Microproellidae phylogeny and evolution

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Acknowledgements

Abstract

Bryozoans are marine invertebrates, who are well suited for investigating evolutionary questions as they have a rich fossil record. Despite this, phylogenetic studies of this group are very limited and many questions regarding interrelations are yet to be explored. The focus of this study has been on exploring phylogenetic relations within the cheilostome family Microproellidae. This family is species rich and morphologically diverse, but little is known about their phylogenetic relationships.

Here I present the most comprehensive phylogeny of Microporellidae to date, as well as the nearly complete mitochondrial genome of *Microporella cf. neocribroides*. The phylogeny is based on six genes, both nuclear and mitochondrial, and includes eleven previously unsampled species. This study finds that Microporellidae is polyphyletic, while *Microporella* and *Fenestrulina* are two clear but separate groups*. Calloporina* is likely closely related to *Fenestrulina*, but increased taxonomic sampling is needed to determine this. The results of this thesis illustrate the challenges of inferring evolutionary relationships within bryozoans based on morphological characters. Furthermore, it adds to the accumulating body of evidence for the high rate of convergent evolution within bryozoans. The partial mitochondrial genome presented in this study reveals a unique gene order in *Microporella*, when compared to other bryozoans. This adds to the mitochondrial story of bryozoans, where extensive mitogenome rearrangements are present in each sampled lineage.

Introduction

Bryozoa is an invertebrate phylum where phylogenetic relationships and evolutionary histories within and between clades are poorly known. To introduce this thesis, I first give a general review of what is known about the evolutionary history of this clade and argue why improved bryozoan phylogenetic hypotheses are needed. I then lay out the aims of my thesis, which is to increase the taxonomic sampling of cheilostomes, the largest extant order, in a phylogenetic framework. This is done through focusing on the family Microporellidae, which is rich in morphological traits as well as species and has a good fossil record, making it an ideal group for testing evolutionary hypotheses.

Bryozoa – a brief overview

Bryozoans are colonial invertebrates that inhabit aquatic, predominantly marine, environments. The colonies exhibit a range of growth forms from erect to free-living, but most encrust benthic substrates (McKinney & Jackson, 1989). Each colony is organized into modular units called zooids, which are the clones of the original zooid (the ancestrula), a metamorphosed sexually produced larvae (Ryland, 1970). The phylum contains close to 6000 described living (Bock & Gordon, 2013) and 15 000 described fossil species (Gordon et al. 2009), although the true diversity is likely to be much higher (Gordon, 2014).

Bryozoans maintain an extensive fossil record, with first occurrences in the Ordovician. The oldest known fossils are found in late Tremadocian (c. 480 million years ago, Myr) outcrops in China and all belong to extinct stenolaemate orders (Xia et al., 2007). Fossil bryozoan faunas were heavily dominated by stenolaemates up until the Cretaceous, but the end-Permian extinction event resulted in the loss of six of seven stenolaemates orders by the Jurassic (Jablonski et al., 1997). Cyclostomes, the sole stenolaemate survivors, experienced a radiation during the Jurassic, but was overshadowed by the major radiation of the gymnolaemate order, Cheilostomata (Jablonski et al., 1997), which constitute over 80 % of the extant bryozoan species (Bock & Gordon, 2013). Cheilostomes appeared in the late Jurassic (Taylor, 1981), but it would take another approximately 50 Ma before the order rapidly diversified (Jablonski et al., 1997; Taylor & Waeschenbach, 2015).

The cheilostome radiation saw the onset of many novel zooidal level phenotypic features (Jablonski et al., 1997), making bryozoans one of the most polymorphic colonial phyla (Harvell, 1994). The highly modular organization allows zooids to take on different roles within the colony (Lidgard et al., 2012). Examples of such polymorphs are autozooids which are the main feeding zooids, avicularia, which are mainly thought to be involved in defending the colony against predators (Carter et al., 2010; Winston, 1986) and ovicells, which serve as brooding chambers (Ostrovsky, 2004). Polymorphs are thought to have played a vital role in the cheilostome radiation and there are two main hypotheses. The first is that the radiation was driven by the evolution of a new larval type, e.g. the lecithotrophic larvae (Taylor, 1988) or, alternatively that it was driven by the evolution of defensive structures (Gordon & Voigt, 1996). Note that these are not mutually exclusive hypotheses but that the relative importance of such traits in the success of this order is yet to be tested in a comparative phylogenetic framework.

Microporellidae – a species rich and highly polymorphic family

My study aims to contribute to our understanding of cheilostome evolution by focussing on the phylogeny of the family Microporellidae (Hincks, 1879). This family belongs to the Schizoporelloidea superfamily within the suborder Neocheilostomina. Herein, Microporellidae is particularly specious (Fig. 1) and has several features that makes it a particularly interesting study group: the group is rich in morphological characters, has a good fossil record and a cosmopolitan distribution today (Fig. 2).
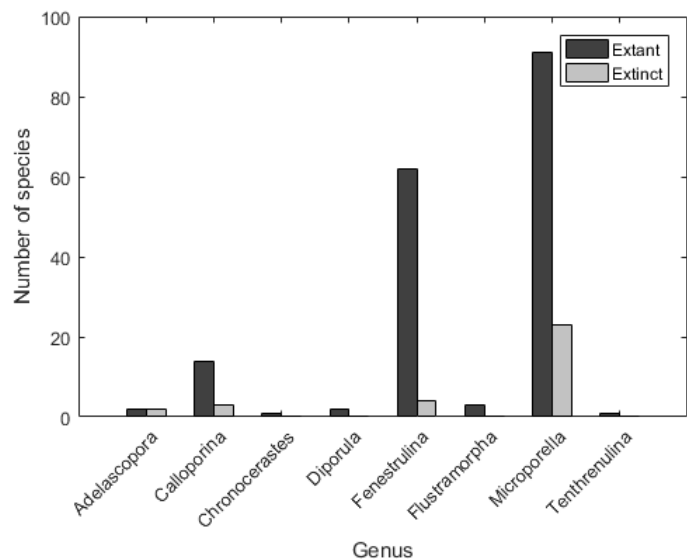
Figure 1 Species richness within genera of Microporellidae. Here it is evident that the three genera *Microporella*, *Fenestrulina* and *Calloporina* are dominant both in terms of extant and extinct species. Red bars represent the described extant species, while the blue bars show described extinct species.

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Microporellids are characterized by a lepralioid frontal shield and consist of 177 described species and eight extant genera (Bock & Gordon, 2013). Species richness among the different genera is highly disparate, where *Microporella, Fenestrulina* and *Calloporina* include over ninety percent of all described species, either extant or extinct (Fig.1). The first appearances of microporellids in the fossil record dates back to the Oligocene (Brown, 1952; Taylor & Mawatri, 2004), although the specious genera *Microporella* and *Fenestrulina* were first recorded in the basal Miocene (Brown, 1952; Guha & Gopikrishna, 2007). By the end of the Miocene, both *Microporella* and *Fenestrulina* had gained a global distribution (Taylor & Mawatri, 2004), which they maintain today (Fig. 2). The smaller genera have more limited distributions, for example *Tenthrenulina* and *Chronocerastes* are endemic to New Zealand, *Adelascopora* is found in and around the Antarctic and *Flustramorpha* is recorded from South Africa. These small ranges impact the ease of getting samples from these species.
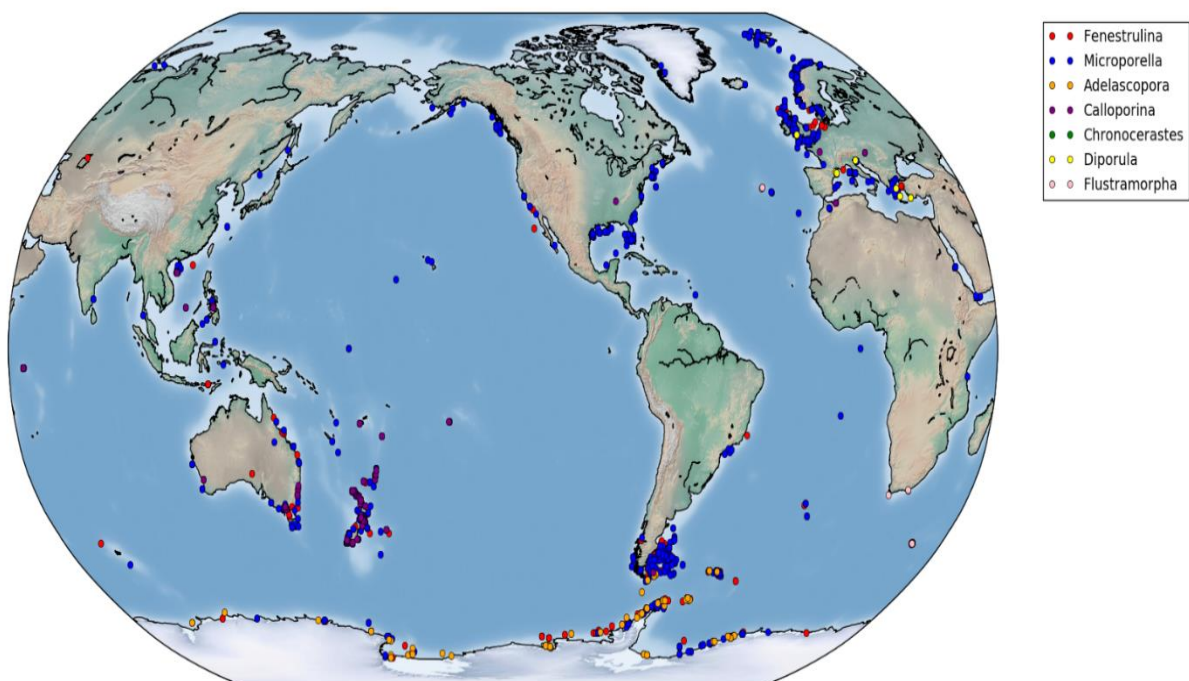
Figure 2 Global distribution of Microporellidae. The figure shows the global distribution of Microporellidae, based on occurrences registered in the Global Biodiversity Information Facility (GBIF) database. Each genus is represented by its own colour, as shown in the legend. This map does not reflect the true distribution of genera, but rather the registered occurrences which may be biased. No *Tenthrenulina* occurrences were registered in GBIF. The map was made using the basemap package in python.

The distinguishing morphological characteristics of Microporellidae is the semicircular primary orifice with a proximal ascopore (Gordon, 1984) (Fig. 3). The orifice relates to the opening in the zooid from which the lophophore is extruded. The ascopore is an opening to the ascus, which in turn is a sac beneath the frontal shield that fills with water as the lophophore is protruded. In most other ascophoran bryozoans the opening to the ascus is directly related to the orifice, while in Microporellidae, as well as Haplopomidae and Calwelliidae, the opening has been separated from the orifice, over evolutionary time, to form the ascopore. This is a highly diagnostic character, on a species level, with variations in shape, size and location relative to the orifice (Fig. 3 and 5). In figure 3, specimens from *Calloporina, Microporella* and *Fenestrulina* exemplify the main morphological characters that are indicative of each genus. Both Microporella and Calloporina have adventitious avicularia, which are avicularia found on the surface of a zooid, while *Fenestrulina* do not have avicularia. *Microporella* is easily distinguishable from the other two by its granular frontal wall, with many simple pores. In *Fenestrulina*, the frontal wall is smooth and has larger, more complex pores – often around the zooid margin. *Calloporina* also has a smooth frontal wall, with large marginal (areolar) pores. Calloporina zooids are often distinguished by being slightly hexagonal in shape. All genera have ovicells and oral spines, although these may not be present in all species.
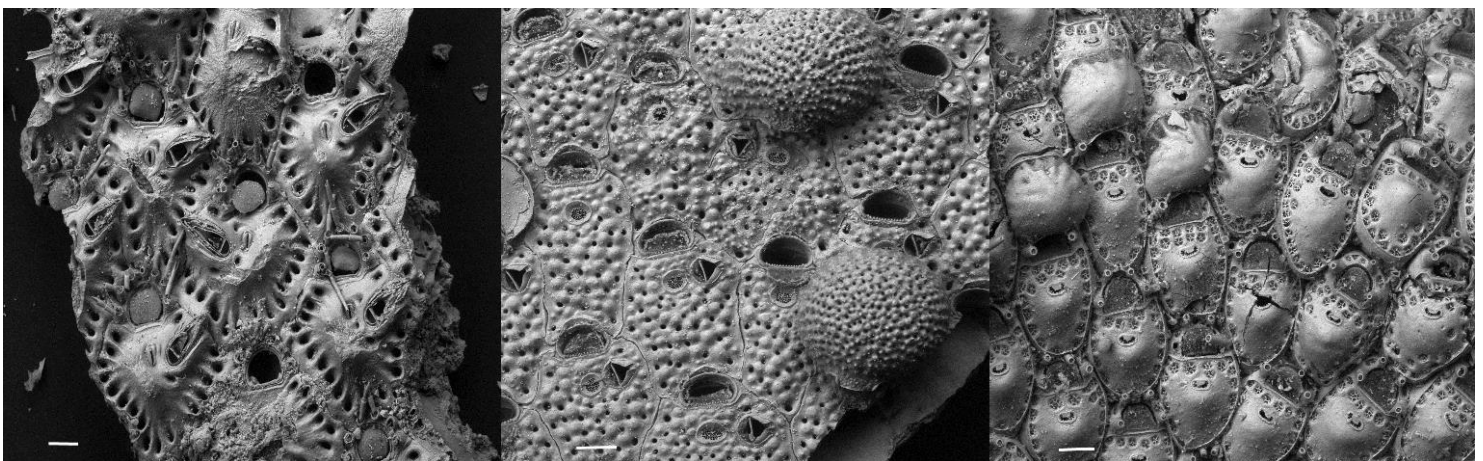
Figure 3 Microporellidae colonies. The figure shows representatives of the three genera; *Calloporina, Microporella* and *Fenestrulina*. From the left: *Calloporina angustipora, Microporella ordo, Fenestrulina cf. orientalis.* The image reflects some differences between the genera, for instance the granular frontal wall of *Microporella*, the complex pores of *Fenestrulina* and large marginal pores of *Calloporina*. Image credit: Dr. Paul Taylor, NHMUK. Scale bars: 100 μm, magnification: 125x.

Current state of phylogenetic relationships

The high morphological diversity within cheilostomes, including Microporellidae, contains major new innovations that have likely evolved several times. For example, the avicularia, which is one of the most extreme forms of polymorphic zooids (Winston, 1986), has evolved independently several times (Cheetham et al., 2006; Lidgard et al., 2012), likewise for the ovicell and lecithotrophic larva (Waeschenbach et al., 2012; Ostrovsky, 2013). The prevalence of such convergent traits highlight the difficulties of inferring evolutionary relationships between taxa based solely on morphology. Molecular phylogenetics has its strength in that it provides evolutionary informative characters that are independent of the morphological traits under study. Indeed, a recent cyclostome phylogeny found that the morphological characters previously used for classification were not supported, leading to a re-evaluation of informative traits (Taylor et al., 2015). Therefore, while the morphological diversity within this group makes it an interesting case study of trait innovation and trait evolution, the morphological characters themselves may not always be the optimal data when the goal is to infer phylogenetic relationships.

McKinney and Jackson (1989) emphasized the importance of using molecular data in resolving evolutionary relations between bryozoans. Since then several molecular phylogenies have appeared, focusing on different taxonomic levels. Some have explored large scale interrelations within the phylum (Fuchs et al., 2009; Knight et al., 2011; Waeschenbach et al., 2012). On lower taxonomic levels, some have focused on specific families and genera (Jagadeeshan & O'Dea, 2012; Fehlauer-Ale et al., 2015), or populations (Dick et al., 2003; Fehlauer-Ale et al., 2014), and some have focused on suspected cryptic species complexes (Gómez et al., 2007; Nikulina et al., 2007; Waeschenbach et al., 2012). These studies have contributed greatly to our knowledge of this group. However, they are few and only represent a small fraction of the known diversity, making bryozoans an under-sampled phylum compared to most other metazoan phyla. Robust and accurate phylogenies are not only important for understanding the relationships between species, they are vital tools in testing evolutionary hypothesis (Felsenstein, 1985).

Not much is known about the interrelations within microporellids. To the best of my knowledge, only one phylogenetic reconstruction of the family has been attempted, a genus

level cladistic analysis based on 37 morphological characters and was conducted by Taylor & Mawatri (2004). In a molecular phylogenetic framework, microporellids are not well sampled, especially considering the size of this family. To date there are sequences available for 5 Microporellidae species; *Microporella ciliata* (*18S, 16S, cox1, cytb*), *Microporella agonistes* (*28S, 18S, 16S, cox1, EF-1alpha*), *Fenestrulina malusii* (*cox1*), *Fenestrulina orientalis* (*18S*) and *Calloporina angustipora* (*28S, 18S, 16S, cox1, EF-1alpha*). However, no phylogenetic analysis has yet included all of these species.

The use of whole mitochondrial genomes has become increasingly popular in inferring evolutionary relationships, particularly in metazoans as they are small and conserved in size (Saccone et al., 2002). Mitgenomes have been useful in resolving relationships among closely related species (Yu et al., 2007; Vilstrup et al., 2011). To date, 8 bryozoan mitochondrial genomes have been published, including one cyclostome (Sun et al. 2011), one ctenostome (Waeschenbach et al., 2006) and otherwise cheilostomes (Jang & Hwang, 2009; Sun et al., 2009; Nesnidal et al., 2011; Waeschenbach et al., 2012; Shen et al., 2012). A further 8 mitochondrial genomes have been successfully sequenced, including *Microporella ciliata*, but are not yet published (pers. comm. Waeschenbach 2016). These have revealed a high rate of gene order rearrangements, comparable to that of molluscs, brachiopods and some annelids (Saccone et al., 2002; Gissi et al., 2008; Ghiselli et al., 2013; Weigert et al., 2016).

The aim of this study is to increase taxonomic sampling of bryozoans in molecular phylogenetics, through targeting the specious and morphologically diverse family Microporellidae, which also has a rich fossil record. Providing a phylogeny is a necessary step in developing this family into a model system for studying trait evolution in bryozoans. The monophyly of Microporellidae and its genera, including their within-family phylogenetic relationships, are discussed. I also aim to add to the mitochondrial story of bryozoans, as these small genomes may prove useful in robust phylogenetic hypothesis.

Materials and Methods

Samples and DNA extractions

The bryozoan samples sequenced in this study were collected at various sites across the world, the details of which are presented in table 1. All samples have been stored on high percentage ethanol in anywhere from a few weeks to 8 years (see table 1). Most colonies were attached to rocks or shells and were scraped off prior to DNA isolation. In some cases colonies of target species grew in close association with other bryozoan species and had to be discarded due to the risk of contamination. Samples that were not successfully isolated and/or amplified, along with details as to why can be found in Appendix 1 (App. Table 1).

Table 1. List of samples and sampling location of species used in this study. All the table were successfully samples isolated and sequenced. The sample id refers to the molecular identification name*. Sampling details include location, the collector and collections dates. Country abbrevations: NZ = New Zealand, SK = South Korea.

Species	Sample ID	Country	Location	Sampling date	Collector
Microporella sp.	AW520	NZ	-46.43; 166.08	20.01.2009	Abigail Smith
Microporella speculum	AW683	NZ	Greta Point, Wellington	11.01.2008	Smith & Porter
Microporella cf discors	LHL5	NZ	-45.52; 171.12	23.01.2010	Abigail Smith
Microporella cf agonistes	LHL11	NZ	-45.84; 170.89	24.01.2010	Abigail Smith
Microporella cf discors	LHL37	NZ	-34.7; 173.31	13.07.2009	Abigail Smith
Microporella ordo	LHL33	NZ	-34.4; 173.03	15.07.2009	Abigail Smith
Microporella cf. neocribroides 1	LHL15	China	Qingdao	20.08.2015	Kamil Zágoršek
Microporella cf. neocribroides 2	LHL25	China	Qingdao	16.09.2015	Kamil Zágoršek
Microporella cf. neocribroides 3	LHL27	China	Qingdao	16.09.2015	Kamil Zágoršek
Microporella cf. neocribroides 4	LHL29	SK	36; 126	24.10.2015	Joungjin
Fenestrulina malusii N	AW218	Norway	Bergen	18.11.2008	Waeschenbach
Fenestrulina cf littoralis	AW423	NZ	Allans Beach, Otago Peninsula	26.02.2011	Waeschenbach
Fenestrulina specca	AW436	NZ	-46.68; 167.88	03.02.2008	Waeschenbach
Fenestrulina thyrephora	LHL40	NZ	-33.9; 171.7	28.03.2011	
Fenestrulina orientalis?	LHL28	China	Qingdao	16.09.2015	Kamil Zágoršek
Fenestrulina malusii O	LHL24	Orkneys	Scapa Flow	20.06.2015	Joanne Porter
Fenestrulina orientalis?	LHL18	China	Qingdao	20.08.2015	Kamil Zágoršek
Fenestrulina sp.	LHL30	SK	35; 126	10.07.2015	Joungjin
Calloporina angustipora	LHL32	NZ	-52.62; 169.32	23.03.2009	Dennis Gordon

*Sample IDs: LHL refers to samples prepared in the BLEED lab (abbreviation: Lee Hsiang Liow). AW are sample IDs from Andrea Waeschenbach.

Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's protocol, with the following modifications: fragments too small to remove from ethanol for drying, were instead rinsed in PBS buffer (3x) to get rid of the ethanol prior to the extraction. Colonies were homogenized, within the lysis buffer, using a pestle.

SEM Imaging

Morphological vouchers were saved for all samples prepared for this study and used for Scanning Electron Microscopy (SEM) imaging. SEM images of samples labelled AW had been taken beforehand by Andrea Waeschenbach. For the samples labelled LHL SEM images were taken by P.D. Taylor at the Natural History Museum in London. To retain as much information as possible, each colony was portrayed both unbleached and bleached, with the exception of colonies growing on soft tissue (algae) as this would dissolve. A voucher for LHL24 was not kept, as the colony only consisted of a few zooids and was too small, and it was identified in the field by J. Porter.

PCR

The genes *18S, 16S, 12S, cytb, cox1* and *cox3,* were amplified using previously published primers, listed in table 2. These genes were chosen as they have been shown to be informative in previous bryozoan phylogenetic studies, and thus they can be used together with published sequences. Two PCR polymerases were used for single gene amplification; DreamTaq (ThermoFisher) and Phusion High Fidelity (ThermoFisher).

For targeting mitochondrial genomes, I used the target and sequenced mitochondrial genes (as described above) to design new primers for long-range amplification. Some primers were designed based on multiple sequence alignments (16S1184 and 12S549), while the rest were species specific, and based on one gene (Table. 2). For the long-range amplification, primers were designed following the optimal criteria for New England Biolabs (NEB) LongAmp polymerase; melting temperature (Tm) range of 58-68 °C, length or nucleotide (nt) range of 20-40 nt and a GC range of 40-60 percent. Fragments that were successfully amplified with LongAmp were used as templated and sequenced by primer walking and primers were thus designed for this purpose, using the default settings in Primer3Plus (Untergasser et al., 2007). These coincide with optimal conditions for Sanger sequencing by GATC; Tm range of

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52-58°C, nt range of 17-20 nt and GC range of 40-60 percent. Primer properties, such as melting temperature and potential hairpin structures, were checked using oligocalc (Kibbe, 2007).

All PCRs, both single gene and long-range, were carried out in 25 µl reaction volumes with 3-5 µl template and 5 % DMSO, otherwise the manufacturer's protocols for the different polymerases were followed. Cycling conditions for the different polymerases and primer (Tm) can be found in the App. Table 2. PCR products were visualized by gel electrophoreses. Genes that were successfully amplified were purified using Wizard SV Gel and PCR Clean-Up kit (Promega), in a 15 µl (x2) volumes of nuclease free water. Prior to sequencing all purified PCR products were checked with a NanoDrop spectrometer (ThermoScientific) to evaluate whether they were inside the reference concentration for sequencing (10-80 ng/µl). Samples > 80 ng/µl were diluted and samples < 10 ng/µl were discarded. Sanger sequencing was performed by GATC Biotech in Germany.

Table 2 PCR primers used to successfully amplify sequences. Primers beginning with LA refers to long-range amplification and are designed specifically for this purpose. PW refers to primer walking, where these primers are designed for Sanger sequences. All primers designed during this study are marked as new.

Primer name	Direction (F, R)	Sequence 5' – 3'	Referance
Bryozoa_16SR	R	ARTCCAACATCGAGGT	Waeschenbach et al. (2012)
Bryozoa_16SF	F	TSKWCCYTGTGTATSATGG	Waeschenbach et al. (2012)
Bryozoa_12SF	F	TGCCAGCANHMGCGG	Waeschenbach et al. (2012)
Bryozoa_12SR	R	YTACTDTGTTACGACTTWTC	Waeschenbach et al. (2012)
Cheilo_12SF	F	AAAGAGCTTGGCGGT	Waeschenbach et al. (2012)
Cheilo_12SR	R	GACGGGCGATTTGT	Waeschenbach et al. (2012)
Cox1F_prifi	F	TTGRTTYTTTGGWCAYCCHGAAG	Waeschenbach et al. (2012)
Cox1R_prifi	R	TCHGARTAHCGNCGNGGTATHCC	Waeschenbach et al. (2012)
Bryozoa_cox3F	F	TGRTGACGAGAYGTNAYHCG	Waeschenbach et al. (2012)
Bryozoa_cox3R_M13F	R	GTAAAACGACGGCCAGACHACRTCWACRAAR TGTCA	Waeschenbach et al. (2012)
Bryozoa_cytbF_B	F	AGGDCAAATRTCWTWYTGRGC	Waeschenbach et al. (2012)
Bryozoa_cytbR	R	GGNAGAAARTAYCAYYCWGG	Waeschenbach et al. (2012)
Gymno300F	F	AAGGGCGCACTTATTAGG	Waeschenbach et al. (2012)
18p	R	TAATGATCCTTCCGCAGGTTCAC	Halanych et al. (1998)
Long-range PCR Primers			
16s_1184	F	CGACCTCGATGTTGGACTAAG	New
12s_549	R	CGCCAAGCTCTTTAGGTT TTAA	New
12S549	F	TTAAAACCTAAAGAGCTT GGCG	New

Primer name	Direction (F, R)	Sequence 5' – 3'	Referance
LA15-1-cytbF	F	CTTACATTACCTCCTCCCTCTCGTAAT	New
LA15-2-cox3R	R	CTAGTTTCTAAGTATTCAGACCCCTGGA	New
LA15-1-cytbR	R	ATTACGAGAGGGAGGAGGTAATGTAAG	New
LA15-1-cox3F	F	AGTACTACTATCCTCAGGAGTCACTGTAA	New
LA25-cytbF	F	CTCACTCGATTTTATGCCTTACATTACCTC	New
LA25-cytbR	R	GAGGTAATGTAAGGCATAAAATCGAGTGAG	New
LA25-cox3F	F	CTCCAGGGGTCTGAATACTTAGAAACTAG	New
LA25-cox3R	R	CTAGTTTCTAAGTATTCAGACCCCTGGAG	New
LA25-16SR	R	ATCTCTTCCTAACTCTTAGCTTATCCCAAG	New
LA11-cox3F	F	GTAATTTGTGACCACCTAAAGGAGTAAACC	New
LA11-cox3R	R	GGTTTACTCCTTTAGGTGGTCACAAATTAC	New
LA11-cytbF	F	ACACGATTTTATGCCCTCCACTATT	New
LA11-cytbR	R	AATAGTGGAGGGCATAAAATCGTGT	New
LA5-cox3F	F	ACTGTAACTTGAGCACATCACTCAATC	New
LA5-cox3R	R	GATTGAGTGATGTGCTCAAGTTACAGT	New
LA5-cytbF	F	CTGCACTATCGATTCTTCACATCATCTTT	New
LA5-cytbR	R	GAAAGATGATGTGAAGAATCGATAGTGCA	New
AW423-16SR	R	TAGGCTTCGTTGCTCCTTTC	New
AW423-12SF	F	TGGCGGCTTTTTACACTACC	New
LA520-cox3F	F	AGCCCATCATTCTCTACTCTCTATAAACCT	New
LA520-cox3R	R	AGGTTTATAGAGAGTAGAGAATGATGGGCT	New
LA520-cytbF	F	CTTAGCTATCGCTGCTCTATCACTCCTAC	New
LA520-cytbR	R	GTAGGAGTGATAGAGCAGCGATAGCTAAG	New
Sanger sequencing primers			
PW15-2cytbF	F	TTCAAAGCGAAGTATGGG	New
PW15-2cox3R	R	CAGTATTAGGGCTCCGGTA	New
PW15-cytbF-2	F	ACCAAGTACATCCCCCTTCC	New
PW15-cytbR-2	R	GCTACGTCCTACCCTGAGGA	New
PW15-cox3F-2	F	CTTGCAGAAACAAATCGTGC	New
PW15-cox3R-3	R	TTAAGGCCTTGGGGCACT	New
PW15-cox3F-3	F	ACTTCATATTGCCGGTGCTT	New
PW15-cox3F-4	F	ACTCTCCATTGCCCTGCTAG	New
PW15-cox3R-4	R	ATGCTGGCACAAAATTTTCC	New
PW15-cytbF-4	F	GGAAGGAACTCGGCAAAAA	New
PW15-cox3F-5	F	CAAAACGTTCACCCAGGTCT	New
PW15-cox3R-5	R	CGAGAGCCATTACCCAGAAA	New
PW15-cytbF-5	F	CTGTGAAGCTAGGCTCGGAC	New
PW15-cox3F-6	F	CTCGAATTATCCCTTTTTGGG	New
PW15-cox3R-6	R	AGGTGAATGATGGTTCCTGC	New
PW15-cytbF-6	F	CAGGATATGAGGGGCACTGT	New

Alignments and Phylogenetic inference

All sequences amplified in this study were queried using BLAST (*Basic Local Alignment Search Tool*) against NCBI, to check for similarities with known bryozoan markers. Sequences that did not hit bryozoans were discarded. Amplicons sequenced with both forward and reverse primers were concatenated using Mesquite version 3.0 (Maddison & Maddison, 2015). The protein coding genes (*cytb, cox1, cox3*) were translated into amino acids with blastx using the invertebrate mitochondrial code, such that translation was guided by the sequence information provided by NCBIs database. Amino acids were favoured for phylogenetic inference over nucleotides, due to the high rate of synonymous substitutions in the third codon and thus saturations. To be included in the dataset a criteria of minimum two genes per taxa was set, both for those generated from this study and sequences downloaded from NCBI. The taxa included in this study (from NCBI) were chosen based on their relatedness to Microporellidae, with a focus on Neocheilostomina species. One non-microporellid sequence was target and sequenced, *Haplopoma graniferum*, as it has previously been included in Microporellidae (Hayward and Ryland 1999) because of its ascopore. *Hornera foliacea*, *Crisia sigmoidea* (both cyclostomes) and *Alcyonidioides mytili* (ctenostome) were chosen as non-cheilostome outgroups. All sequence data used in this study is presented in App. Table 3

Alignments of the six genes were carried out separately in MAFFT version 7 (Katoh & Standley, 2013), using the E-INS-I algorithm with default settings. The alignments were thereafter evaluated and manually edited using Mesquite V3.0. The cox1 alignment was partitioned into two, as it was clear that two different regions of the gene had been amplified (due to different primer pairs). The dataset was refined using Gblocks v0.91b (Castresana, 2000), under the least stringent parameters. This programme detects and removes sites that lack phylogenetic signal, due to them being poorly aligned or too divergent.

To determine which model of sequence evolution fit the datasets, nucleotide alignments were run separately through jModelTest (Guindon & Gascuel, 2003; Darriba et al., 2012) and amino acid through ProtTest 3 (Darriba et al., 2011) to determine the best fitting model of

evolution (in both cases maximum likelihood (ML) was used). Both the Akiakes information criteria (AIC) and the Bayesian information criteria (BIC) indicated that the General Time Reversible model with a gamma distributed rate variation among sites (GTR+G) fitted the ribosomal genes best, while the MtArt+G model had the best fit for all three protein alignments. As the MtArt model is not implemented in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), the second most favoured model rtREV was used for ML and Bayesian analyses.

Refinements to the dataset were done by ML inference for each single gene alignment using RAxML v8 (Stamatakis, 2014). This was done on the same dataset that is used throughout the study, with the exception of the non-bryozoan taxa *Terebratalia transversa, Laqueus spp.* And *Phoronis spp.,* that were included as outgroups, to determine if there were any non-bryozoan sequences in the dataset (App. Table 3). The analyses was carried out with 50 topology searches and 100 bootstrap replicates, using the models specified above. The best tree and bootstrap values for each dataset were submitted to RoguNaRok (Aberer et al., 2013) to identify genes that jump between different positions in the tree topology. Rogues (including eventual gene paralogues and non-bryozoan sequences) that were discarded are indicated in App. Table 3.

When the final refinements were completed, a concatenated matrix was constructed in Mesquite. This supermatrix was analysed using both maximum likelihood and Bayesian approaches. The RAxML analysis was run, using 100 topology searches and 500 bootstrap replicates and previously defined evolutionary models (GTR and rtREV). An additional RAxML analysis was carried out, using the same conditions as previously mentioned, but with the MtART model instead of rtREV. The topological congruence between the two trees were calculated using the I_{cong} index (de Vienne et al., 2007), this was done to test the effect of using a sub optimal evolutionary model. Bayesian inferences were performed with MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using the same dataset and the same evolutionary models. Two independent runs both included two chains, one heated and one cold, and were run for forty million generations and sampled every thousand generations. The analysis ran until the average standard deviation of split frequencies reached 0.01. The first 2048500 generations were discarded as burn-in, as determined in

Tracer v1.6 (Rambaut & Drummond, 2013). Trees were summarized post burn-in and a majority rule consensus tree was inferred.

Mitochondrial genomes

The mitochondrial genome of *Microporella cf. neocribroides* (LHL15) was amplified with long range polymerase in two fragments; *cytb* forward – *cox3* reverse and *cytb* reverse – *cox3* forward. This was done using species specific primers, shown in table 2. Gel electrophoresis images showed approximate fragment sizes of 7 kb each. The two fragments were used as template for primer walking and all recovered sequences were assembled in Mesquite v3.0. Gene annotation was carried out in MITOS (Bernt et al., 2013) using the invertebrate mitochondrial code. Pairwise gene order comparisons of the 8 published bryozoan mitogenomes, *M. cf. neocribroides* and the hypothesized basal Lophotrochozoan gene order (Podsiadlowski et al., 2009; Bernt et al., 2013) were carried out in CREx (Bernt et al., 2007), computed as breakpoints . This was done using protein coding genes and rRNAs, and not the highly variable tRNAs (as several tRNAs were missing from *M. cf neocribroides*).

Results

Eighty-six new sequences from twenty samples were recovered in this study (Table 3), including eleven species that have not been sequenced before. Of these eleven species there are six species of *Microporella* and six *Fenestrulina* species.

Table 3. Successfully amplified genes during this study. The table shows the successfully amplified and sequenced genes from nineteen Microporellidae samples as well as *Haplopoma graniferum*. Eleven of the samples have not been sequenced before (indicated as new). All ticks represent successful genes.

genus	species	Sample nr	new	18S	16S	12S	cox1	cox3	cytb
Haplopoma	*graniferum*				✓	✓			
Calloporina	*angustipora*					✓		✓	✓
Fenestrulina	*cf. littoralis*		✓	✓	✓	✓		✓	✓
	specca		✓	✓	✓			✓	✓
	malusii O		✓	✓	✓	✓		✓	✓
	malusii N		✓	✓	✓			✓	
	cf. orientalis	1	✓	✓	✓	✓		✓	
	cf. orientalis	2	✓	✓	✓	✓		✓	
	sp.		✓	✓	✓	✓		✓	
	thyrephora		✓	✓	✓			✓	✓
Microporella	*sp.*		✓	✓	✓	✓	✓	✓	✓
	speculum		✓	✓	✓	✓			✓
	*cf. discors1**		✓	✓	✓	✓	✓	✓	✓
	cf. agonistes			✓	✓	✓	✓	✓	✓
	cf. neocribroides	1	✓	✓	✓	✓	✓	✓	✓
	cf. neocribroides	2	✓	✓	✓	✓		✓	✓
	cf. neocribroides	3	✓	✓	✓	✓		✓	
	cf. neocribroides	4	✓	✓	✓	✓		✓	
	ordo		✓		✓	✓		✓	✓
	*cf. discors2**		✓		✓	✓		✓	

* There are two samples labelled *Microporella cf. discors* in this study, however these are not the same species, see appendix 2 for a further discussion.

Phylogenetic inference

The multigene dataset used for phylogenetic inference in this study included 51 taxa, 6 genes and was 3060 bp long. These were based on a combination of new sequences (from this study) and vetted sequences from genbank (fig. 4, see app tab 3 for full dataset). I also present ML (RAxML) inferences for single gene datasets and the concatenated dataset based

on the rtREV model and the MtArt model (see methods; Alignments and Phylogenetic inference) to test the effect of using a sub optimal model (App. Fig. 1-7). Statistical support for nodes, based on posterior probability (PP) and bootstrap percentage (BP), are defined as follows: full support 1 PP/100 BP, high support >90 BP (99 PP), moderate support >65 (>95 PP), low support >50 BP (>90PP) and anything under this is not supported.

This phylogeny supports the suborder Neocheilostomina (d'Hondt, 1903) and herein there is high to moderate support (0.99 PP/89 BP) for the separation of two clades, one including Hippotoomorpha and Flustrina (clade 1, fig. 4) and the other including Lepraliamorpha and Umonulomorpha (clade 2, fig. 4). Within clade 1 there is strong support for a Hippothoomorpha group consisting of *Celleporella, Haplopoma* and *Antarctothoa*. *Chorizopora*, another Hippothoomorph, is embedded within Flustrina. Clade 2 includes the three superfamilies Lepralielloidea (*Umbonula, Exochella, Celleporaria, Escharoides, Escharella*), Schizoporelloidea (*Microporella, Fenestrulina, Calloporina, Schizoporella, Cryptosula*) and Smittinoidea (*Watersipora, Smittina, Schizomavella*), all appear to be polyphyletic. *Microporella* and *Fenestrulina,* traditionally placed in the same family, are clearly separated into two different clades. There is strong support for *Microporella* as a monophyletic genus (0.99/92, where the first value is the Bayesian posterior probability and the second bootstrap value). *Fenestrulina* is polyphyletic, its species interspersed with *Calloporina, Escharoides* and *Escharella.* However this grouping has no support at all (0.72/-). The inferred topology suggests that, *Microporella* is sister group to a clade/group including *Watersipora, Umbonula, Crytopsula, Smittina*, *Schizomavella, Celleporaria* and *Schizoporella* (fig. 4), although this node is also not supported (0.72/-). The clade including *Fenestrulina*, *Calloporina, Escharoides* and *Escharella* is sister group to the previously mentioned groups. Note that the morphological grades Hippothooorpha, Flustrina, Umbonulamorpha and Lerpraliamorpha were all found to be polyphyletic. Several species are represented by multiple samples. The four samples of *M.* cf. *neocribroides* grouped together, indicating monophyly. In contrast, samples of *M. agonistes* and *M. discors* did not cluster as expected. *Fenestrulina malusii* from Norway and the Orkneys are strongly monophyletic (0.99/82) and consistent throughout trees. The two *F. orientalis* samples, believed to be the same species, do not cluster but group with *F. malusii* and with *F. cf. littoralis*.
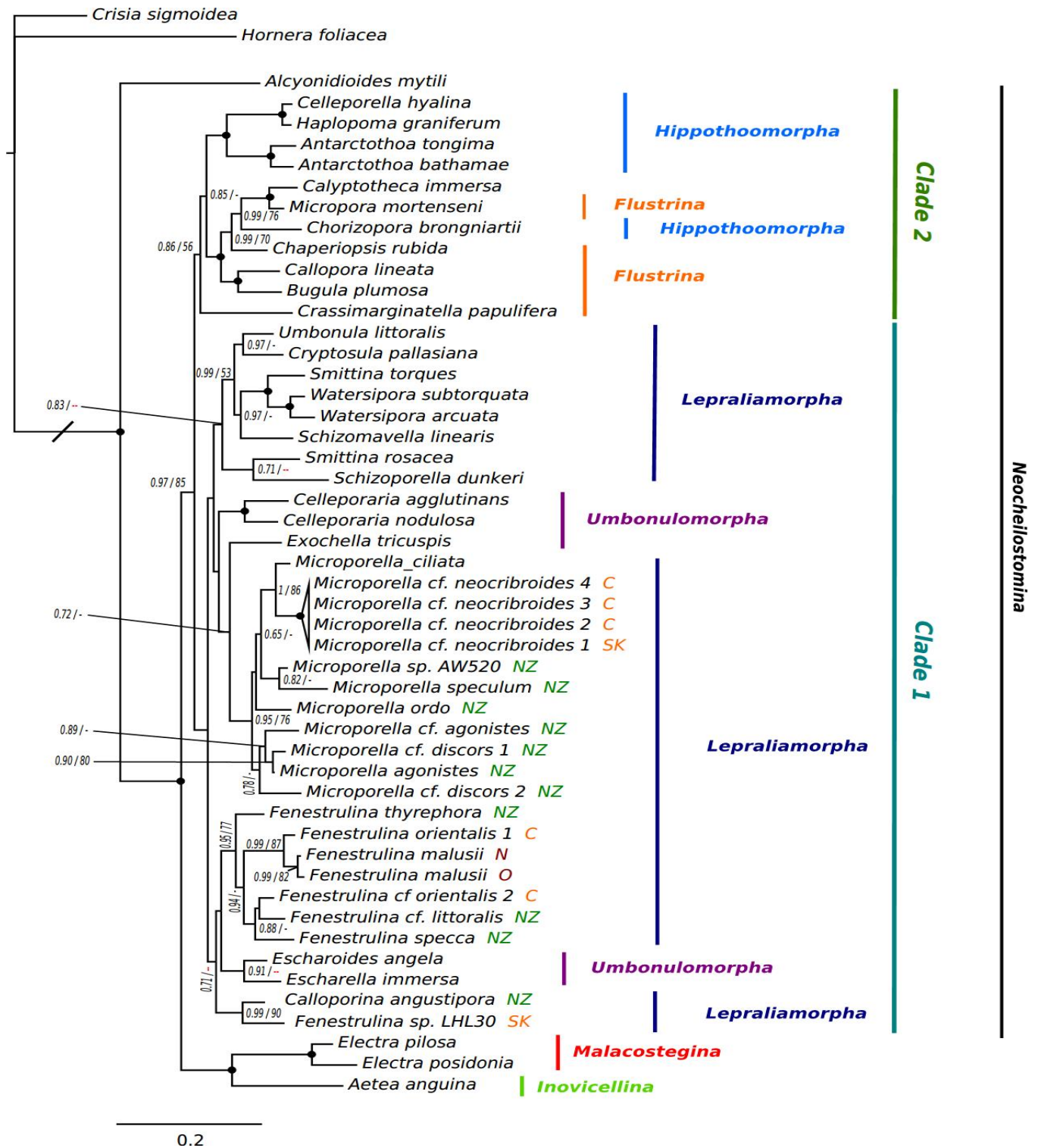
Figure 4. Phylogenetic inference of 51 taxa based on a six gene dataset (18S+16S+12S+cox1+cox3+cytb) including 3060 characters using the rtREV model of evolution. The topology is constructed from Bayesian inference using MrBayes. Node support values are shown as posterior probability/ bootstrap. Bootstrap values were generated under Maximum likelihood analysis of the same dataset using RAxML. Circles at nodes indicate a posterior probability of 1 and >95% bootstrap support. Nodes without numbers indicate support lower than 0.5 posterior probability and/or 50% bootstrap support. Red dashes indicate topological difference between Bayesian and ML trees. Scale bar indicates number of substitutions per site. The abbrevations behind species names refer to the collection site, NZ = New Zealand, SK = South Korea, C = China, N = Norway, O = Orkneys.

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Neocheilostomina, Malacostegina and Inovicellina are cheilostome suborders. Umbonulamorpha, Lepraliamorpha, Hippothoomorpha and Flustrina are grades.

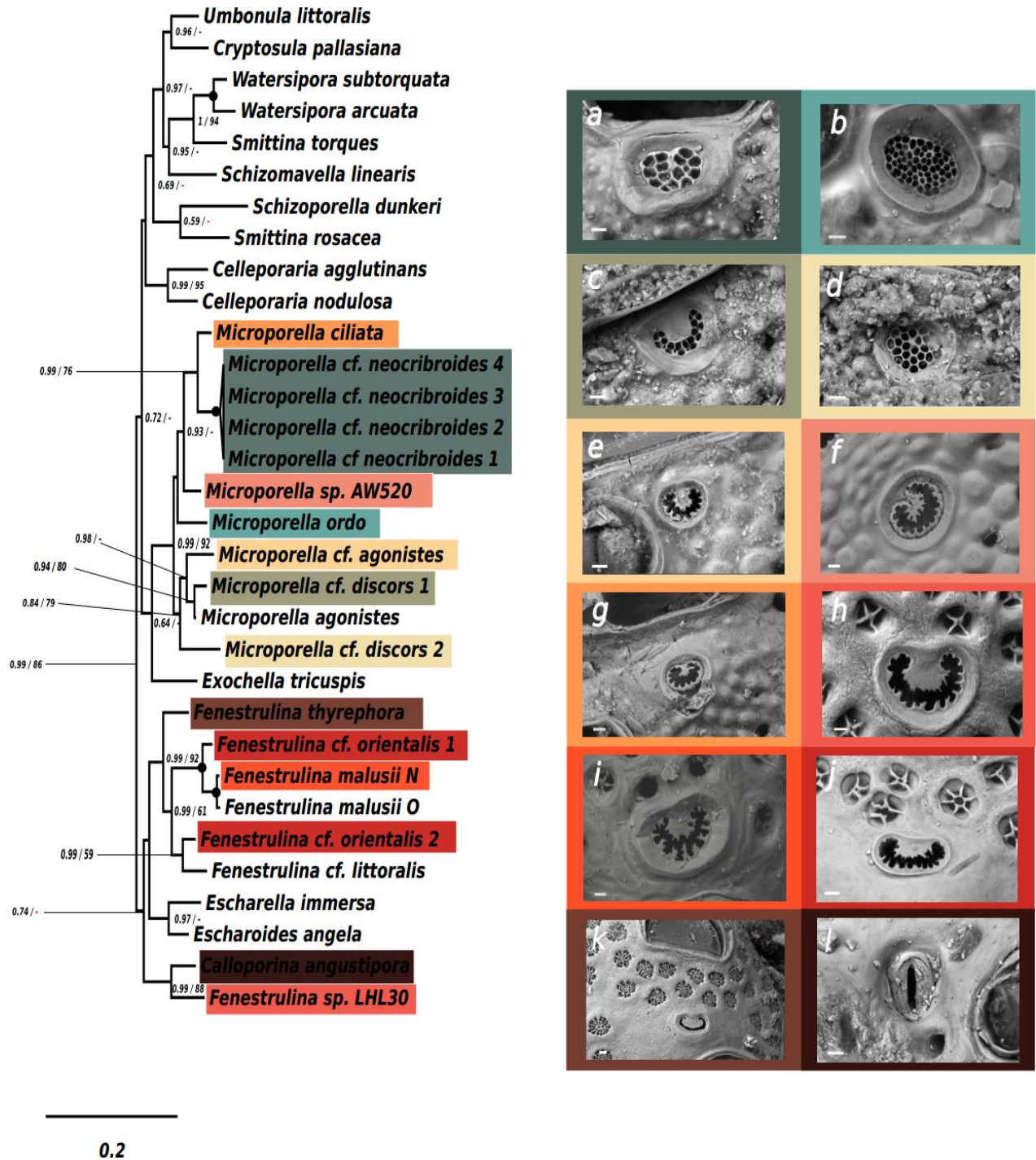**Figure 5. Variation in ascopore morphology among Microporellidae species.** The variation in ascopore morphology is shown together with a subset of the tree topology recovered in Figure 4. Ascropores can be circular (a, b, d), crescentic (c, e-k) or slit-like (l). The ascopore rim may be smooth (k, l) or denticulate (e-j), in some species, the ascopore has a porous plate (a-d). Scalebars: 10 μm.

There is no clear relationship between ascopore morphology and species relatedness (fig. 5), given that the inferred tree topology is true. For instance, the porous ascopore (fig 5a-d) may have evolved several times

Mitochondrial genome

The mitochondrial genome of *Microporella cf. neocribroides* (LHL15) was amplified and the close to fully sequenced, by primer walking. *M. cf. neocribroides* (LHL25) and *Microporella cf. discors* (LHL5) were successfully amplified but not yet sequenced, due to time constraints. The mitogenome of *M. cf. neocribroides* LHL15 was amplified in two non-overlapping fragments and the recovered sequences make up 12877 bp. Of the 13 expected protein coding genes only *nad2* is missing. Both expected rRNAs and 19 of the 22 expected tRNAs were recovered (fig. 6). All of the recovered genes are transcribed from the plus strand, with exception of trnV. The gene order of *M. cf. neocribroides* is the same as that found in *M. ciliata* (pers. comm. Waeschenbach, unpublished dataset). However, it differs from the gene orders of other bryozoans (fig. 7, tab 4). The pairwise distance matrix presented in table 4 shows the number of breakpoints in the gene order between each pair of species. Here a high score indicates dissimilarity in gene order and low scores similarities. There are no conserved blocks of genes across all bryozoans, however the block *cox1+atp8* is found in 7 of the 9 bryozoans (fig. 7). No blocks found across species exceed 4 genes, most contain 2 or 3 genes;

cytb+nad4l+nad4+nad5 (Lophotrochozoa, *F. foliacea* and *B. neritina*), *nad4+nad5+nad6* (*M. grandicella* and *F. foliacea*) and *nad4+nad6+nad5* (*C. hyalina* and *M. cf. neocribroides*) (fig. 7).

Table 4. Pairwise comparison of gene order breakages presented as a distance matrix.

The comparison of the gene orders of bryozoan mitochondrial genomes and the hypothesized Lophotrochozoan ground pattern, reveal highly divergent gene order patterns within this phylum – event to the exclusion of the highly variable tRNAs. Low scores indicate similarity in gene order, where a score of zero means that there has not been any breakages and the gene order is identical. A high

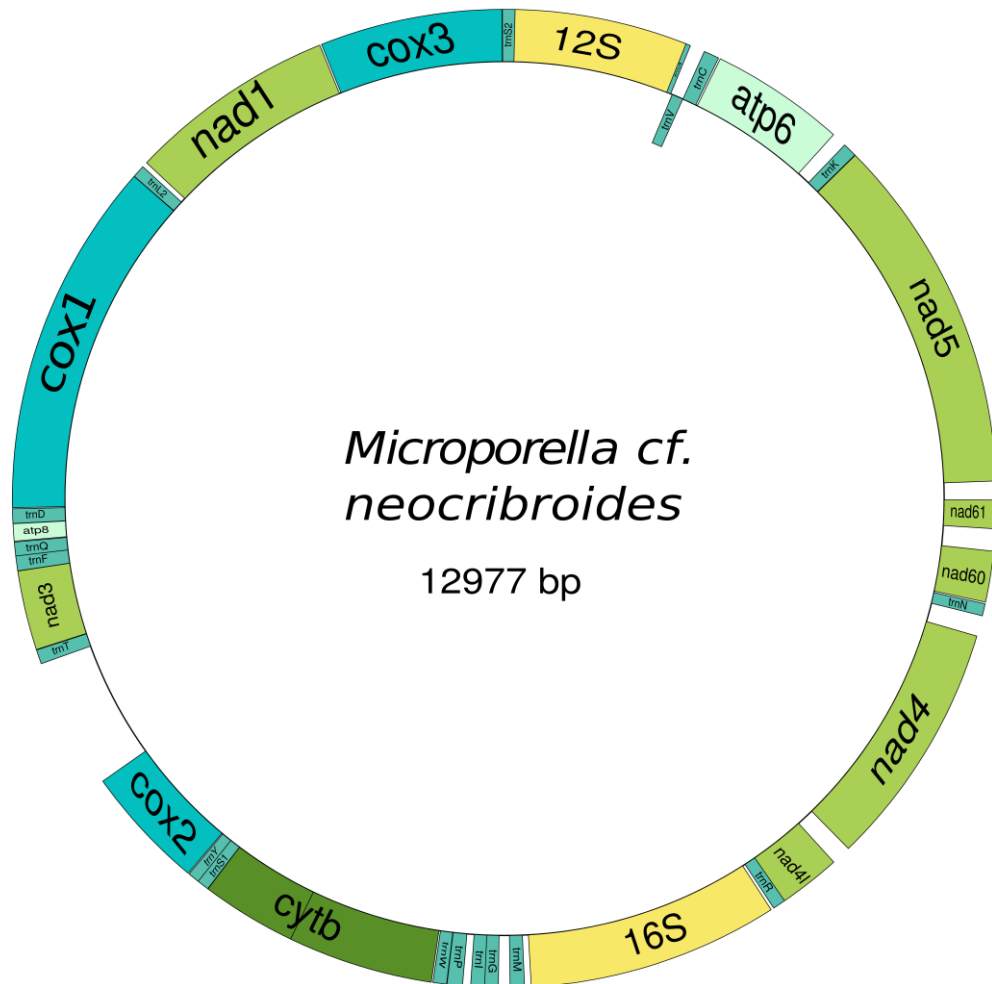|	L	Tf	Fh	Mg	Ch	Ff	Bn	Ws	M
Lophotrochozoa	0	12	13	14	14	12	9	14	12
Tubulinpora flabellaris		0	13	14	13	14	11	14	13
Flustrellidra hispidia			0	13	12	13	12	13	12
Membranipora grandicella				0	13	12	13	14	11
Celleporella hyalina					0	14	12	14	12
Flustra foliacea						0	12	12	11
Bugula neritina							0	12	10
Watersipora subtorquata								0	11
Microporella cf. neocribroides									0

Figure 6. Almost complete mitochondrial genome of *Microporella cf. neocribroides.* The recovered sequences from the nearly complete mitogenome include 12 of the 13 expected protein coding genes, missing only *nad2*. Both rRNAs and 19 of the 22 tRNAs are recovered. All genes are transcribed from the same strand, except for *trnV*.
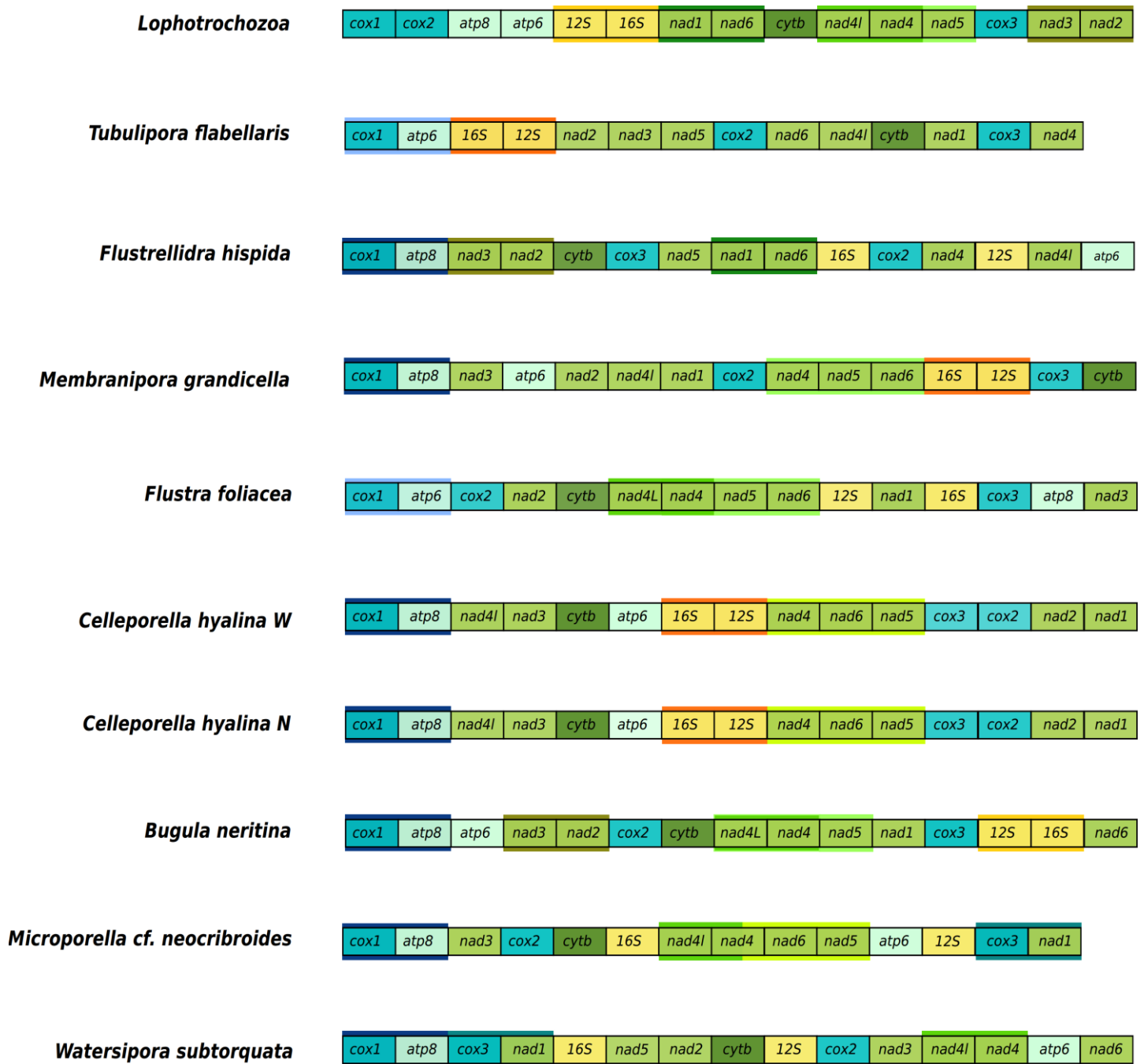
Figure 7. Mitochondrial gene order in bryozoans and the putative basal gene order of Lophotrochozoa. This comparison of bryozoan gene orders show a high degree of rearrangements within bryozoans and a divergencs from the putative Lophotrochozoan ground pattern. No large blocks of genes are shared among all bryozoans, and no conserved block exceeds four genes. From the present taxa, closely related species do not share more conserved blocks than they do with more distantly related taxa. The gene orders only include protein coding genes and rRNAs, not tRNAs. Genes are not to scale and information on reading direction is not included. Blocks of genes that are shared across species are colour coded. The gene orders of the different bryozoans are ordered according to relatedness, according to the phylogeny recovered by Waeschenbach et al. (2012).

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SEMs and species identifications

SEM images of the species used in this study have revealed the possibility of an undescribed species (*Microporella sp.* AW520, App. Figure 14), as well as some samples that resemble described species but vary in some characters. Images of all samples as well as comments on the samples that are not identified with certainty are presented in Appendix 2, this includes 14 samples (App. Fig. 8-21). Unfortunately the colony of *M. sp.* (AW520) does not have ovicells, which are need for a proper species description. *F. cf. littoralis* (App. Fig. 15), *M. cf. agonistes* (App. Fig. 10) and *M. cf. discors* (App. Fig. 8-9) show some variation from the type species, this may be due to intraspecific variation or complexes of sibling species.

Discussion

Interrelationships of Neocheilostomina

This study supports the monophyly of the suborder Neocheilostomina. This suborder includes most cheilostome taxa and reflects the bulk of cheilostome morphological diversity. It includes some anascans (taxa with membranous frontal-walls) and all ascophorans (taxa with calcified frontal shields). Within Neocheilostomina, my study recovered two clades, whose composition are in broad agreement with Waeschenbach et al. (2012) and Tsyganov-Bodounov et al. (2009), but not Knight et al. (2011). Clade 1 includes the anascan infraorder Flustrina and the morphological grade Hippothoomorpha, characterized by gymnocystal frontal shields. I inferred *Haplopoma graniferum* as a member of the Hippothoomorpha clade and note here that it also has a gymnocystal frontal shield, unlike *Microporella*, *Fenestrulina* and *Calloporina,* which are lepraliomorphs. *Haplopoma* was previously included in Microporellidae by Hayward & Ryland (1999), however the results of my study are more in agreement with the suggestions of Bock & Gordon (2013), who left *Haplopoma* out of *Microporellidae*. Clade 2 includes the two grades Lepraliomorpha and Umbonulpmorpha, found among the three superfamilies Lepralielloidea (e.g. *Umbonula, Exochella, Celleporaria, Escharoides, Escharella*), Schizoporelloidea (e.g. *Microporella, Fenestrulina, Calloporina, Schizoporella, Cryptosula*) and Smittinoidea (e.g. *Watersipora, Smittina, Schizomavella*). All superfamilies within clade 2 appear to be polyphyletic, and have been found so also in previous studies (Tsyganov-Bodounov et al., 2009; Knight et al., 2011; Waeschenbach et al., 2012).The consistent polyphyly of the above-mentioned superfamilies highlights the challenges of bryozoan systematics. It is clear that many more taxa, including representatives from the 23 Neocheilostomina superfamilies of which only seven were included here, must be sampled and sequenced to resolve intra. and inter-superfamilial relationships. The morphological grades (Umbonulamorpha, Lepraliamorpha, Hippothoomorpha) are unequivocally found to be polyphyletic in previous studies, and further supported by new data in my study. The tree presented in my study is broadly congruent with more recent phylogenies, which lends credence to the recovered topology.

Polyphyly of Microporellidae

Species assigned to Microporellidae share many morphological traits, such as the lepralioid frontal shield and the ascopore proximal to a semi-circular orifice. Despite this, there has been doubt among bryozoan systematists as to the legitimacy of this grouping. For instance Brown (1952) remarked "*Microporella* is not at all closely related to *Calloporina*", and Gordon has questioned the relationship between *Microporella* and *Fenestrulina* (Dennis Gordon pers. comm. 2015). These concerns have been confirmed by the phylogenetic inferences presented in this study. Microporellidae is not a monophyletic or even a paraphyletic clade. However the genera *Fenestrulina* and *Microporella* both constitute separate and highly supported clades/genera. *Fenestrulina* maybe paraphyletic, as suggested by the placement of *Fenestrulina sp.* (LHL30) + *Calloporina angustipora*, outside of the other samples of *Fenestrulina* examined in my study. However, the phylogenetic placement of this group is inconsistent and has little statistical support. Greater taxa-sampling among *Calloporina* will be needed to explore the phylogenetic position of *Calloporina* further.

Exochella tricuspis as sister to *Microporella* is consistent between Bayesian (Fig. 4) and maximum likelihood (Fig. A7) inferences, but received no statistical support in both cases. *E. tricuspis* is umbonuloid-shielded and belongs to the Lepralielloidae superfamily and does not resemble *Microporella* morphologically. The *Fenestrulina* clade is grouped with *Escharoides angela* and *Escharella immersa* which are in turn placed in the umbonuloid family Romancheinidae together with *Exochella tricuspis* (Bock & Gordons, 2013). The polyphyly of both umbonuloid and lepralioid taxa is by now uncontroversial as this has been found in several molecular phylogenies (Tsyganov-Bodounov et al., 2009; Knight et al., 2011; Waeschenbach et al., 2012). Nevertheless, whether the Romancheinidae genera should be split up as suggested by my results is more questionable. Not only do they differ from Microporellidae in frontal shield morphology, but also in ooecial morphology. According to Ostrovsky's (2013) classification of ooecial morphology, microporellids are microporelliform (although *Fenstrulina* seems to be a mix of microporelliform and lpralielliforme) and the Romancheinidae genera are escharelliform. Despite these differences, similar relationships to those found in this phylogeny have been recovered earlier. In Knight et al. (2011) *Exochellea tricuspis* was recovered as a sister group to *Microporella* and *Escharoides angela* clustered with *Calloporina anugustipora*. In Waeschenbach et al. (2012) *Microporella ciliata*

is closely related to *Escharoides coccinea* and *Escharella immersa*. This consistency across studies suggests that there may well be a close relationship between these genera. However, the various bryozoan analyses are based on overlapping datasets, in terms of both genes and taxa. Therefore, to understand if these unexpected relationships hold merit, more taxa (especially *Calloporina*, *Exochellea, Escharoides* and *Escharella* as well as other Romancheinidae genera) and genes should be included. Furthermore, there are three taxa in particular that should be included in future microporellid phylogenies as they resemble different microporellid genera, namely *Chiastosella,* Calwellidae and *Escharina*. Brown (1952) pointed out the similarities, and hence a likelihood of a close relationship, between the genus *Chiastosella* and *Calloporina.* Gordon (1984) has remarked on the similarities between Calwelliidae and Microporellidae and *Fenestrulina* in particular. Taylor & Mawatari (2004) remarked on Gordon's comment on the similarities between *Microporella and Escharina waiparaensis*, the latter now included in the newly erected genus *Taylorius* (Dennis P. Gordon 2014). Jullien (1888) erected the family Fenestrulinidae as he found *Fenestrulina* species to be sufficiently different from *Microporella* as to merit its own family. Whether or not this family should be reinstated is up for debate. However, before this question can receive proper consideration, a better understanding of the relationships among the taxa in the two superfamilies, Schizoporelloidea and Romancheinidae, is needed. Even more critically, the five remaining microporellid genera (*Diporula, Flustramorpha, Tenthrenulina, Adelascopora* and *Chronocerastes*) should be included in future phylogenetic analyses. This, however, will be challenging as they are rare and more even more difficult to sample. Gordon (1984) remarked that *Tenthrenulina* is likely closely related to *Fenestrulina,* however the cladistics analysis by Taylor and Mawatari (2004) find *Tenthrenulina* more closely related to *Microporella*. Based on the polyphyly of the microporellid ascopore and the high propensity for convergent traits observed in bryozoans, it would be unsurprising if a phylogenetic analysis that included all genera ended up splitting the family further.

Trait evolution: ascopores and avicularia

The ascopore is an established polyphyletic trait, even prior to the fragmentation of Microporellidae, as it is also found in Haplopomidae and Calwelliidae (Gordon, 1984). Nevertheless, this rare feature, apparently an innovation in the mechanism of water

expulsion, has apparently evolved several times, among closely related species. This begs the question as to what (if any) adaptive advantage this trait holds and what costs are involved in maintaining it. Ascopore morphology varies among species (fig. 5), and based on my inferred tree topology there is no clear pattern in the distribution of the different types, as one could expect. I suggest preliminarily that ascopore types do not hold phylogenetic signal at an interspecific level. The presence or absence of avicularia does not seem to hold any phylogenetic signal either: *Fenestrulina* is characterized as being avicularia-free, but was inferred as closely related to *Escharella* without avicularia, as well as *Escharoides* and *Calloporina* which do have this zooid type.

Mitochondrial genomes

The nearly complete mitochondrial genome presented in this study has provided additional evidence of the high degree of gene order rearrangements in bryozoan mitochondrial genomes. The gene order of *M. cf. neocribroides* differs greatly from the 8 published mitochondrial genomes, even when the highly variable tRNAs are excluded. The closest relative to *M. cf neocribroides* of the taxa with previously sequenced mitochondrial genomes is *Watersipora subtorquata*. These two species share 3 blocks of genes (all blocks contain 2 genes), where the block *cox3+nad1* is exclusive to them. Gene order data has been suggested as useful phylogenetic information (Boore et al., 1995; Boore & Brown, 1998), as rearrangements are thought to be rare events and thus similar patterns should reflected relatedness. There are no clear patterns in gene order that distinguish more closely related species from more distantly related species within the sampled bryozoans, except for very closely related species (within genus) such as two *Celleporella* and two *Microporella*. Based on this, gene order does not seem to hold any phylogenetic signal between higher taxonomic levels. However, how useful gene order is as a phylogenetic trait for congeneric analyses of bryozoans remains to be seen.

I estimate that not more than 1 kb of the mitochondrial genome remains to be sequenced in *M.* cf. *neocribroides.* The, so far, the identical gene order between *M. cf. neobribroides* and *M. ciliata* is promising as it indicates that the mitogenomes are not too divergent. However a more in-depth comparison between the two mitochondria (e.g. nucleotide composition and

substitution rates) is needed to determine the value of using mitochondria as phylogenetic markers for this group.

Best practice

In this section, I strongly suggest some best practices, especially when working with neglected taxa such as bryozoans. First, due to the difficulty of species identification and the prevalent revisions and re-descriptions of species, when adding sequences from new samples and making them publically available, images of the samples (SEMs in the case of bryozoans) should also be publically available. This would help avoid misidentifications to accumulate and be "planted" down the line. Second, information on sampling location should also be made available, as many species are misidentified. For instance, the sequence for *Microprella ciliata* is problematic, where the four sequences available in NCBI are from three different publications, the *18S* sequence is from South Wales (Tsyganov-Bodounov et al., 2009), *16S* is from Maine (Dick et al., 2000) and *cox1* and *cytb* from Norway (Waeschenbach et al., 2012). *Microporella ciliata* is, in all likelihood, endemic to the Mediterranean (Kuklinski & Taylor, 2008), thus the public sequences cannot be material stemming from this species. Likewise, while the *Fenestrulina malusii* accession in genbank is from China, the species *F. malusii* is likely restricted to the Mediterranean and NE Atlantic (Hayward & Ryland, 1999). Third, taxonomists describing new species should include sequence information or at least keep molecular vouchers Bickford et al. (2007).

Concluding remarks

Microporellidae is not a monophyletic clade, thus there is need for a systematic revision of the genera included in this family. For further exploration of microporellid (sensu lato) relationships to other cheilostomes, future phylogenies should include representatives from *Taylorius, Chiastosella* and Calwelliidae, as well as representatives from the remaining genera currently assigned to Microporellidae. The convergent evolution of phenotypic traits (such as the ascopore and frontal field types) signifies the value of molecular markers, in addition to morphology, in bryozoan systematics. However, recovering robust bryozoan molecular phylogenies is not an unproblematic endeavour. Due to the small size of bryozoans and ethanol preserved samples (often over long periods of time), the recovered DNA concentration is often very low. Several nodes in the recovered tree topology have low to no support, which reflects a need for a larger dataset, in terms of both taxa and genes. In this study I opted for a target approach, using long range amplification followed by primer walking, to recover mitochondrial genomes. This proved to be a time-consuming endeavour, with varying results. Thus, for future work on bryozoan mitochondrial genomes I would recommend trying a different approach, for example using multiple displacement amplification (MDA) to amplify mitogenomes before high-throughput sequencing. Alternatively, one could also sequence the full genomes (on a high-throughput sequencing platform) and recover the mitochondria bioinformatically, post sequencing. The most optimal approach would of course be to use fresh material. However, sampling is time consuming and costly, especially when the goal is extensive taxonomic sampling with a broad geographical distribution. A largely unexplored area that would solve some of the problems when it comes to taxonomic sampling, is the use of the treasure-trove of information museum collections hold, by using new techniques within ancient DNA.

There are many interesting aspects of microporellid evolution and taxonomy that should be considered further in addition to those I have begun to discuss in this thesis. For example New Zealand is a hotspot when it comes to bryozoan diversity, and harbours many endemic microporellid species. The fossil presence of microporellids from the basal Miocene in New Zealand suggests that this could be the birthplace of *Microporella* and *Fenestrulina*. However, to resolve this a broader geographical sampling is needed, and in particular

samples from the tropics and the west coast of America should be represented. The finding of a potentially undescribed species in this study indicates that the true species diversity of *Microporella* and probably also *Fenestrulina* is likely underestimated, especially when considering that New Zealand has one the world's best described bryofaunas. Furthermore, the cryptic species complexes *M. ciliata* and *F. malusii* should be revised both morphologically and molecularly.

References

Aberer, Andre J., Denis Krompass, and Alexandros Stamatakis. 2013. "Pruning Rogue Taxa Improves Phylogenetic Accuracy: An Efficient Algorithm and Webservice." *Systematic Biology* 62 (1): 162–66. doi:10.1093/sysbio/sys078.

Bernt, Matthias, Christoph Bleidorn, Anke Braband, Johannes Dambach, Alexander Donath, Guido Fritzsch, Anja Golombek, et al. 2013. "A Comprehensive Analysis of Bilaterian Mitochondrial Genomes and Phylogeny." *Molecular Phylogenetics and Evolution* 69 (2): 352–64. doi:10.1016/j.ympev.2013.05.002.

Bernt, Matthias, Alexander Donath, Frank Jühling, Fabian Externbrink, Catherine Florentz, Guido Fritzsch, Joern Pütz, Martin Middendorf, and Peter F. Stadler. 2013. "MITOS: Improved de Novo Metazoan Mitochondrial Genome Annotation." *Molecular Phylogenetics and Evolution* 69 (2): 313–19. doi:10.1016/j.ympev.2012.08.023.

Bernt, Matthias, Daniel Merkle, Kai Ramsch, Guido Fritzsch, Marleen Perseke, Detlef Bernhard, Martin Schlegel, Peter F. Stadler, and Martin Middendorf. 2007. "CREx: Inferring Genomic Rearrangements Based on Common Intervals." *Bioinformatics* 23 (21): 2957–58. doi:10.1093/bioinformatics/btm468.

Bickford, David, David J. Lohman, Navjot S. Sodhi, Peter K L Ng, Rudolf Meier, Kevin Winker, Krista K. Ingram, and Indraneil Das. 2007. "Cryptic Species as a Window on Diversity and Conservation." *Trends in Ecology and Evolution*. doi:10.1016/j.tree.2006.11.004.

Bock, Philip E., and Dennis P. Gordon. 2013. "Phylum Bryozoa Ehrenberg, 1831." *Zootaxa* 3703 (1): 67–74. doi:10.11646/zootaxa.3703.1.14.

Boore, J L, T M Collins, D Stanton, L L Daehler, and W M Brown. 1995. "Deducing the Pattern of Arthropod Phylogeny from Mitochondrial DNA Rearrangements." *Nature*. doi:10.1038/376163a0.

Boore, Jeffrey L., and Wesley M. Brown. 1998. "Big Trees from Little Genomes: Mitochondrial Gene Order as a Phylogenetic Tool." *Current Opinion in Genetics and Development* 8 (6): 668–74. doi:10.1016/S0959-437X(98)80035-X.

Brown, D. A. 1952. *The Tertiary Cheilostomatous Polyzoa of New Zealand*. London, UK: British Museum (Natural History).

Carter, Michelle C., Dennis P. Gordon, and Jonathan P A Gardner. 2010. "Polymorphism and Vestigiality: Comparative Anatomy and Morphology of Bryozoan Avicularia." *Zoomorphology* 129 (3): 195–211. doi:10.1007/s00435-010-0113-9.

Castresana, J. 2000. "Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis." *Molecular Biology and Evolution* 17 (4): 540–52. doi:10.1093/oxfordjournals.molbev.a026334.

Cheetham, Alan H., Joann Sanner, Paul D. Taylor, and Andrew N. Ostrovsky. 2006.

"Morphological Differentiation of Avicularia and the Proliferation of Species in Mid-Cretaceous Wilbertopora Cheetham, 1954 (Bryozoa: Cheilostomata)." *Journal of Paleontology* 80 (1): 49–71. doi:10.1666/0022-3360(2006)080[0049:MDOAAT]2.0.CO;2.

Darriba, Diego, Guillermo L Taboada, Ramón Doallo, and David Posada. 2012. "jModelTest 2: More Models, New Heuristics and Parallel Computing." *Nature Methods* 9 (8): 772–772. doi:10.1038/nmeth.2109.

Darriba, Diego, Guillermo L. Taboada, Ramón Doallo, and David Posada. 2011. "ProtTest-HPC: Fast Selection of Best-Fit Models of Protein Evolution." In *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*, 6586 LNCS:177–84. doi:10.1007/978-3-642-21878-1_22.

Darwin, Charles. 1859. *On the Origin of the Species*. *Darwin*. Vol. 5. doi:10.1016/S0262-4079(09)60380-8.

de Vienne, Damien M., Tatiana Giraud, and Olivier C. Martin. 2007. "A Congruence Index for Testing Topological Similarity between Trees." *Bioinformatics* 23 (23): 3119–24. doi:10.1093/bioinformatics/btm500.

Dick, M.H., J.R. Freeland, L.P. Williams, and M. Coggeshall-Burr. 2000. "Use of 16S Mitochondrial Ribosomal DNA Sequences to Investigate Sister-Group Relationships among Gymnolaemate Bryozoans." In *Proceedings of the 11th International Bryozoology Association Conference*, edited by A. Herrera Cubilla and J.B.C. Jackson, 197–210. Balboa: Smithsonian Tropical Research Institute.

Dick, Matthew H., Amalia Herrera-Cubilla, and Jeremy B C Jackson. 2003. "Molecular Phylogeny and Phylogeography of Free-Living Bryozoa (Cupuladriidae) from Both Sides of the Isthmus of Panama." *Molecular Phylogenetics and Evolution* 27 (3): 355–71. doi:10.1016/S1055-7903(03)00025-3.

Fehlauer-Ale, Karin H., Joshua A. Mackie, Grace E. Lim-Fong, Ezequiel Ale, Marcio R. Pie, and Andrea Waeschenbach. 2014. "Cryptic Species in the Cosmopolitan Bugula Neritina Complex (Bryozoa, Cheilostomata)." *Zoologica Scripta* 43 (2): 193–205. doi:10.1111/zsc.12042.

Fehlauer-Ale, Karin H., Judith E. Winston, Kevin J. Tilbrook, Karine B. Nascimento, and Leandro M. Vieira. 2015. "Identifying Monophyletic Groups within Bugula Sensu Lato (Bryozoa, Buguloidea)." *Zoologica Scripta*, no. May: n/a – n/a. doi:10.1111/zsc.12103.

Felsenstein, Joseph. 1985. "Phylogenies and the Comparative Method." *The American Naturalist*. doi:10.1086/284325.

Fuchs, Judith, Matthias Obst, and Per Sundberg. 2009. "The First Comprehensive Molecular Phylogeny of Bryozoa (Ectoprocta) Based on Combined Analyses of Nuclear and Mitochondrial Genes." *Molecular Phylogenetics and Evolution* 52 (1). Elsevier Inc.: 225–33. doi:10.1016/j.ympev.2009.01.021.

Ghiselli, Fabrizio, Liliana Milani, Davide Guerra, Peter L. Chang, Sophie Breton, Sergey V. Nuzhdin, and Marco Passamonti. 2013. "Structure, Transcription, and Variability of Metazoan Mitochondrial Genome: Perspectives from an Unusual Mitochondrial Inheritance System." *Genome Biology and Evolution* 5 (8): 1535–54. doi:10.1093/gbe/evt112.

Gissi, C, F Iannelli, and G Pesole. 2008. "Evolution of the Mitochondrial Genome of Metazoa as Exemplified by Comparison of Congeneric Species." *Heredity* 101 (4): 301–20. doi:10.1038/hdy.2008.62.

Gómez, Africa, Roger N. Hughes, Peter J. Wright, Gary R. Carvalho, and David H. Lunt. 2007. "Mitochondrial DNA Phylogeography and Mating Compatibility Reveal Marked Genetic Structuring and Speciation in the NE Atlantic Bryozoan Celleporella Hyalina." *Molecular Ecology* 16 (10): 2173–88. doi:10.1111/j.1365-294X.2007.03308.x.

Gordon, D P., and E Voigt. 1996. *The Kenozooidal Origin of the Ascophorine Hypostegal Coelom and Associated Frontal Shield*. Edited by D. P. Gordon, A. M. Smith, and J. A. Grant-Mackie. *Bryozoans in Space and Time*. Wellington, New Zealand: National Institute of Water & Atmospheric Research.

Gordon, Dennis P, Paul D Taylor, and Françoise P Bigey. 2009. *Moss Animals, Sea Mats, Lace Corals*. Edited by Dennis P. Gordon. New Zealand inventory of biodiversity: 1. Kingdom Animalia: Radiata, Lophotrochozoa, Deuterostomia.

Gordon, Dennis P. 1984. *The Marine Fauna of New Zealand: Bryozoa: Gymnolaemata from the Kermadec Ridge*. New Zealand Oceanographic Institute Memoir 91.

Gordon, Dennis P. 2014. "Apprehending Novel Biodiversity-Fifteen New Genera of Zealandian Bryozoa." *Journal of the Marine Biological Association of the United Kingdom* 94 (8): 1597–1628. doi:10.1017/S0025315414000599.

Guha, A. K., and K. Gopikrishna. 2007. "Some Fossil Ascophoran Bryozoan Taxa from Tertiary Sequences of Western Kachchh, Gujarat." *Journal of the Palaeontological Society of India* 2: 195–222.

Guindon, Stéphane, and Olivier Gascuel. 2003. "A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood." *Systematic Biology* 52 (5): 696–704. doi:10.1080/10635150390235520.

Halanych, Kenneth M., Richard A. Lutz, and Robert C. Vrijenhoek. 1998. "Evolutionary Origins and Age of Vestimentiferan Tube-Worms." *Cahiers de Biologie Marine* 39 (3-4): 355–58.

Harvell, C.Drew. 1994. "The Evolution of Polymorphisms in Colonial Invertebrates and Social Insects." *The Quarterly Review of Biology* 69 (2): 155–85.

Hayward, PJ, and JS Ryland. 1999. *Cheilostomatous Bryozoa, Part 2. Hippothooidea-Celleporoidea*. 2nd ed. Synop Br Fauna.

Huelsenbeck, J. P. & Ronquist, F. 2001. "MRBAYES: Bayesian 17:754-755., Inference of

Phylogeny. Bioinformatics."

Jablonski, David, Scott Lidgard, and Paul D Taylor. 1997. "Comparative Ecology of Bryozoan Radiations : Origin of and Cheilostomes Novelties in Cyclostomes." *PALAIOS* 12 (6): 505–23.

Jagadeeshan, Santosh, and Aaron O'Dea. 2012. "Integrating Fossils and Molecules to Study Cupuladriid Evolution in an Emerging Isthmus." *Evolutionary Ecology* 26 (2): 337–55. doi:10.1007/s10682-011-9522-6.

Jang, Kuem Hee, and Ui Wook Hwang. 2009. "Complete Mitochondrial Genome of Bugula Neritina (Bryozoa, Gymnolaemata, Cheilostomata): Phylogenetic Position of Bryozoa and Phylogeny of Lophophorates within the Lophotrochozoa." *BMC Genomics* 10: 167. doi:10.1186/1471-2164-10-167.

Jullien, J. 1888. *Mission Scientifique Du Cap Horn*. VI. Paris: Zoologie, Bryozoaires.

Katoh, Kazutaka, and Daron M. Standley. 2013. "MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability." *Molecular Biology and Evolution* 30 (4): 772–80. doi:10.1093/molbev/mst010.

Kibbe, Warren A. 2007. "OligoCalc: An Online Oligonucleotide Properties Calculator." *Nucleic Acids Research* 35 (SUPPL.2). doi:10.1093/nar/gkm234.

Knight, Sarah, Dennis P Gordon, and Shane D Lavery. 2011. "A Multi-Locus Analysis of Phylogenetic Relationships within Cheilostome Bryozoans Supports Multiple Origins of Ascophoran Frontal Shields." *Molecular Phylogenetics and Evolution* 61 (2). Elsevier Inc.: 351–62. doi:10.1016/j.ympev.2011.07.005.

Kuklinski, Piotr, and Paul D. Taylor. 2008. "Arctic Species of the Cheilostome Bryozoan Microporella, with a Redescription of the Type Species." *Journal of Natural History* 42 (27-28): 1893–1906. doi:10.1080/00222930802126904.

Lidgard, Scott, Michelle C. Carter, Matthew H. Dick, Dennis P. Gordon, and Andrew N. Ostrovsky. 2012. "Division of Labor and Recurrent Evolution of Polymorphisms in a Group of Colonial Animals." *Evolutionary Ecology* 26 (2): 233–57. doi:10.1007/s10682-011-9513-7.

Maddison, W. P., and D.R. Maddison. 2015. "Mesquite: A Modular System for Evolutionary Analysis. Version 3.04."

McKinney, F.K., and J.B.C. Jackson. 1989. *Bryozoan Evolution*. London, UK: Unwin Hyman Ltd.

Nesnidal, Maximilian P, Martin Helmkampf, Iris Bruchhaus, and Bernhard Hausdorf. 2011. "The Complete Mitochondrial Genome of Flustra Foliacea (Ectoprocta, Cheilostomata) - Compositional Bias Affects Phylogenetic Analyses of Lophotrochozoan Relationships." *BMC Genomics* 12 (1). BioMed Central Ltd: 572. doi:10.1186/1471-2164-12-572.

Nikulina, E. a., R. Hanel, and P. Schäfer. 2007. "Cryptic Speciation and Paraphyly in the

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Cosmopolitan Bryozoan Electra Pilosa-Impact of the Tethys Closing on Species Evolution." *Molecular Phylogenetics and Evolution* 45: 765–76. doi:10.1016/j.ympev.2007.07.016.

Ostrovsky, A. N. 2004. "Brood Chambers (Ovicells) of Cheilostome Bryozoans (Bryozoa: Gymnolaemata): Structure, Research History, and Modern Problematics." *Russian Journal of Marine Biology* 30 (SUPPL. 1). doi:10.1007/s11179-005-0025-6.

Ostrovsky, Andrew N. 2013. *Evolution of Sexual Reproduction in Marine Invertebrates. Example of Gymnolaemate Bryozoans*. Dordrecht: Springer.

Podsiadlowski, Lars, Anke Braband, Torsten H Struck, Joern von Doehren, and Thomas Bartolomaeus. 2009. "Phylogeny and Mitochondrial Gene Order Variation in Lophotrochozoa in the Light of New Mitogenomic Data from Nemertea." *BMC Genomics* 10 (1): 364. doi:10.1186/1471-2164-10-364.

Rambaut, A, and A J Drummond. 2013. "Tracer v1.6." *Available from Http://tree.bio.ed.ac.uk/software/tracer/*.

Ronquist, Fredrik, and John P. Huelsenbeck. 2003. "MrBayes 3: Bayesian Phylogenetic Inference under Mixed Models." *Bioinformatics* 19 (12): 1572–74. doi:10.1093/bioinformatics/btg180.

Ryland, J.S. 1970. *Bryozoans*. London, UK: Hutchinson University Library.

Saccone, Cecilia, Carmela Gissi, Aurelio Reyes, Alessandra Larizza, Elisabetta Sbisà, and Graziano Pesole. 2002. "Mitochondrial DNA in Metazoa: Degree of Freedom in a Frozen Event." *Gene* 286 (1): 3–12. doi:10.1016/S0378-1119(01)00807-1.

Shen, Xin, Mei Tian, Xueping Meng, Huilian Liu, Hanliang Cheng, Changbao Zhu, and Fangqing Zhao. 2012. "Complete Mitochondrial Genome of Membranipora Grandicella (Bryozoa: Cheilostomatida) Determined with next-Generation Sequencing: The First Representative of the Suborder Malacostegina." *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics* 7 (3). Elsevier Inc.: 248–53. doi:10.1016/j.cbd.2012.03.003.

Stamatakis, Alexandros. 2014. "RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies." *Bioinformatics* 30 (9): 1312–13. doi:10.1093/bioinformatics/btu033.

Sun, Ming'an, Xin Shen, Huilian Liu, Xixing Liu, Zhigang Wu, and Bin Liu. 2011. "Complete Mitochondrial Genome of Tubulipora Flabellaris (Bryozoa: Stenolaemata): The First Representative from the Class Stenolaemata with Unique Gene Order." *Marine Genomics* 4 (3). Elsevier B.V.: 159–65. doi:10.1016/j.margen.2011.03.006.

Sun, Ming'an, Zhigang Wu, Xin Shen, Jianfeng Ren, Xixing Liu, Huilian Liu, and Bin Liu. 2009. "The Complete Mitochondrial Genome of Watersipora Subtorquata (Bryozoa, Gymnolaemata, Ctenostomata) with Phylogenetic Consideration of Bryozoa." *Gene* 439 (1-2). Elsevier B.V.: 17–24. doi:10.1016/j.gene.2009.03.003.

Suwa, T., and S. F. Mawatari. 1998. "Revision of Seven Species of Microporella (Bryozoa, Cheilostomatida) from Hokkaido, Japan, Using New Taxonomic Characters." *Journal of Natural History* 32 (April): 895–922. doi:10.1080/00222939800770461.

Taylor, P. D. 1981. "Bryozoa from the Jurassic Portland Beds of England." *Palaeontology* 24: 863–75.

Taylor, P.D., and S.F. Mawatri. 2004. "Preliminary Overveiw of the Cheilostome Bryozoan Microporella." In *Bryozoan Studies 2004*, edited by Cancino & Wyse Jackson (eds). Moyano. Tylor & Francis Group.

Taylor, Paul D. 1988. "Major Radiation of Cheilostome Bryozoans: Triggered by the Evolution of a New Larval Type?" *Historical Biology* 1 (1): 45–64.

Taylor, Paul D., and Andrea Waeschenbach. 2015. "Phylogeny and Diversification of Bryozoans." *Palaeontology* 58 (4): 585–99. doi:10.1111/pala.12170.

Taylor, Paul D., Andrea Waeschenbach, Abigail M. Smith, and Dennis P. Gordon. 2015. "In Search of Phylogenetic Congruence between Molecular and Morphological Data in Bryozoans with Extreme Adult Skeletal Heteromorphy." *Systematics and Biodiversity* 2000 (July): 1–20. doi:10.1080/14772000.2015.1049673.

Tsyganov-Bodounov, Anton, Peter J. Hayward, Joanne S. Porter, and David O F Skibinski. 2009. "Bayesian Phylogenetics of Bryozoa." *Molecular Phylogenetics and Evolution* 52 (3). Elsevier Inc.: 904–10. doi:10.1016/j.ympev.2009.05.010.

Untergasser, Andreas, Harm Nijveen, Xiangyu Rao, Ton Bisseling, René Geurts, and Jack A M Leunissen. 2007. "Primer3Plus, an Enhanced Web Interface to Primer3." *Nucleic Acids Research* 35 (SUPPL.2). doi:10.1093/nar/gkm306.

Vilstrup, Julia T, Simon Yw Ho, Andrew D Foote, Phillip A Morin, Danielle Kreb, Michael Krützen, Guido J Parra, et al. 2011. "Mitogenomic Phylogenetic Analyses of the Delphinidae with an Emphasis on the Globicephalinae." *BMC Evolutionary Biology* 11 (1): 65. doi:10.1186/1471-2148-11-65.

Waeschenbach, Andrea, Joanne S. Porter, and Roger N. Hughes. 2012. "Molecular Variability in the Celleporella Hyalina (Bryozoa; Cheilostomata) Species Complex: Evidence for Cryptic Speciation from Complete Mitochondrial Genomes." *Molecular Biology Reports* 39 (9): 8601–14. doi:10.1007/s11033-012-1714-9.

Waeschenbach, Andrea, Paul D Taylor, and D Timothy J. Littlewood. 2012. "A Molecular Phylogeny of Bryozoans." *Molecular Phylogenetics and Evolution* 62 (2). Elsevier Inc.: 718–35. doi:10.1016/j.ympev.2011.11.011.

Waeschenbach, Andrea, Maximilian J. Telford, Joanne S. Porter, and D. T J Littlewood. 2006. "The Complete Mitochondrial Genome of Flustrellidra Hispida and the Phylogenetic Position of Bryozoa among the Metazoa." *Molecular Phylogenetics and Evolution* 40 (1): 195–207. doi:10.1016/j.ympev.2006.03.007.

Weigert, Anne, Anja Golombek, Michael Gerth, Francine Schwarz, Torsten H. Struck, and Christoph Bleidorn. 2016. "Evolution of Mitochondrial Gene Order in Annelida." *Molecular Phylogenetics and Evolution* 94: 196–206. doi:10.1016/j.ympev.2015.08.008.

Winston, E. 1986. "Victims of Avicularia." *Marine Ecology* 7 (3): 193–99.

Xia, Feng-Sheng, Sen-Gui Zhang, and Zong-Zhe Wang. 2007. "The Oldest Bryozoans: New Evidence From the Late Tremadocian (Early Ordovician) of East Yangtze Gorges in China." *Journal of Paleontology* 81 (6): 1308–26. doi:10.1666/04-128.1.

Yu, Li, Yi-Wei Li, Oliver a Ryder, and Ya-Ping Zhang. 2007. "Analysis of Complete Mitochondrial Genome Sequences Increases Phylogenetic Resolution of Bears (Ursidae), a Mammalian Family That Experienced Rapid Speciation." *BMC Evolutionary Biology* 7: 198. doi:10.1186/1471-2148-7-198.

Appendix 1

Table 5 Unsuccessful samples. Samples that were not successful, either in isolation or amplification. NZ = New Zealand. Country abbreviation: NZ = New Zealand. There are two main reason that the samples did not succeed: (1) Low DNA concentration and no success in amplification or (2) Contamination, the colonies grew on other bryozoans.

Species	Country	Reason sample did not succeed	sampling date	collected by
Microporella agonistes	NZ	1	26.01.2010	Smith & Gordon
Diporula verrucosa	Italy	1	2015	Antonietta Rosso
Fenestrulina cf. Incompta	NZ	2	04.07.2009	Smith & Gordon
Microporella luella	Japan	1	-	Matt Dick
Microporella trigomellata	Japan	1	-	Matt Dick
Calloporina triporosa	NZ	1	14.07.2009	Smith & Gordon
Fenestrulina disjuncta	NZ	2	2010	Smith & Gordon
Fenestrulina reticulata	NZ	2	27.01.2010	Smith & Gordon

Table 2 PCR cycling conditions. Cycling conditions for the three different polymerases and the annealing temperature (Tm) for each primer pair used to amplify genes. Each primer pair is listed along with its target gene.

Polymerase/primer pair	Gene		Cycling conditions			
			35 cycles			
dream taq		95°C: 3 min	95°C: 30 s	Tm (°C) - 30 s	72°C: 1 min	72°C: 10 min
Bryozoa_16SF+Bryozoa_16SR	*16S*			49		
Bryozoa_12SF+Bryozoa_12SR	*12S*			54		
Gymno300F+18pb	*18S*			54		
Bryozoa_cox3F+Bryozoa_cox3R	*cox3*			54		
Cox1F_prifi+Cox1R_prifi	*cox1*			53		
Bryozoa_cytbF_B+Bryozoa_cytbR	*cytb*			48		
phusion high-fidelity		98°C: 3 min	98°C: 30 s	Tm (°C) - 10 s	72°C: 1 min	72°C: 10 min
Bryozoa_16SF+Bryozoa_16SR	*16S*			49		
Bryozoa_12SF+Bryozoa_12SR	*12S*			54		
Gymno300F+18pb	*18S*			54		
Bryozoa_cox3F+Bryozoa_cox3R	*cox3*			54		
Cox1F_prifi+Cox1R_prifi	*cox1*			53		
Bryozoa_cytbF_B+Bryozoa_cytbR	*cytb*			48		

LongAmp	94°C: 30 s	94°C: 30 s	Tm (°C) - 1 min	65°C: 12 min	65°C: 10 min
16S1184F+12S549R			51		
LA15-1-cytbF+LA15-2-cox3R			58		
LA15-1-cox3F+LA15-1-cytbR			58		
LA25-cytbF+LA25-cox3R			57		
LA25-cox3F+LA25-cytbR			57		
LA5-cytbF+LA5-cox3R			55		
LA5-cox3F+LA5-cytbR			55		

Table 3. All taxa included in this study. The taxa downloaded from NCBI are listed with accession numbers for the six genes (*18S, 16S, 12S, cox1, cox3 and cytb*). Sequences that were sequenced in this study are indicated by ticks. The genes that were excluded from the dataset because they were misaligned or jumped in the tree topology (found by RogueNaRok) are coloured grey.

taxa	18S	16S	12S	cox1	cox3	cytb
Non Bryozoa						
Terebratalia transversa*	FJ196115	NC_003086	NC_003086	NP_203506	NP_203509	NP_203507
Laqueus spp.*	U083231	NC_002322	NC_002322	NP_058502	NP_058509	NP_058503
Phoronis spp*	U36271	AY368231	AY368231	AAR13390	AAR13386	AAR13396
Class Stenolaemata						
Order cyclostomata						
Crisia sigmoidea	FJ409608	JN681067	JN681100		AEV21523	AEV21444
Hornera foliacea	FJ409613	JN681068		AEV21488	AEV21526	AEV21446
Class Gymnolaemata						
order Ctenostomata						
Alcyonidium mytili	JN680936	JN681069	JN681102	AEV21493	AEV21531	AEV21452
order Cheilostomata						
Suborder Neocheilostomina						
Superfamily Hippothooidea						
Family Hippothoidae						
Celleporella hyalina		NC_018344	NC_018344	AFJ53903	AFJ53912	AFJ53907
Antarctothoa tongima	JF950364	JF950308		AEL29593		
Antarctothoa bathamae	JF950365	JF950303		AEL29591		
Family Chorizoporidae						
Chorizopora brongniartii	JF950366	JF950324		AEL29595		
Family Haplopomidae						
Haplopoma graniferum	FJ152037	New	New			
Superfamily Lepralielloidea						
Family Lepraliellidae						
Celleporaria agglutinans	JF950355	JF950310		AEL29617		
Celleporaria nodulosa	JF950357	JF950329		AEL29609		
Family Umbonulidae						
Umbonula littoralis	JN680953	JN681082	JN681116	AEV21508	AEV21542	AEV21469

taxa	18S	16S	12S	cox1	cox3	cytb
Superfamily Smittinoidea						
Family Smittinidae						
Smittina rosacea	JF950377	JF950318		AEL29603		
Smittina torques	JF950375	JF950352		AEL29582		
Family Watersiporidae						
Watersipora subtorquata	JN680947			ABY55219	ABY55220	ABY55224
Watersipora arcuata	FJ009090	AY789107		AAM46672		
Family Bitectiporidae						
Schizomavella linearis	JN680946	JN681077	JN681111	AEV21500	AEV21538	AEV21462
Family Schizoporellidae						
Schizoporella dunkerii	JN680955		JN681118	AEV21509		AEV21470
Family Lanceoporidae						
Calyptotheca immersa	JF950374	JF950327		AEL29584		
Family Cryptosulidae						
Cryptosula pallasiana	JN680940	JN681073	JN681107	AEV21496		AEV21457
Escharella immersa	FJ196116			AEV21501		AEV21463
Exochella tricuspis	JF950361	JF950341		AEL29599		
Escharoides angela	JF950360	JF950338		AEL29587		
Family Microporellidae						
Calloporina angustipora	JF950388	JF950321	√	AEL29577	√	√
Fenestrulina littoralis	√	√	√		√	√
Fenestrulina specca	√	√			√	√
Fenestrulina malusii O	√	√	√		√	√
Fenestrulina malusii N	√	√			√	
Fenestrulina cf. orientalis 1	√	√	√		√	
Fenestrulina cf. orientalis 2	√	√	√		√	
Fenestrulina sp.	√	√	√		√	
Fenestrulina thyrephora	√	√			√	√
Microporella ciliata	FJ152038	AF156286		AEV21504		AEV21465
Microporella agonistes	JF950387	JF950343		AEL29613		
Microporella sp.	√	√	√	√	√	√
Microporella speculum	√	√	√			√
Microporella cf. discors1	√	√	√	√	√	√
Microporella diademata	√	√	√	√	√	√
Microporella cf. neocribroides 1	√	√			√	√
Microporella cf. neocribroides 2	√	√	√		√	√
Microporella cf. neocribroides 3	√	√	√		√	
Microporella cf. neocribroides 4	√	√	√		√	
Microporella ordo		√	√		√	√
Microporella cf. discors2		√	√		√	
Superfamily Calloporoidea						
Family Calloporidae						

taxa	18S	16S	12S	cox1	cox3	cytb
Callopora lineata	JN680949	JN681080	JN681114	AEV21506	AEV21540	AEV21467
Family Chaperiidae						
Chaperiopsis rubida	JF950394	JF950332		AEL29600		
Superfamily Buguloidea						
Family Bugulidae						
Bugula plumosa	JN680951	JX183888		AFZ78225		
Superfamily Microporoidea						
Family Microporoidea						
Micropora mortenseni	JF950371	JF950345		AEL29610		
Suborder Malacostegina						
Family Electridae						
Electra pilosa	JN680944	JN681076	JN681110	AEV21499	AEV21536	AEV21460
Electra posidonia	AM886850	AM747486	FR754514			
Suborder Inovicellina						
Superfamily Aeteoidea						
Family Aeteidae						
Aetea anguina	JN680942	JN681074	JN681108		AEV21535	

*The non-bryozoan outgroups were used to check for if there were any non-bryozoan genes in the dataset, they were not included in further refinements of the dataset, or the concatenated dataset.
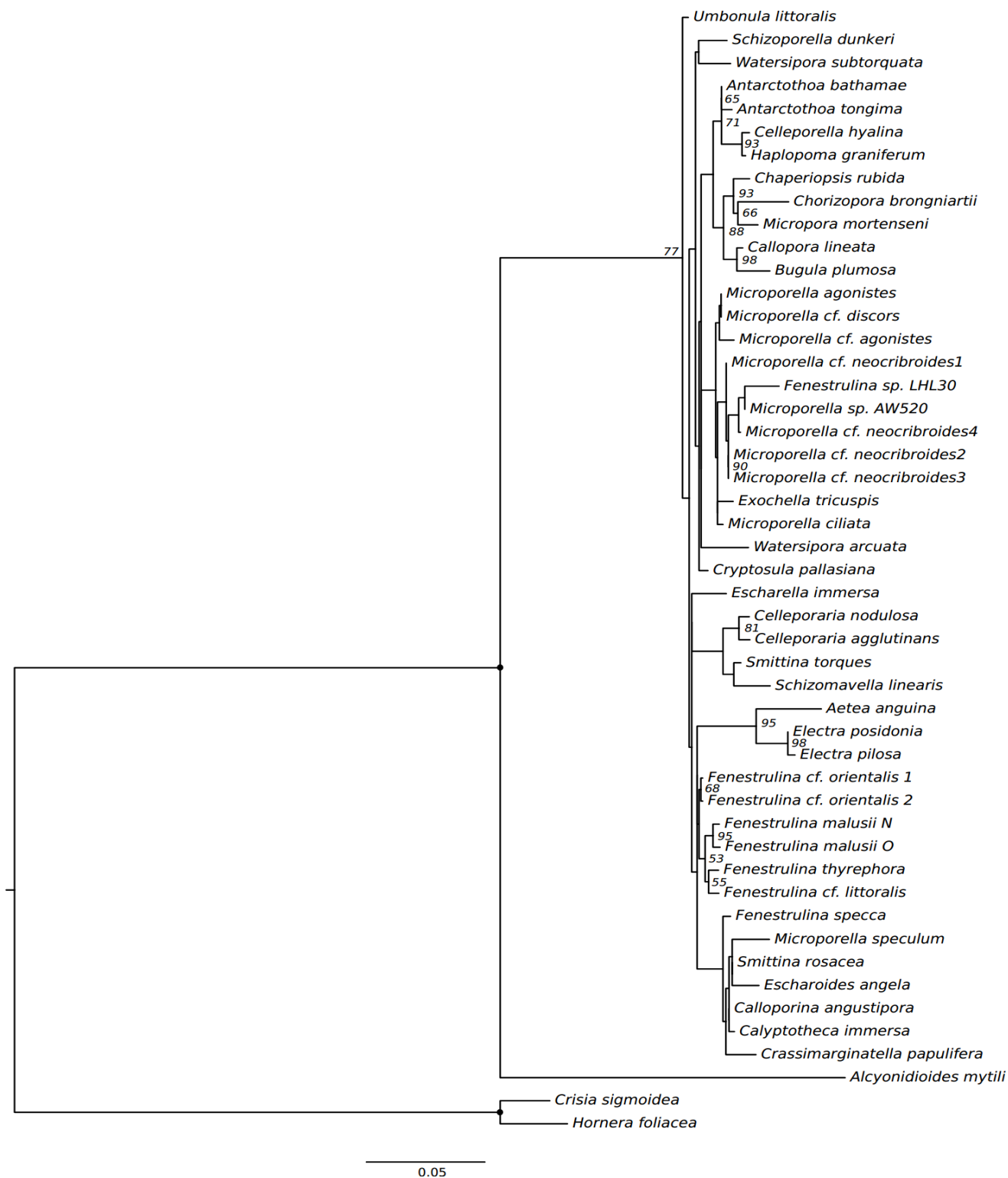
Figure 1. Single gene phylogeny for 49 species inferred from *18S* (1500 characters). The tree was reconstructed with ML inference (RAxML) using the GTR+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.
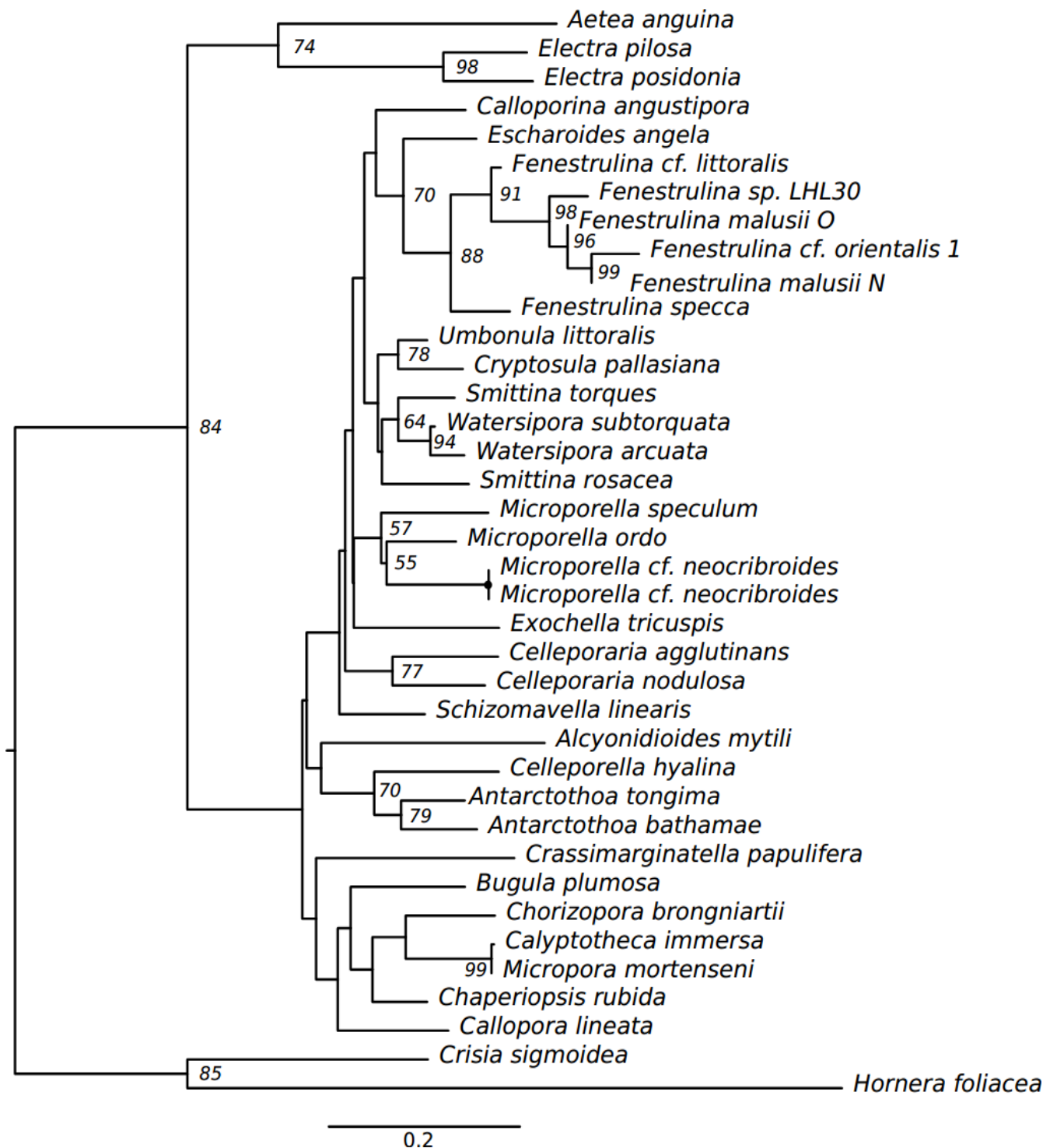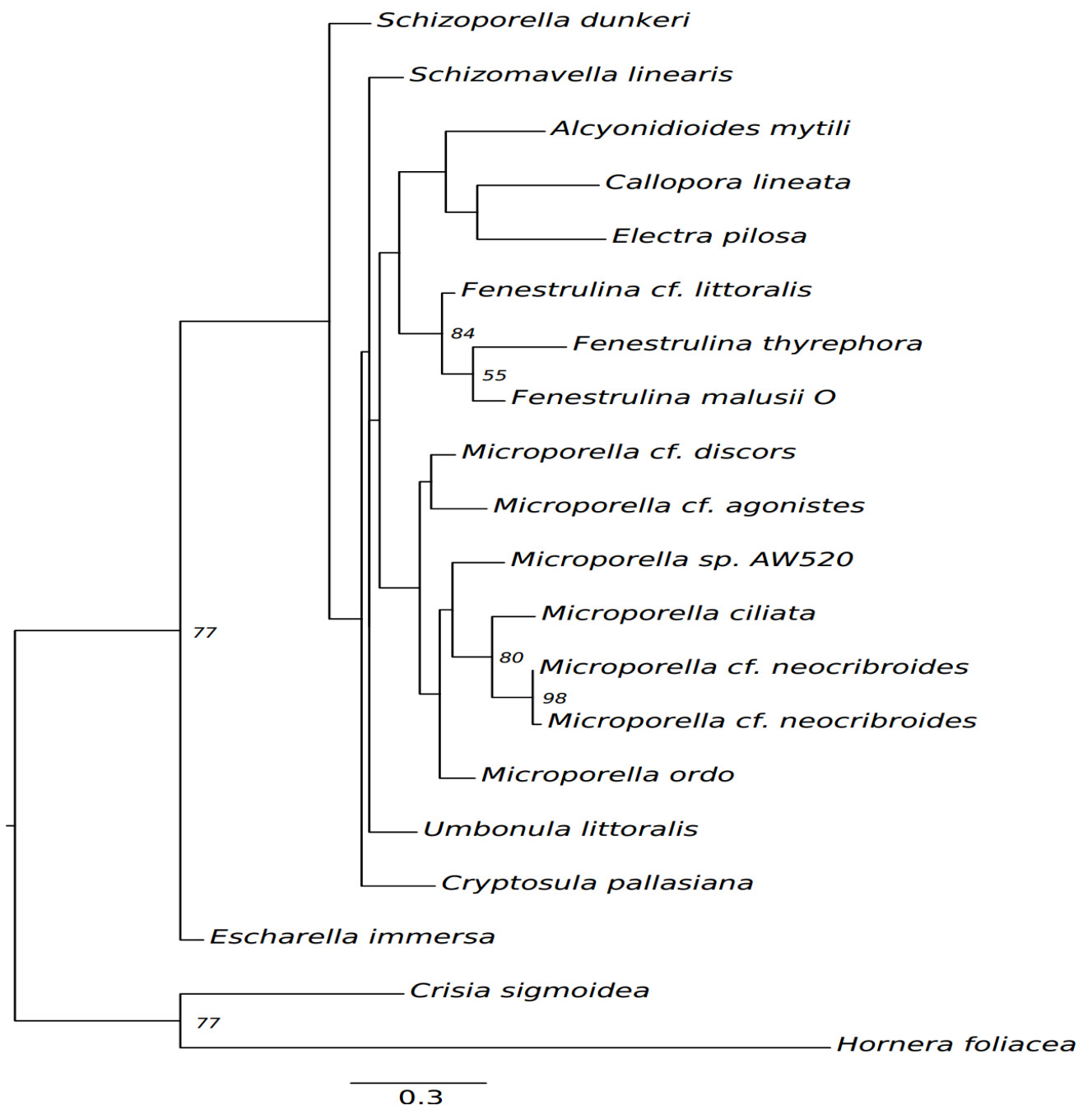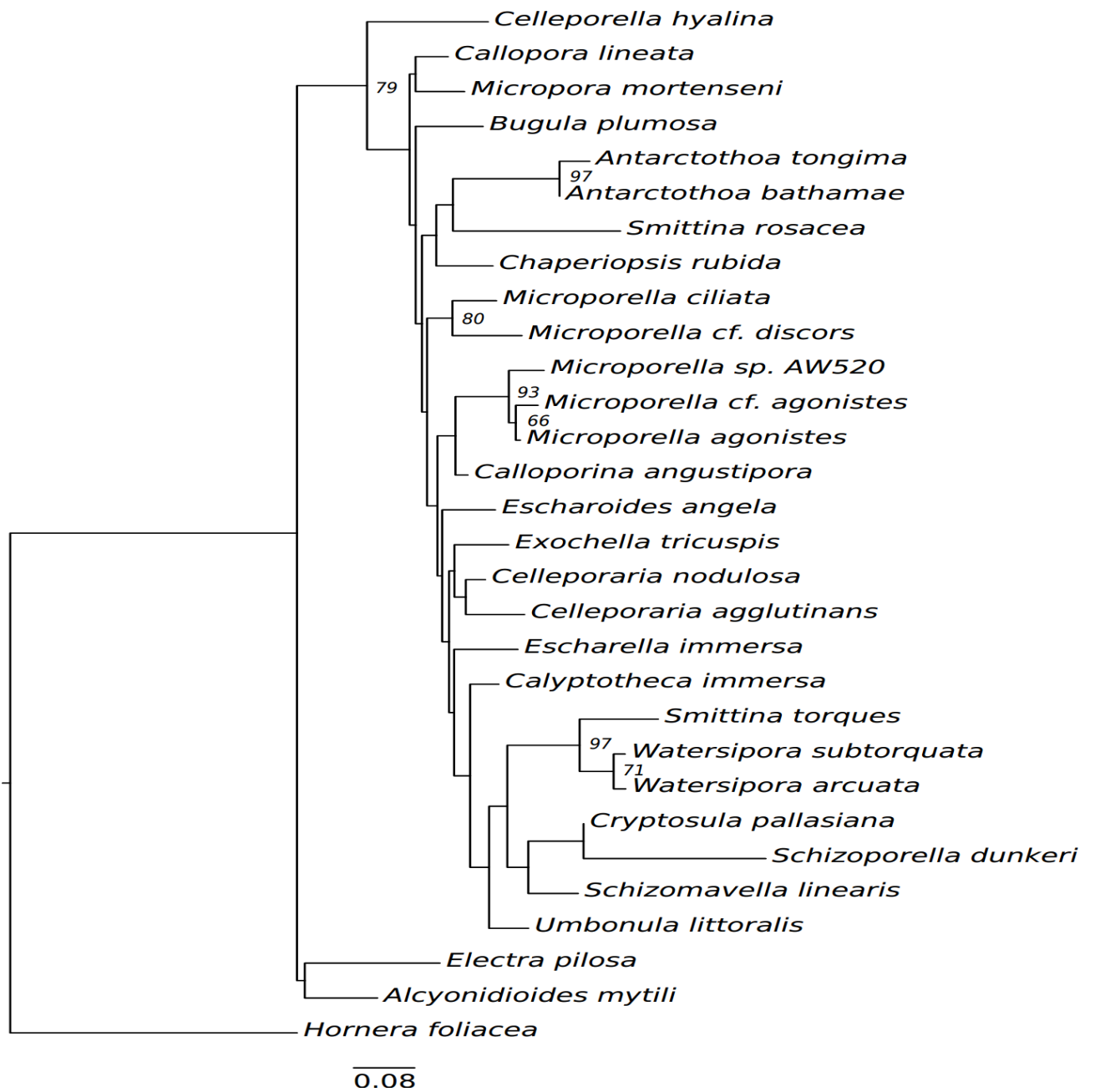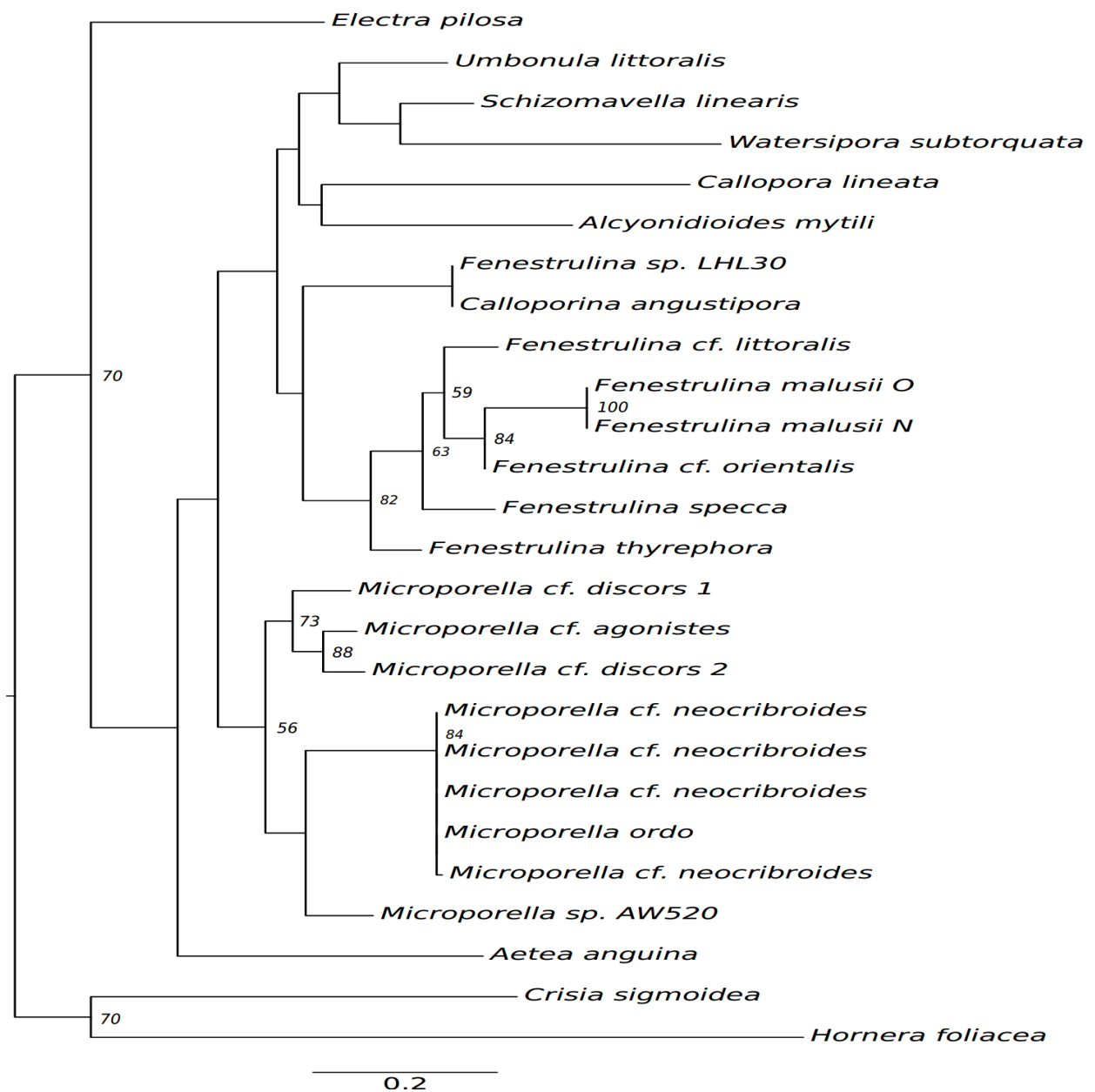
Figure 2. Single gene phylogeny for 38 species inferred from *16S* (414 characters). The tree was reconstructed with ML inference (RAxML) using the GTR+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.
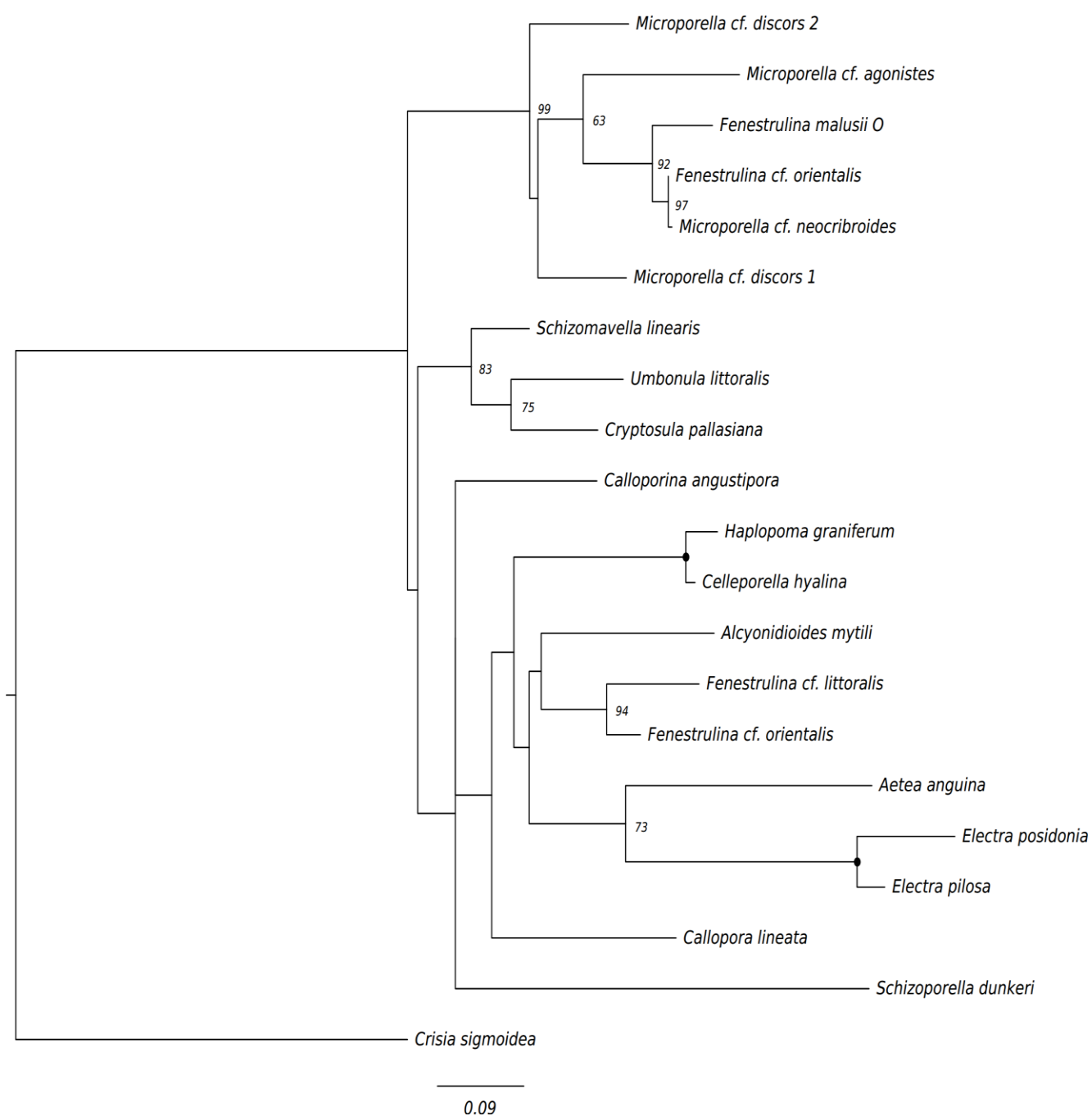
Figure 3. Single gene phylogeny for 21 species inferred from *12S* (420 characters). The tree was reconstructed with ML inference (RAxML) using the GTR+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.

Figure 4. Single gene phylogeny for 20 species inferred from amino acid alignment of *cytb* (137 characters). The tree was reconstructed with ML inference (RAxML) using the rtREV+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.

Figure 5. Single gene phylogeny for 30 species inferred from amino acid alignment of *cox1* (414 characters). The tree was reconstructed with ML inference (RAxML) using the rtREV+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.

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Figure 6. Single gene phylogeny for 26 species inferred from amino acid alignment of *cox3* (174 characters). The tree was reconstructed with ML inference (RAxML) using the rtREV+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site.
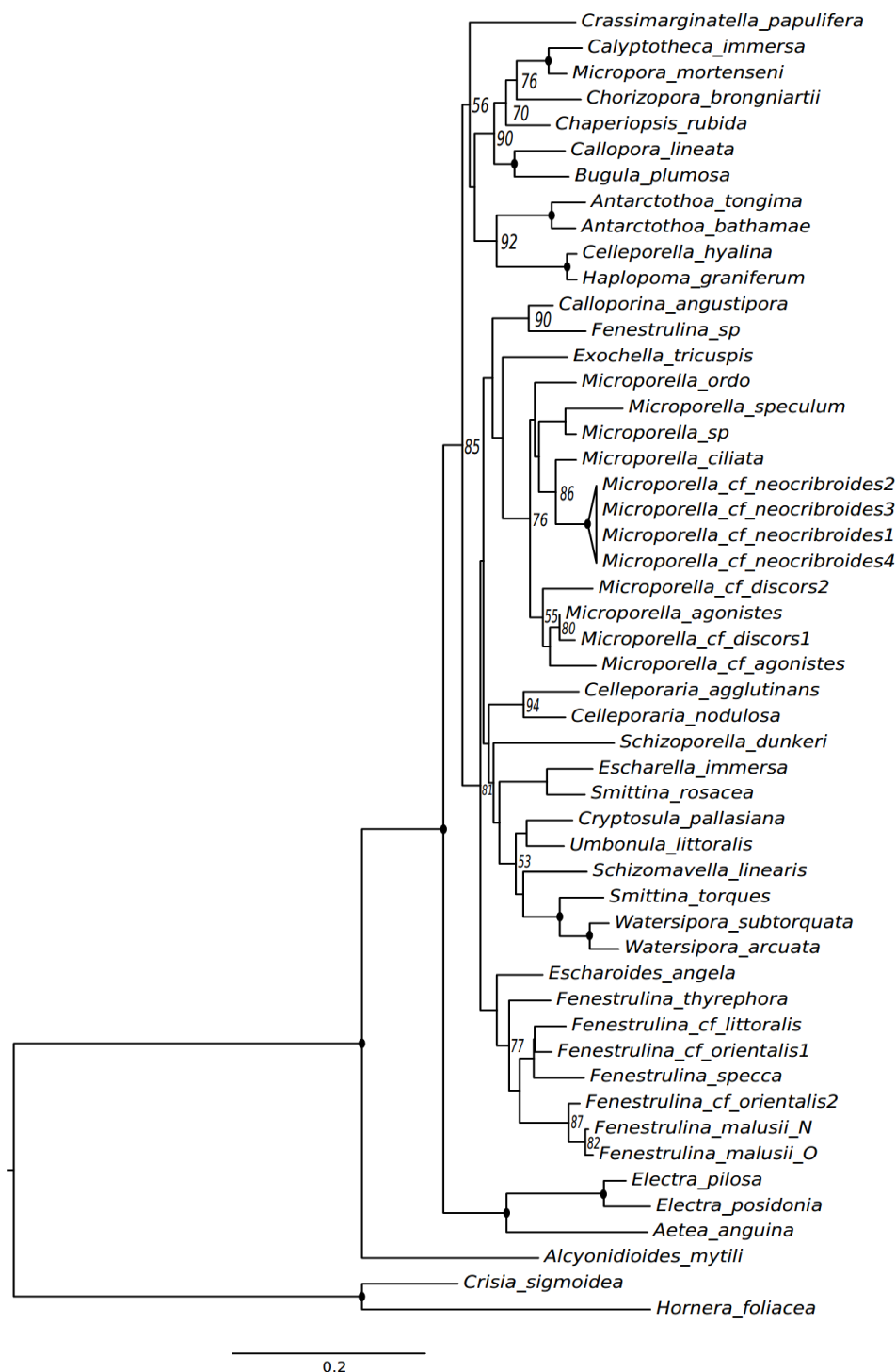
Figure 7. Phylogenetic inference of multigene dataset (*18S+16S+12S+cox1+cox3+cytb*), based on 51 species and 3060 characters. The tree was reconstructed with ML inference (RAxML) using the *GTR+G and* rtREV+G model of evolution. Bootstrap values for node support are shown for values above >50%, black circles indicate full support (100%). Scale bar shows estimated substitutions per site. The Congruence test between the presented tree topology using rtREV and a tree topology inferred using the MtArt model found that there are 11 differences between the two tree topologies. This is likely due to the ambiguous placement of several taxa.

Appendix 2

Microporell cf discors Uttley & Bullivant, 1972

Specimen ID: LHL5

Locality: New Zealand

Remarks: This specimen resembles *M. discors,* in that the orifice rim is minutely serrated, the ascopore is crescentic and porous and the avicularia is placed level to the ascopore.

Avicularia: single with long setiform mandibles.
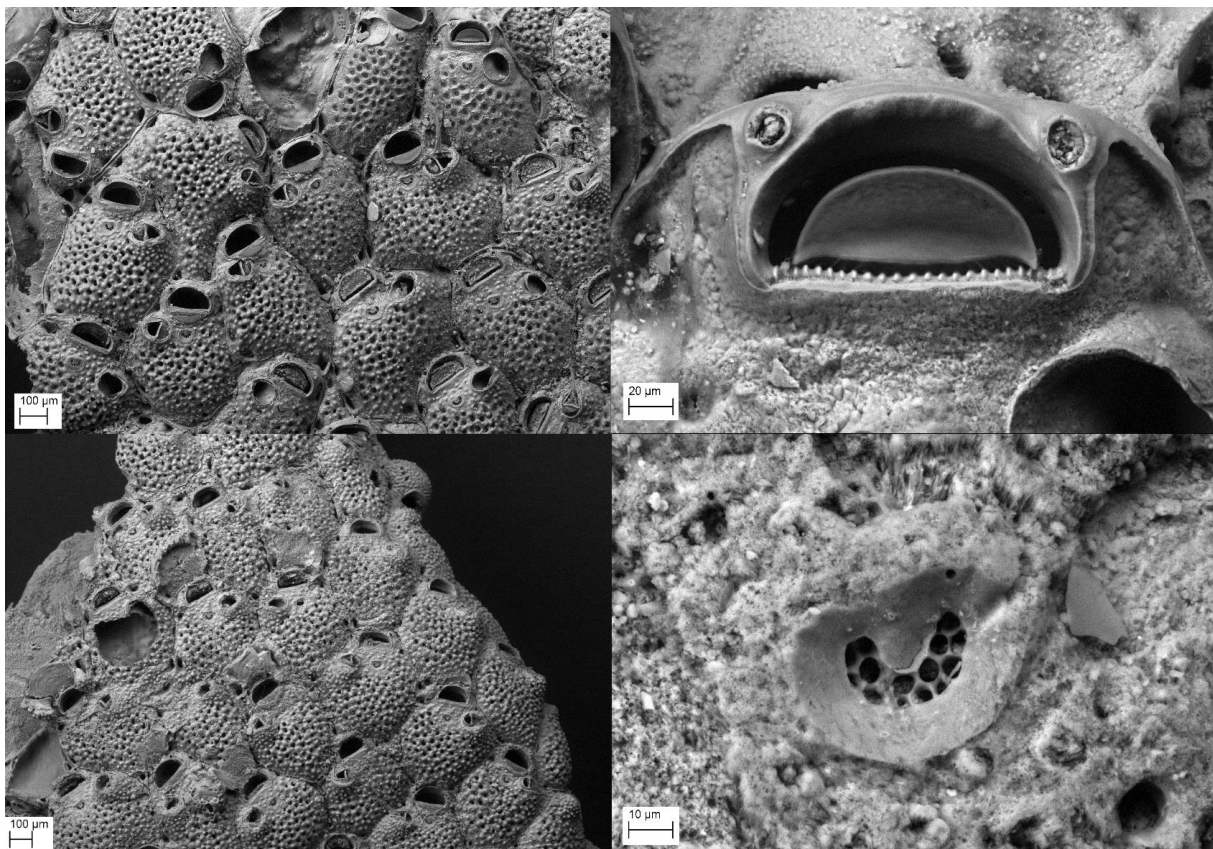
Figure 8. *Microporell cf discors.* Images to the left: images include several zooids, scale bars: 100 μm. Top right: orifice with denticulate proximal rim and 2 oral spines, scale bar: 20 μm. Bottom right: crescentic and porous ascopore, scale bar: 10 μm. Image credit: P. D. Taylor, NHMUK.

Microporella cf. discors Uttley & Bullivant, 1972

Specimen ID: LHL37

Locality: New Zealand

Remarks: This specimen greatly resembles *M. discors*, however it differs in having a large and close to circular ascopore, rather than a crescentic one. Both have porous ascopores. The avicularia in *M. discors* is described as level with the ascopore, while in this specimen the ascopore is mostly placed proximal to the ascopore. These differences could well be due to intraspecific variation.

Figure 9. *Microporell cf discors.* Top left: colony including several zooids, scale bar: 100 μm. Bottom left: zooids with ooecia, scale bar: 100 μm. Top right: orifice with denticulate proximal rim and 4 oral spines. Ascopore circular to crescentic and porous. Scale bar: 20 μm. Bottom right: zooid showing avicularia with long setiform mandible, scale bar: 100 μm. Image credit: P. D. Taylor, NHMUK.

Microporella cf. agonistes Gordon, 1984

Specimen ID: LHL11

Locality: New Zealand

Remarks: Gordon remarked that this specimen *M. agonistes* as it has proportionately large avicularia. However this specimen differs in having 5 oral spines and not 4. Unfortunately the specimen does not have ooecium, thus a certain identification cannot be made.
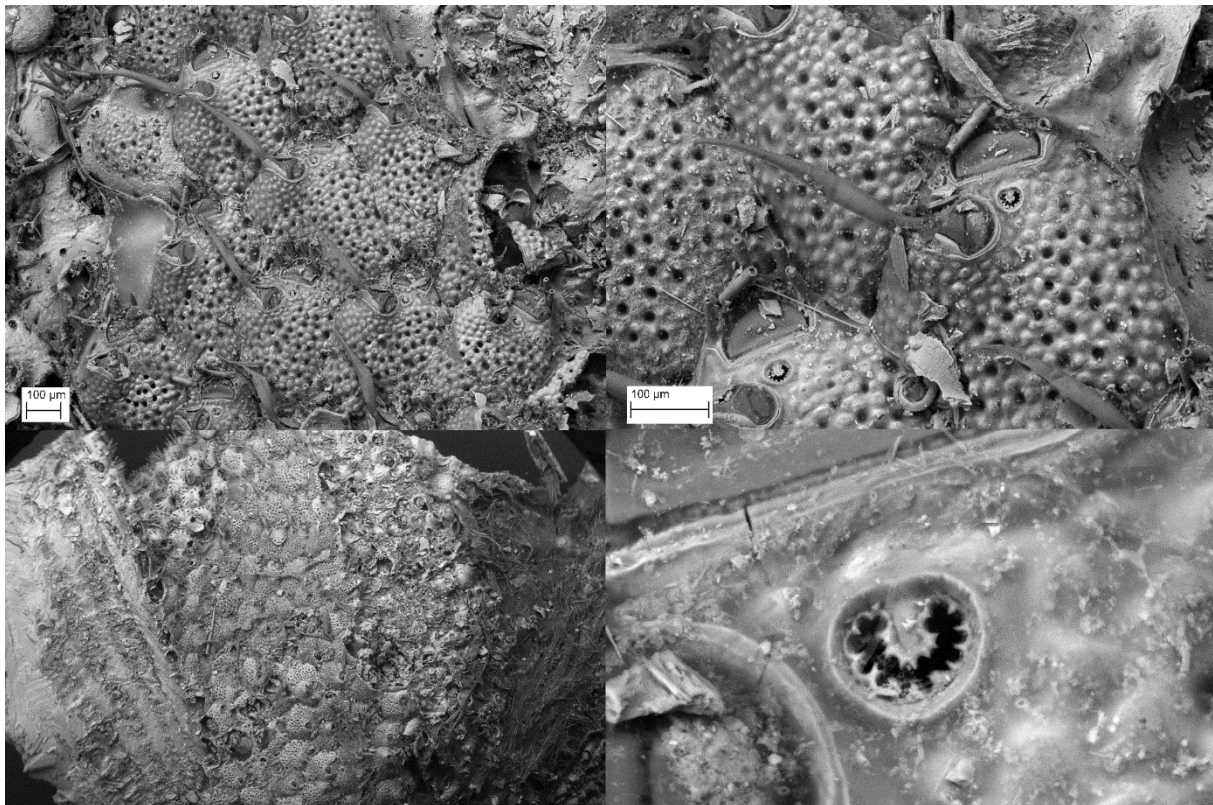
Figure 10. *Microporell cf. agonistes.* Top and bottom left: colony including several zooids, scale bars: 100 μm (top), 1 mm (bottom). Top right: zooid showing avicularia with long setiform mandible, 5 oral spines. Scale bar: 100 μm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 μm. Image credit: P. D. Taylor, NHMUK.

Microporella ordo Brown, 1952

Specimen ID: LHL33
Locality: New Zealand
ID verification: Dennis Gordon
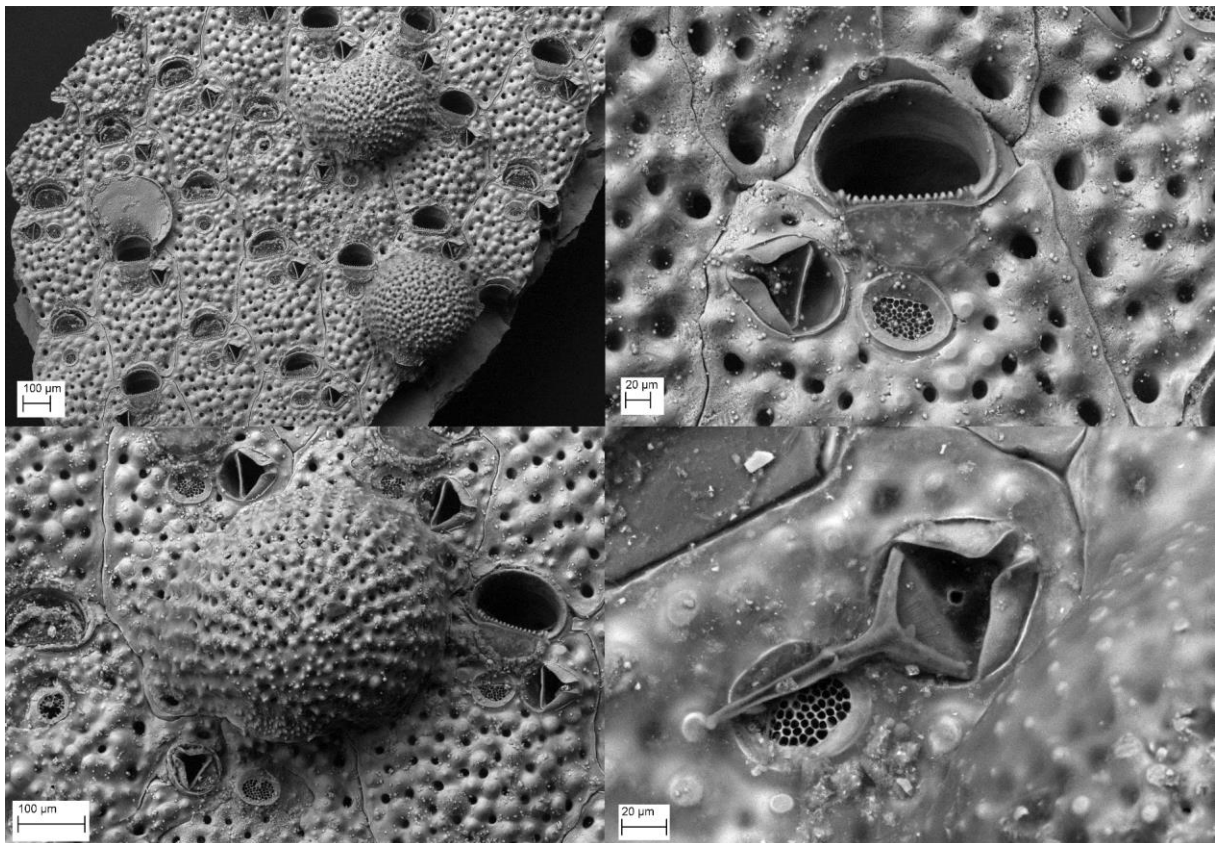
Figure 11. ***Microporell ordo.*** Top left: colony including several zooids, scale bar: 100 μm (top). Bottom left: ooecium. Top right: orifice with serrated proximal rim, no oral spines. Ascopore circular and porous, avicularia single. Scale bar: 20 μm. Bottom right: acvicularia with short mandible, scale bar: 20 μm. Image credit: P. D. Taylor, NHMUK.

***Microporella* cf. *neocribroides* Dick and Ross, 1988**

Specimen ID: LHL15, 25, 27 and 29

Locality: China and South Korea

Comment: *M. neocribroides* has its type location in Alaska, but has also been recorded in Japan (Suwa & Mawatari, 1998). This specimen resembles the one recovered from Japan in that it has a circular and porous ascopore, which is relatively small, with few pores. Similarly to the one recovered from Japan, the ascopore is "raised on a distinctively elevated plate-like mucro". The original description sites a single avicularia, while this specimen and the one from Japan may have single or paired avicularia. Unfortunately this specimen did not have any ovicells.

The specimen also resembles *Microporella inermis* Liu & Liu, 2001.
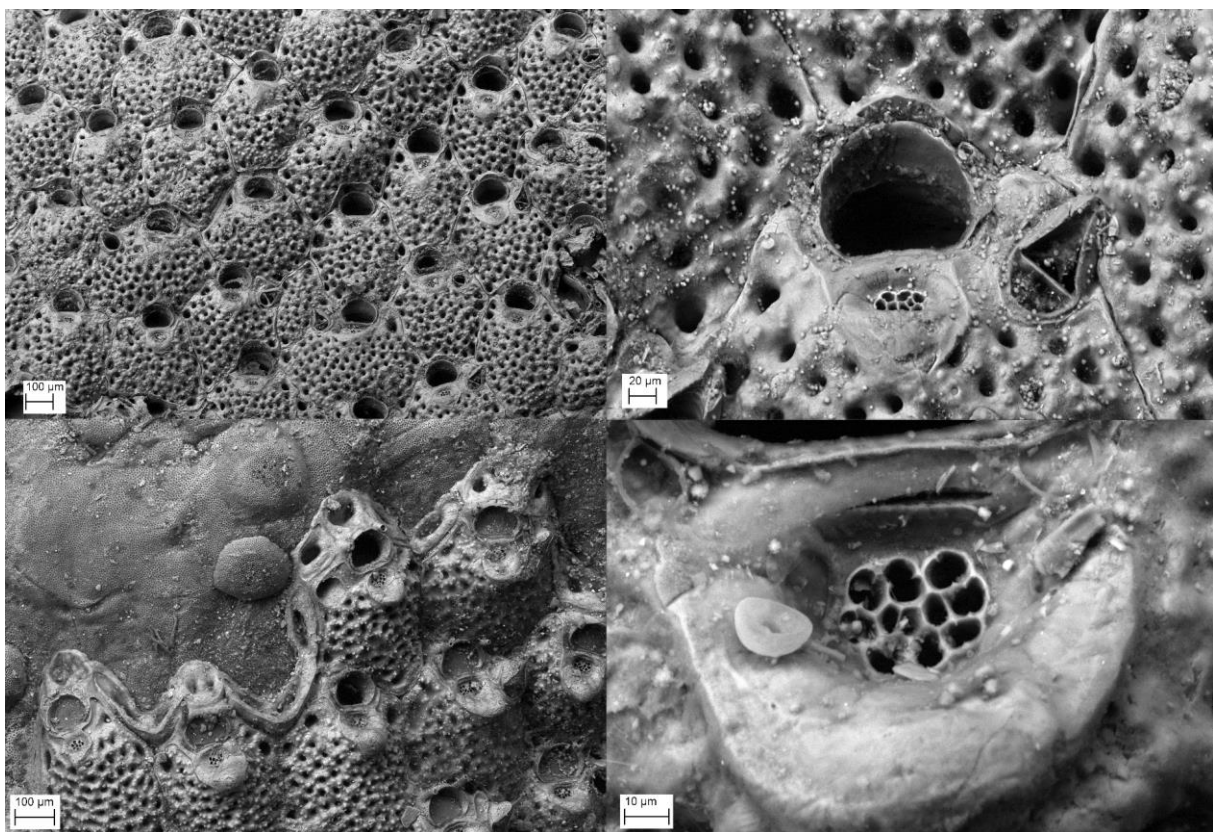
Figure 12. *Microporell cf neocribroides.* Top left: colony including several zooids. Scale bar: 100 μm. Bottom left: zooids at colony margin. Scale bar: 100 μm. Top right: zooid with smooth orifice, similar in length and width. Avicularia small, close to orifice.Scale bar: 20 μm. Bottom right: circular and porous ascopore, set within a "plate-like mucro". Scale bar: 10 μm. Image credit: P. D. Taylor, NHMUK.

Microporella speculum Brown 1952

Specimen ID: AW683-B
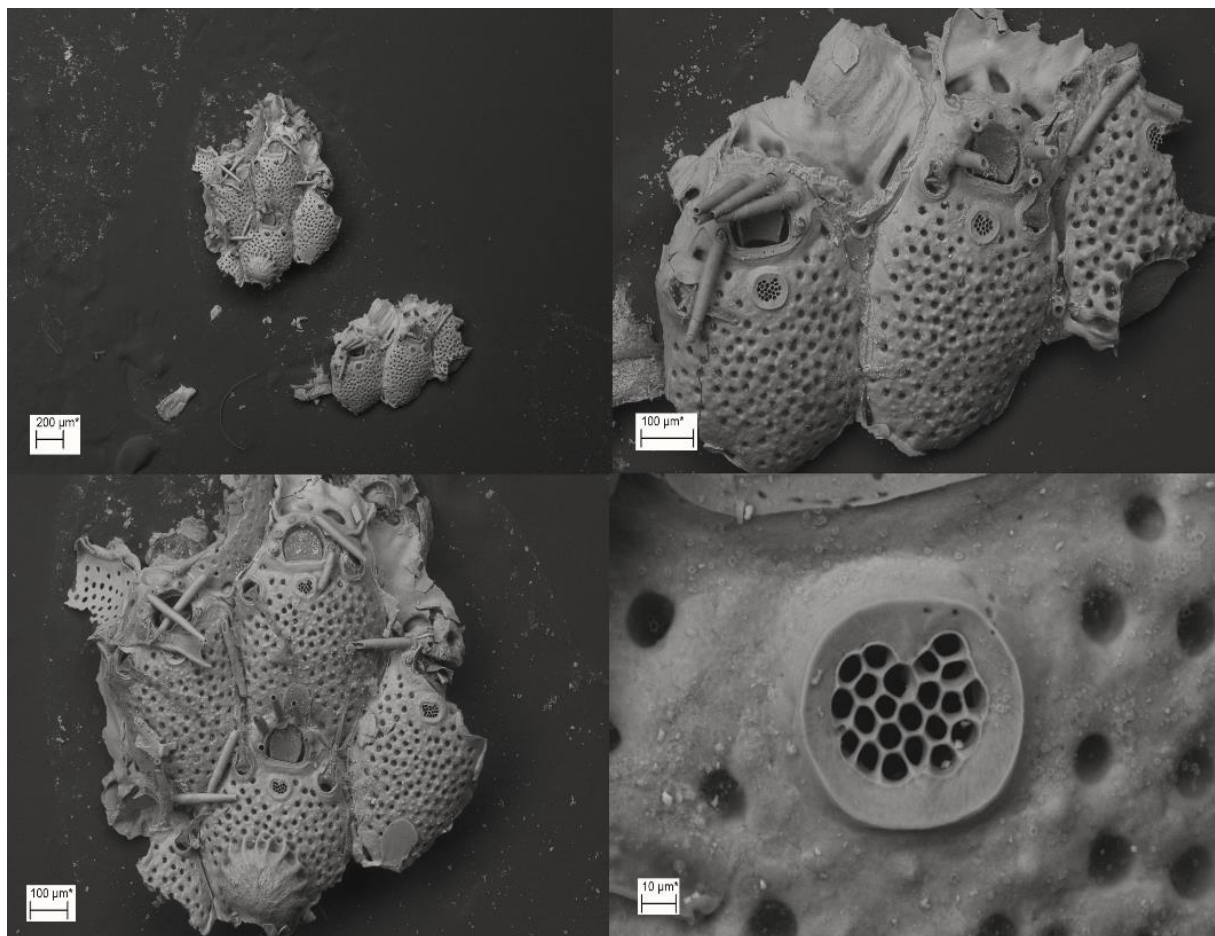Locality: New Zealand
ID verification: Dennis Gordon

Figure 13. *Microporell cf* **agonistes.** Top left: colony including a few zooids. Scale bar: 200 µm. Bottom left: Zooid showing avicularia with long setiform mandible, one ovicell present. Scale bar: 100 µm. Top right: 2 zooid showing 6 oral spines. Scale bar: 100 µm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 µm. Image credit: A. Waeschenbach, NHMUK.

Microporella sp.

Specimen ID: AW520

Locality: New Zealand

Comment: Gordon commented that this is likely an undescribed species. Unfortunately the specimen does not have ooecia, which is need for a species description.
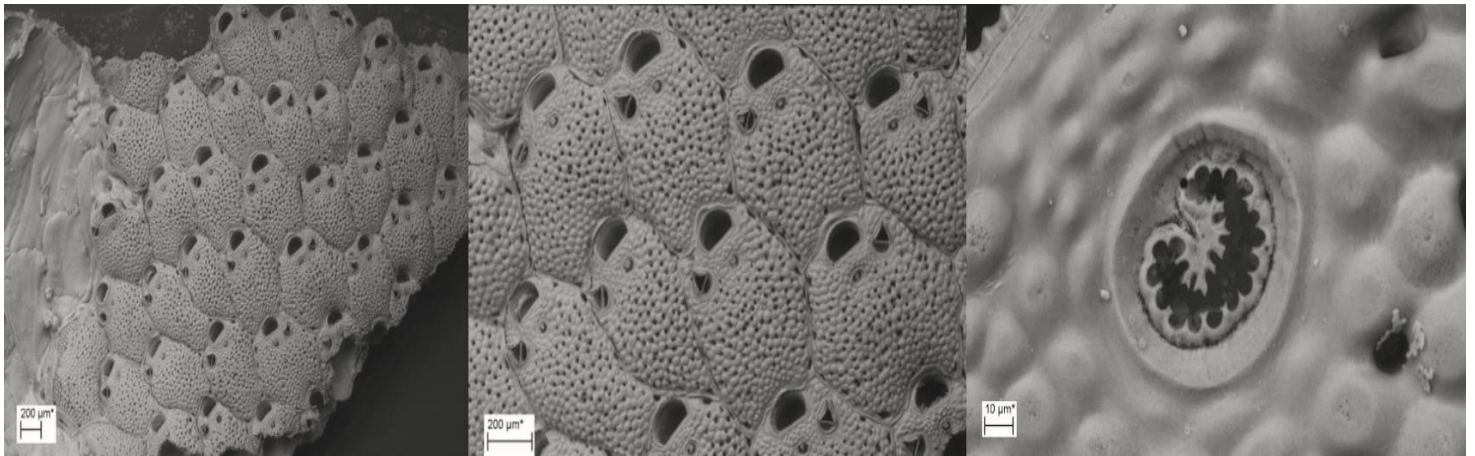
Figure 14. *Microporell sp.* Left: colony including several zooids, scale bar: 200 μm. Middle: zooids showing single avicularia and no oral spines. Scale bar: 200 μm. Right: crescentic and denticulate ascopore. Scale bar: 10 μm. Image credit: A. Waeschenbach, NHMUK.

Fenestrulina cf. littoralis Gordon 2009

Specimen ID: AW423

ID verification: Dennis Gordon

Locality: New Zealand

Comment: Gordon commented that this specimen has much in common with F. littoralis, however it differs from in that the ascopore is more complex and set within a slightly elevated disk-like area, and there is no umbo on the frontal shield. And that this could conceivably be a different but closely related species.

Figure 15. *Fenestrulina cf. littoralis.* Left: colony including several zooids, scale bar: 100 µm. Middle: zooids with 4 oral spines, smooth ooecia and large pores around the zooid margin. Scale bar: 100 µm. Right: crescentic and denticulate ascopore, set within a slightly elevated disk-like area. Scale bar: 10 µm. Image credit: A. Waeschenbach, NHMUK.

Fenestrulina sp.

Specimen ID: LHL30

Locality: South Korea

Comment: This specimen has large complex pores that cover most of the zooid frontal shield. The ascopore is crescentic and denticulate. The orifice is approximately equal in length and width and has 3 oral spines. This specimen has yet to be identified.
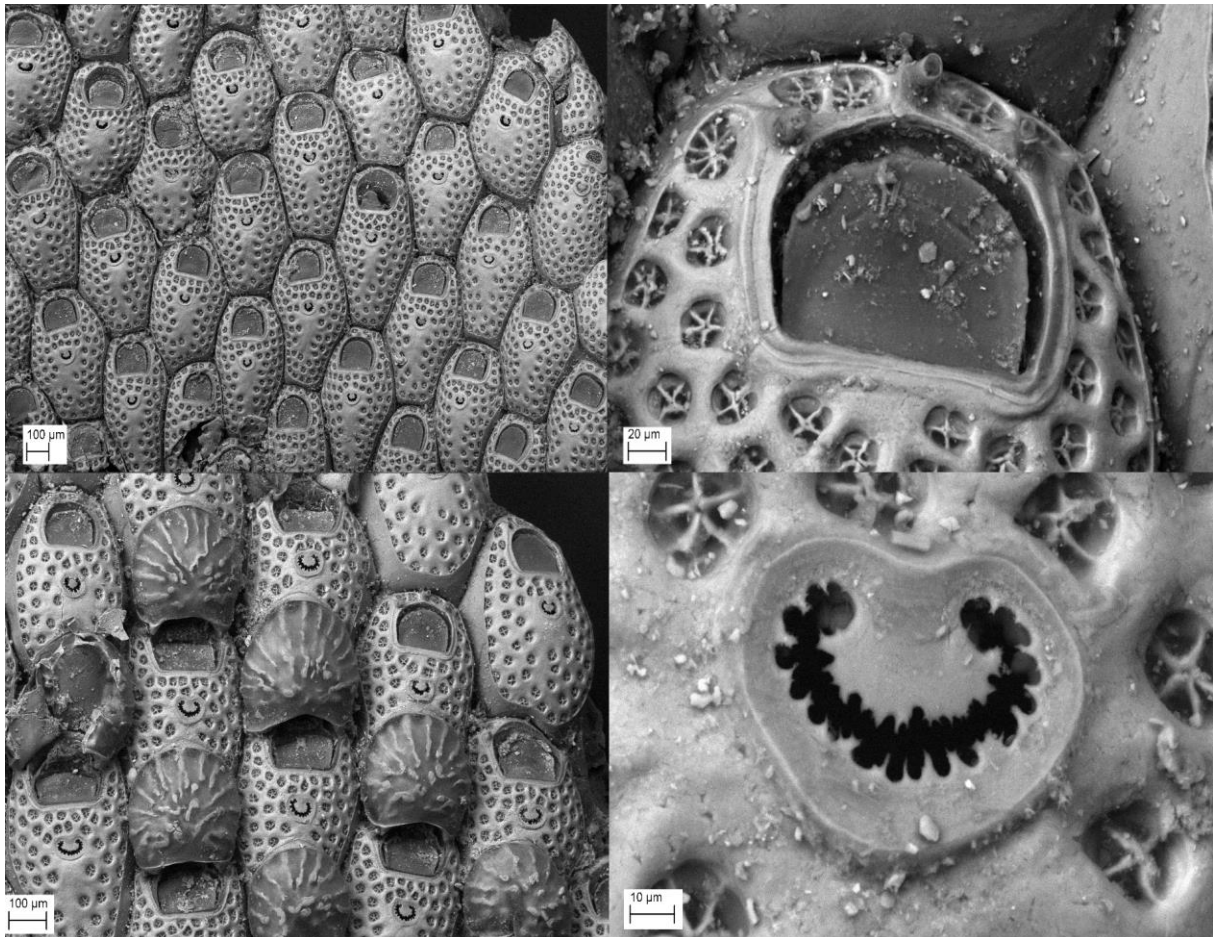

Figure 16. *Fenestrilna sp.* Top left: colony including several zooids, the zooid frontal walls are almost completely covered in pores. Scale bar: 100 µm. Bottom left: Ooecia with ridges. Scale bar: 100 µm. Top right: 2 zooid orifice, smooth, with 3 oral spines. Scale bar: 20 µm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 µm. Image credit: P. D. Taylor, NHMUK.

Fenestrulina malusii Audouin, 1826

Specimen ID: AW218
Locality: Norway

Comment: *F. malusii* is found in the NE Atlantic and Mediterranean. This specimen resembles the F. malusii described by Hayward & Ryland (1999) in that it has 2-3 oral spines, a broad ascopore which is crescentic and denticulate. The ovicell has ridges around the margin.
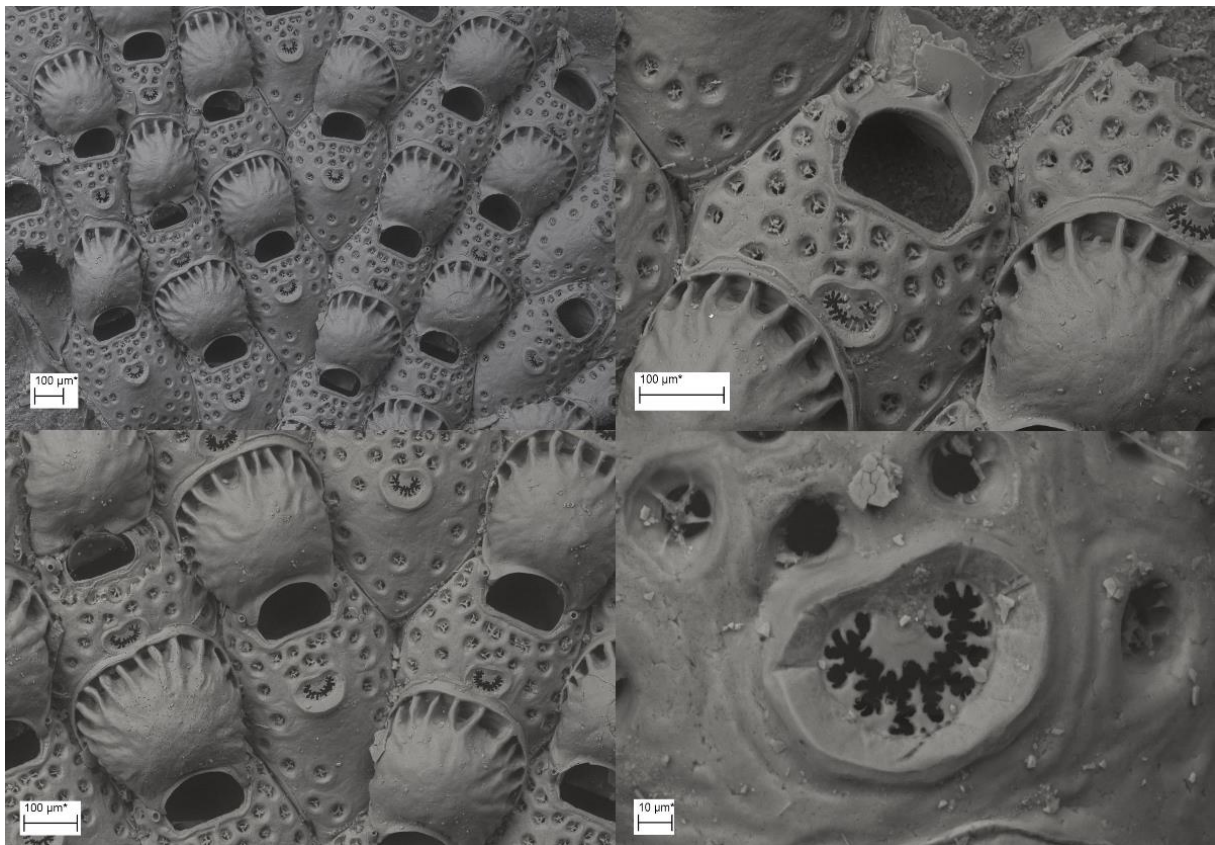

Figure 17. *Fenestrilna malusii.* Top left: colony including several zooids with many small pore. Scale bar: 100 μm. Bottom left: Zooids with ovicells and 2 oral spines. Scale bar: 100 μm. Top right: 2 zooid with smooth orifice. Scale bar: 100 μm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 μm. Image credit: A. Waeschenbach, NHMUK.

Fenestrulina specca Gordon, 1989

Specimen ID: AW436
ID verification: Dennis Gordon
Locality: New Zealand

Figure 18. *Fenestrilna specca.* Top left: colony including a few zooids. Scale bar: 200 μm. Bottom left: zooids with smooth ooecia smooth. Scale bar: 100 μm. Top right: zooid with large simple pores. 4 oral spines. Scale bar: 100 μm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 μm. Image credit: A. Waeschenbach, NHMUK.

Fenestrulina cf. orientalis 1 Liu & Liu, 2001

Specimen ID: LHL18
Locality: China

Comment: This sample is similar to *F. cf. orientalis* in that it has up to six oral spines in non-brooding zooids. Zooids commonly have two rows of marginal pores between the ascopore and the orifice. The ascopore is crescentic with a proximal umbo.
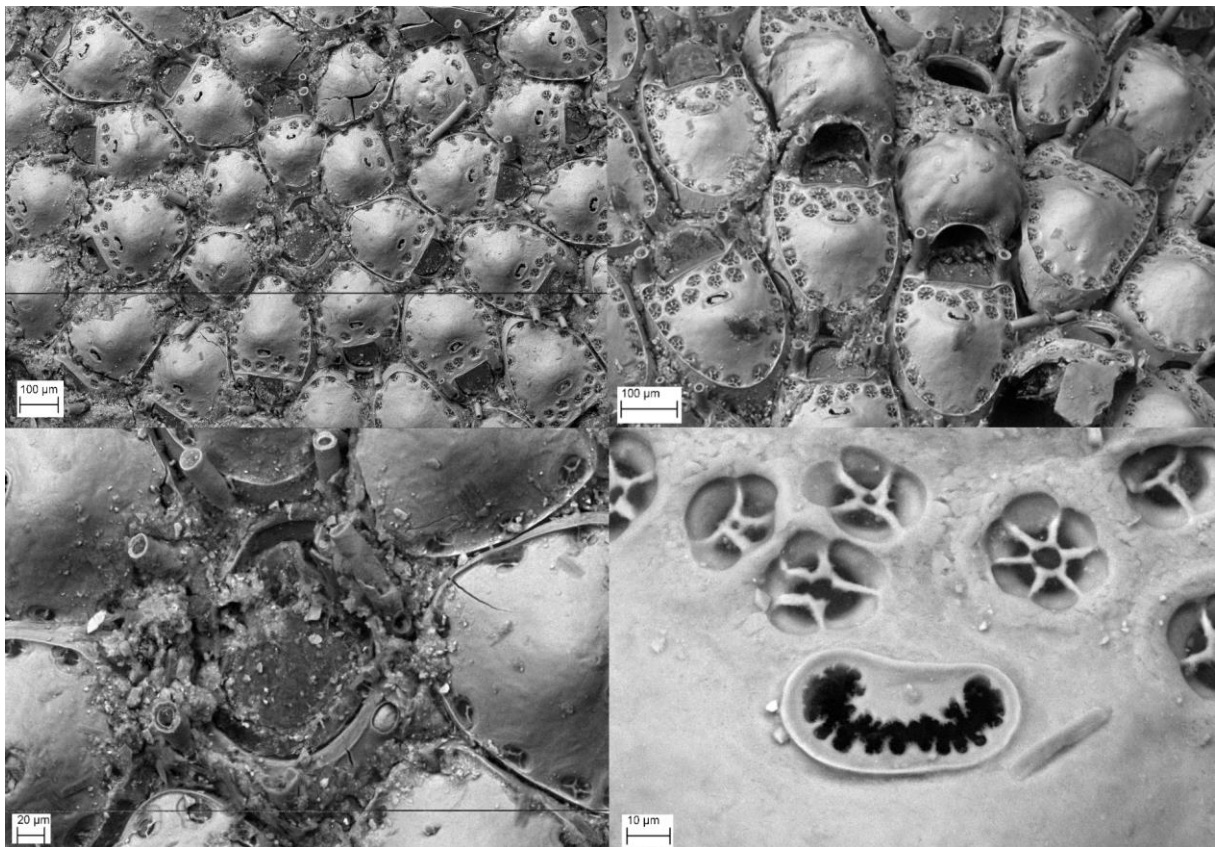

Figure 19. *Fenestrilna cf. orientalis.* Top left: ancestrula and early generation zooids. Scale bar: 100 µm. Bottom left: ancestrula. Scale bar: 20 µm. Top right: zooid with large complex pores. 3-4 oral spines. Scale bar: 100 µm. Bottom right: crescentic and denticulate ascopore, scale bar: 10 µm. Image credit: P. D. Taylor, NHMUK.

Fenestrulina cf. orientalis 2 Liu & Liu, 2001

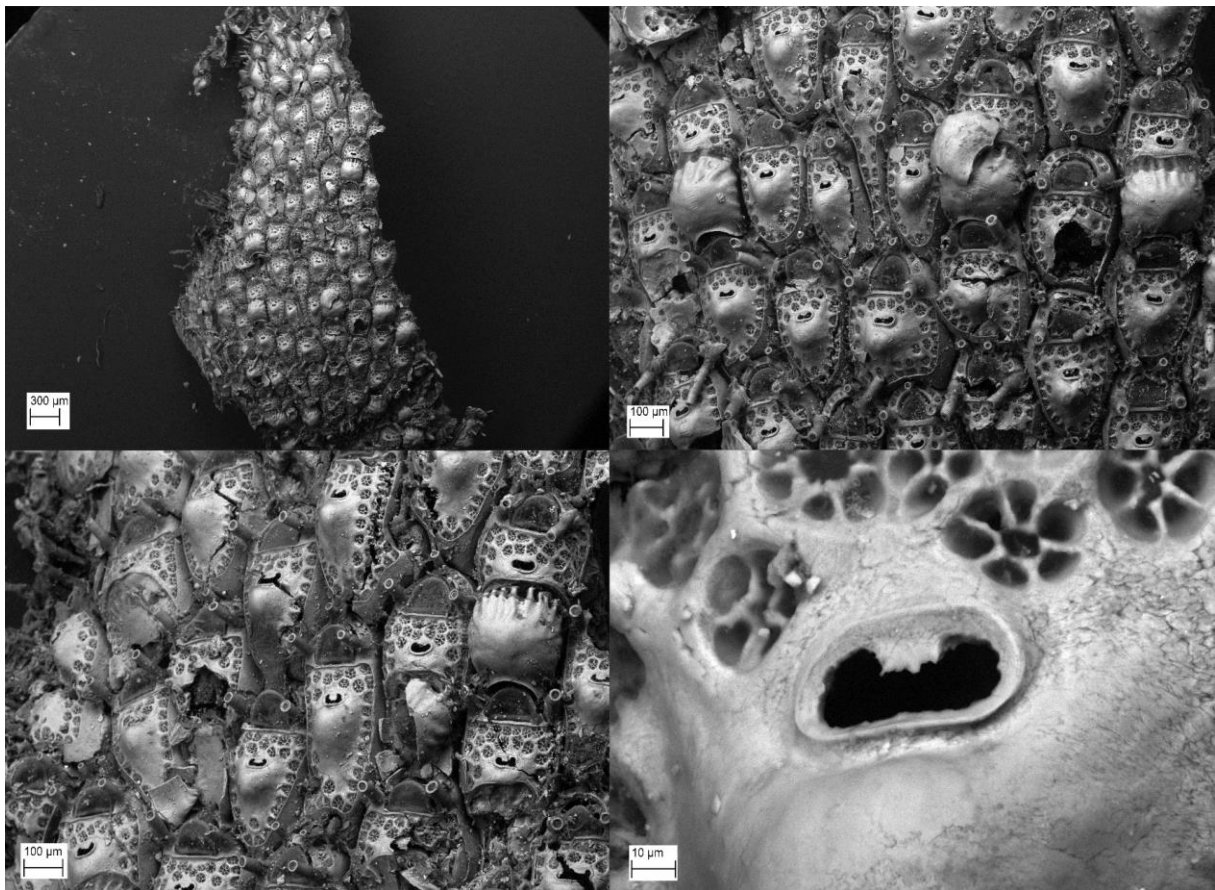Specimen ID: LHL28
Locality: China

Comment: see previous page.

Figure 20. ***Fenestrilna cf. orientalis.*** Top left: colony overview. Scale bar: 100 µm. Bottom left: zooids with smooth ooecium, ovicellate zooids with 2 oral spines, non-ovicellate zooids with 3. Complex pores around margin. Zooids with prominent umbo. Scale bar: 100 µm. Top right: zooid with large complex pores. 3-4 oral spines. Scale bar: 100 µm. Bottom right: rectangular to crescentic ascopore, scale bar: 10 µm. Image credit: P. D. Taylor, NHMUK.

Calloporina angustipora Hincks, 1885

Specimen ID: LHL31
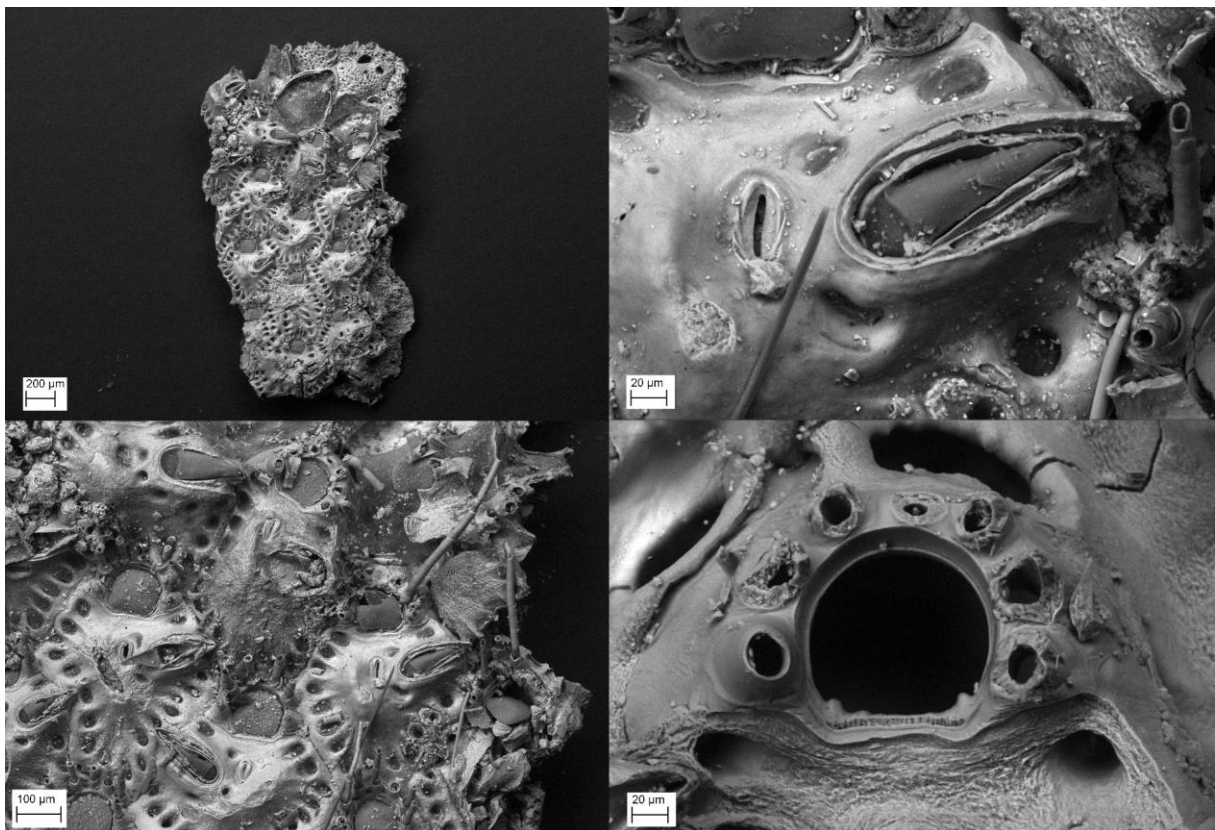Locality: New Zealand
ID verification: Dennis Gordon

Figure 21. *Calloporina angustipora.* Top left: colony overview. Scale bar: 200 μm. Bottom left: zooids diamond-shaped, with prominent areolar pores along the margin, Single avicularia. Long oral spines. Scale bar: 100 μm. Top right: Ascopore slit-like, large avicularia. Scale bar: 20 μm. Bottom right: orifice with condyles in corners, 7 oral spines. Scale bar: 20 μm. Image credit: P. D. Taylor, NHMUK.