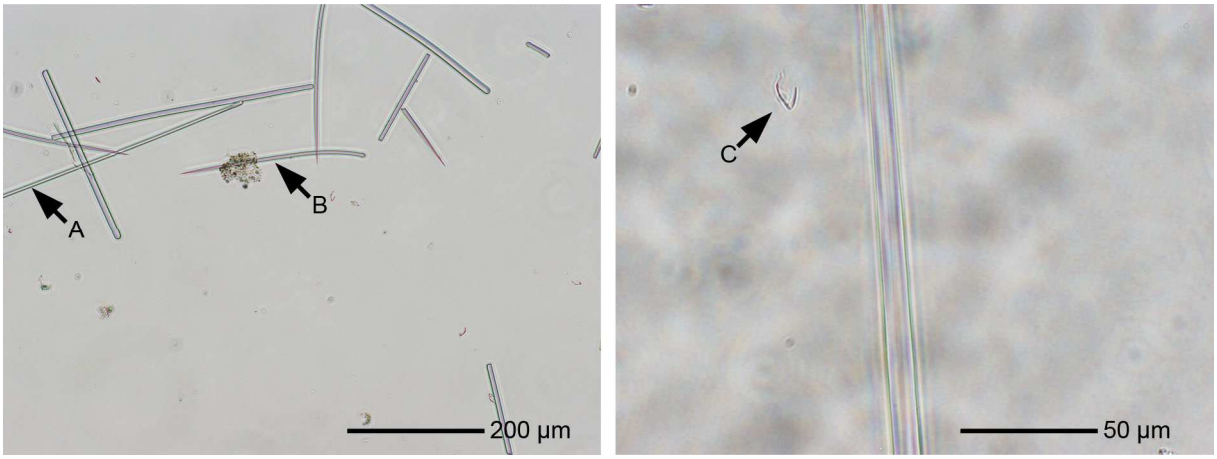

Figure C.6. Microscopy pictures of *Asbestopluma* sp. 1 aff. *lycopodium* at 100 and 400X. (A) Style, (B) subtylostyle, (C) palmate anisochela.

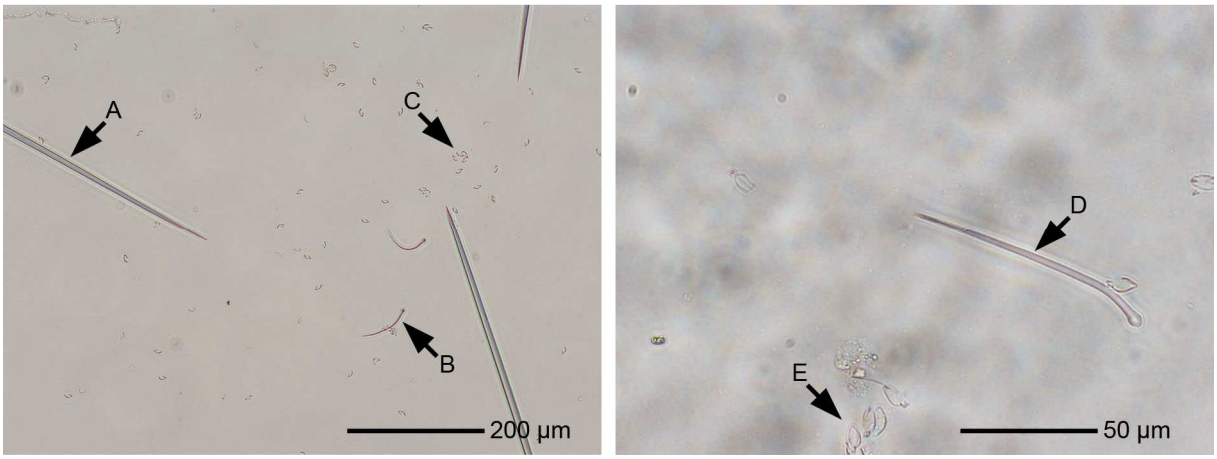

Figure C.7. Microscopy pictures of *Asbestopluma* sp. 2 aff. *lycopodium* at 100 and 400X. (A) Style, (B, D) short subtylostyli, (C, E) palmate anisochelae.

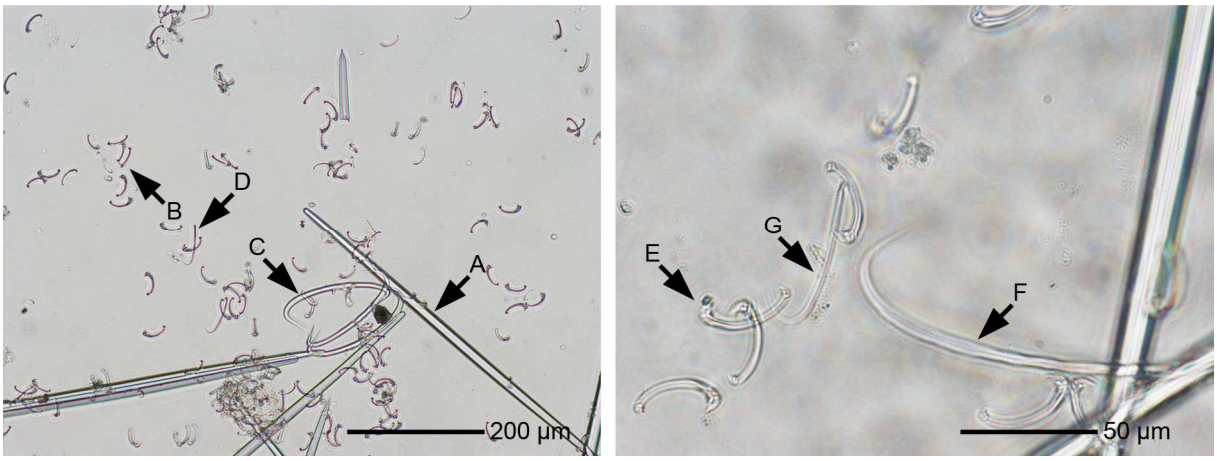

Figure C.8. Microscopy pictures of *Cladorhiza corticocancellata* at 100 and 400X. (A) Style, (B, E) anchorate anisochelae, (C, F) large sigmata, (D, G) small sigmata.

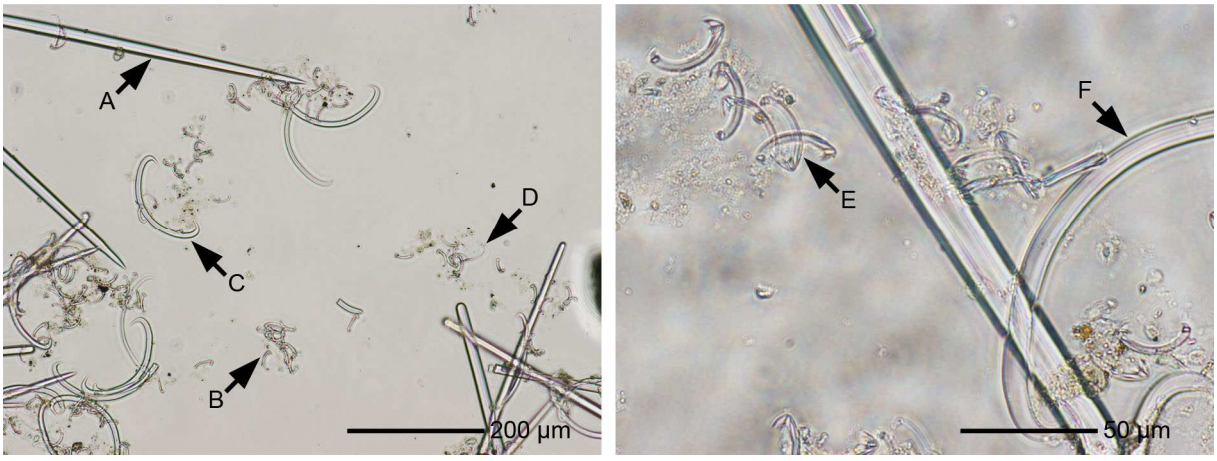

Figure C.9. Microscopy pictures of *Cladorhiza gelida* at 100 and 400X. (A) Style, (B, E) anchorate anisochelae, (C, F) large sigmata, (D) small sigma.

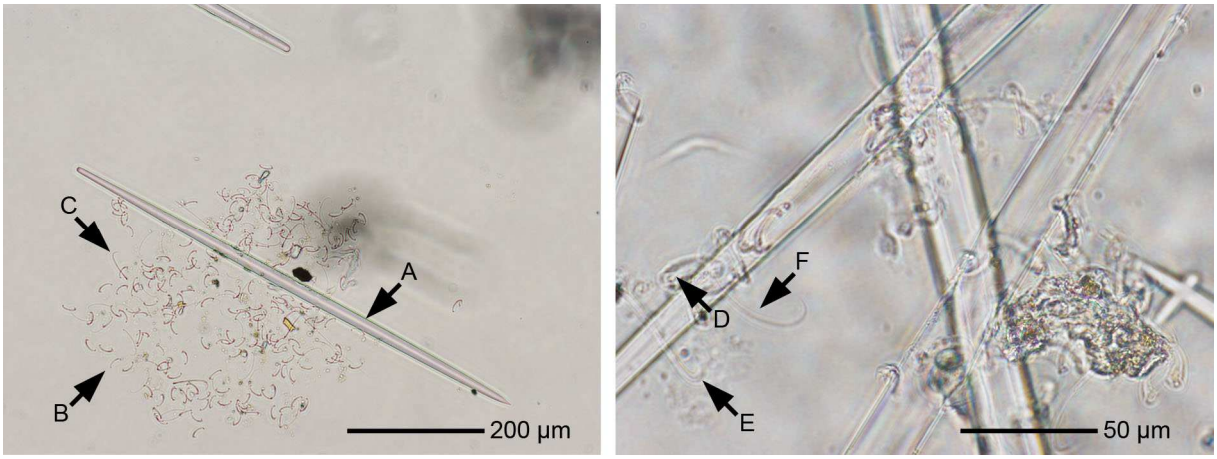

Figure C.10. Microscopy pictures of *Cladorhiza tenuisigma* at 100 and 400X. (A) Style, (B, D) anchorate anisochelae, (C, E) large sigmata, (F) small sigma.

Appendix D: List of species sequences from GenBank

GenBank sequence species	GenBank accession number
COI	
Artemisina melana	EF519575.1
Biemna fistulosa	AM076982.1
Clathria oxeota	EF519605.1
Clathria schoenus	EF519607.1
Crambe crambe	AF526297.1
Diacarnus spinipoculum	AY561975.1
Eurypon cf. *clavatum*	AJ843893.1
*Geodia (Sidonops) neptuni**	NC_006990.1
Holopsamma helwigi	EF519627.1
Iotrochota birotulata	AY561963.1
Lissodendoryx isodictyalis	EF519638.1
Lissodendoryx sigmata	EF519643.1
Microciona prolifera	AJ704978.1
Monanchora arbuscula	EF519645.1
Mycale fibrexilis	AJ843890.1
Mycale laxissima	EF519649.1
Negombata magnifica	AM076981.1
Neofibularia nolitangere	EF519653.1
Pandaros acanthifolium	EF519662.1
Suberites aurantiacus	EF519681.1
Strongylacidon bermudae	AJ843889.1
Tedania ignis	AJ704976.1
Tedania klausi	DQ133898.1
Tethya actinia	NC_006991.1
ITS	
Crambe crambe	AY319396.1
Eurypon cf. *clavatum*	AJ621547.1
Microciona prolifera	AJ705047.1
Mycale fibrexilis	AJ627185.1
Suberites ficus	AJ627184.1
Tedania ignis	AJ704975.1
Tethya sp.	AJ633835.1

*Wrongly identified as *Geodia* instead of *Sidonops* in GenBank.