Bryozoan genera *Fenestrulina* and *Microporella* no longer confamilial; multi-gene phylogeny supports separation

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Bryozoans are a moderately diverse, mostly marine phylum with a fossil record extending to the Early Ordovician. Compared to other phyla, little is known about their phylogenetic relationships at both lower and higher taxonomic levels. Hence, an effort is being made to elucidate their phylogenetic relationships. Here, we present newly sequenced nuclear and mitochondrial genes for 21 cheilostome bryozoans. Combining these data with existing orthologous molecular data, we focus on reconstructing the phylogenetic relationships of *Fenestrulina* and *Microporella*, two species-rich genera. They are currently placed in Microporellidae, defined by having a semicircular primary orifice and a proximal ascopore. Our six-gene phylogenetic analysis reveals that the genera *Fenestrulina* and *Microporella* are each monophyletic, with the sister clade to *Microporella* comprising non-microporellids. Our result hence supports the reinstatement of the family Fenestrulinidae Jullien, 1888 for *Fenestrulina* and genera with comparable frontal shield and ooecial morphologies. Our well-supported phylogeny, based on independent molecular data, lends credit to existing phylogenetic hypotheses based on morphological observations but does not conform to the current classification of these bryozoans. This illustrates the general need for a rethink of bryozoan higher level systematics, ideally based on both morphological and molecular data.

ADDITIONAL KEYWORDS: Bryozoa – cheilostomes – mitochondria – phylogenetic reconstruction – rRNA.

INTRODUCTION

Bryozoa is a metazoan phylum that boasts 6601 described extant species (pers. comm. to Phil Bock 2018) and a superb fossil record ranging back to the Ordovician (Ma et al., 2015). While their colonies are often small and inconspicuous, bryozoans are important members of benthic communities and contribute significantly to species diversity and sometimes to biomass, whilst providing food and habitat for diverse marine organisms. Yet, despite their ecological and evolutionary importance, not only are the phylogenetic relationships among bryozoan species and higher taxa uncertain, and currently largely based on morphological traits (Taylor & Waeschenbach, 2015), the exact placement of these colonial organisms in the metazoan tree also remains contentious (Ostrovsky, ...
1999; Cheetham et al. investigate ecological and evolutionary questions in the fossil record, providing an excellent system in which to study macroevolutionary questions. However, there is mounting evidence for a monophyletic Lophophorata in which Bryozoa and Phoronida are sister taxa (Nesnidal et al., 2013; Laumer et al., 2013; Kocot, 2016). As most bryozoans have a calcified skeleton, they have a relatively high preservation potential in the fossil record, providing an excellent system in which to investigate ecological and evolutionary questions in the deep past (Cheetham, 1986; Jackson & Cheetham, 1990, 1999; Cheetham et al., 1993, 1994; Liow et al., 2017). The study of macroevolutionary questions is greatly enhanced when addressed in a phylogenetic context, yet, only a handful of bryozoans have been sequenced for a few genes (Knight et al., 2011; Waeschenbach et al., 2012). As a comparison, there are about 7000 described species in the phylum Echinodermata, and a nucleotide search for ‘Echinodermata’ in the NCBI database returned 2.6 million hits, while ‘Bryozoa’ returned only 4622 hits (09.02.2018).

In the most comprehensive published bryozoan phylogeny to date, Waeschenbach et al. (2012) sequenced the nuclear genes’ small ribosomal subunit RNA (SSU/18S) and large ribosomal subunit RNA (LSU/28S), and five mitochondrial genes (Cytochrome c oxidase subunit 1 (COX1), Cytochrome c oxidase subunit 3 (COX3), Cytochrome b (Cytb), small ribosomal subunit (rrnS/12S), and large ribosomal subunit (rrnL/16S)) and incorporated published orthologous data in their phylogenetic inferences. Despite the increasing universality of including sequence data in evolutionary and ecological studies of other groups of organisms, only a handful of these studies added limited information to the pool of molecular sequence data for the four extant clades of bryozoans, namely: cyclostomes (e.g. Waeschenbach et al., 2009; Taylor et al., 2015); ctenostomes (e.g. Waeschenbach et al., 2015); phylactolaemates (e.g. Hartikainen et al., 2013); and cheilostomes (e.g. Vieira et al., 2012; Fehlauer-Ale et al., 2015). The challenge of generating molecular sequences of this understudied phylum is exacerbated by the small colony size of many taxa and their living in close proximity with other biota. As a result, many of the sequences deposited in GenBank that are attributed to bryozoans have been shown to be contaminants (see supplementary material in Waeschenbach et al., 2012).

Here, we aim at resolving the phylogenetic relationships of two target genera in a large cheilostome bryozoan family, Microporellidae Hincks, 1879, currently comprising nine genera (Taylor & Mawatari, 2005). The traditional main defining traits of this family are semicircular orifices and an ascopore (entrance to a hydrostatic compensation sac for tentacle-crown eversion) in the frontal wall (Hincks, 1879; Hayward & Ryland, 1999). The two most species-rich genera in the family Microporellidae are Fenestrulina Jullien, 1888 and Microporella Hincks, 1877, both of which are globally distributed in the marine realm today (Hincks, 1877; Jullien, 1888).

The main differences in morphology between Fenestrulina and Microporella are the form of the skeletal frontal shield, pseudopores and ooecium, and the presence or absence of avicularia (Fig. 1). Microporella accommodates those microporellids having an evenly pseudoporous and granulartubercular frontal shield, a calcified endooecium that is connected with the proximal part of the frontal shield of the distal zooid (Ostrovsky, 2013) and single or paired avicularia that extend to the basal wall (Hastings, 1963).

The frontal shield in Fenestrulina is much more diverse than in Microporella; it is frequently less evenly pseudoporous and sometimes has a variably developed area of gymnocyst laterally and proximally. Pseudopores in Fenestrulina typically have complex radii and the calcified endooecium is almost never evenly pseudoporous. Note that the ascopore is often close to the orifice in Microporella, with no intervening pseudopores, and more distant in Fenestrulina, which has one or more rows of pseudopores between the orifice and ascopore.

Moreover, the ooecial coelomic cavity (the space between the membranous ectoecium and the calcified endooecium) is confluent with the hypostegal coelom of distal autozooids in Microporella, whereas the ooecial coelomic cavity communicates with the zooidal visceral coelom via a special pore in Fenestrulina (Ostrovsky, 2013). In fact, these differences are substantial enough to support, on morphological grounds, assigning the two genera to different families. In addition, avicularia have been reported in only one species of Fenestrulina (as Fenestraloides (Soule, Soule & Chaney, 1995)) in an extreme proximal position, rather than latero-suborally.

We know from other molecular studies that the phylogenetic positioning of bryozoan taxa is often incongruent when based on simple shared morphology (Waeschenbach et al., 2012; Taylor & Waeschenbach, 2015; Taylor et al., 2015). Given suspicions, based on morphological observations, that Fenestrulina and Microporella should not be congeneric, we tested whether the genera Fenestrulina and Microporella are each monophyletic, and whether they belong in the same family, using molecular data generated in this study.

**METHODS**

**SAMPLES**

Cheilostome bryozoans sequenced in this study were mainly from New Zealand, with additional samples from China, Norway and Scotland (Supporting
Information, Table S1). Samples were exported to Norway according to the protocols of the countries of origin and all lab work was conducted at the University of Oslo. Morphological vouchers from the sequenced colonies were dried and scanning electron micrographs (SEM) were taken (Supporting Information, Fig. S8). Metadata associated with our samples are reported in Supporting Information, Table S1.

### DNA ISOLATION AND SEQUENCING

Ethanol-preserved samples were dried and rinsed in phosphate-buffered saline before genomic DNA was isolated using the DNeasy Blood and Tissue kit following manufacturer’s instructions (QIAGEN, Germantown, MD, USA). Colonies were homogenized in lysis buffer, using a pestle, in the presence of proteinase K (50 µg/mL). DNA templates were either targeted for PCR and Sanger sequencing, or sequenced directly by high-throughput sequencing, HTS, using Illumina HiSeq (Table 1).

For PCR and subsequent Sanger sequencing, genes 18S, 12S, 16S, COX1, COX3 and Cytb were targeted for amplification using primers outlined in Waeschenbach et al. (2012), and those specifically designed for this study (Supporting Information, Table S2). Primers were designed with Primacleade (Gadberry et al., 2005), and OligoCalc (Kibbe, 2007) was applied to check self-complementarity and to calculate primer annealing temperature (Tm). PCR was performed with DreamTaq DNA polymerase or Phusion high-fidelity DNA polymerase (Thermofisher Scientific) in the presence of 2.5% DMSO; PCR conditions are outlined in Supporting Information, Table S3. PCR products were purified with Wizard SV gel and PCR Clean-up system (Promega) following standard protocol. Sanger sequencing was performed by GATC Biotech (Konstanz, Germany).

For HTS, samples were processed at the Norwegian Sequencing Centre (Oslo, Norway) using Illumina HiSeq2500 125 bp paired-end (PE) sequencing with a 350 bp insert size (see Table 1).

### SEQUENCE ASSEMBLY AND ALIGNMENT

Sanger reads from 14 species (Table 1) were quality trimmed using a Phred quality score of 40 (Ewing & Green, 1998). Contig assembly was performed in SEQUENCHER 5.1 (GeneCodes Corporation, Ann Arbor, MI, USA).

Illumina HiSeq reads were quality and adapter trimmed using TrimGalore v.0.4.4 (https://www.bioinformatcs.babraham.ac.uk/projects/trim_galore/) and assembled with SPAdes 3.11.1 (Bankevich et al., 2012) with k-mers of 33, 55, 77, 99 and 121, before final genome polishing with Pilon (Walker et al., 2014).

Orthologous genomic sequences were identified with blastn in CLC main workbench 7 (Qiagen, Hilden, Germany).

Protein-coding genes (COX1, COX3 and Cytb) were translated into amino acids according to the invertebrate mitochondrial code using the ‘translate’ tool in ExPASy (https://web.expasy.org/translate/). Suitable orthologous sequences for each gene deposited in the NCBInr database were downloaded and aligned with MAFFT (Katoh & Standley, 2013) using default parameters: for the rRNA genes (18S, 12S and 16S), the Q-INS-i model, considering secondary RNA structure, was utilized; for the protein-coding genes, the G-INS-I model was used. The six separate alignments were edited manually using MESQUITE v.3.1 (Maddison & Maddison, 2017).

Ambiguously aligned characters were removed from each alignment using Gblocks (Talavera & Castresana, 2007) with the least stringent parameters.

A sampling rule was established to limit the amount of missing data in the matrix, whilst maintaining a broad taxon sample; Fenestrulina or Microporella (our target genera) needed three of the six genes to be included in the analysis. All other taxa needed 18S rRNA and at least three other genes.

The six single-gene alignments were concatenated using catfasta2phyml perl script (https://github.com/nylander/catfasta2phyml). The final dataset consisted of 38 taxa and 3726 characters, of which 749 were amino acids. The alignments (both masked and unmasked) have been made freely available through Dryad (https://datadryad.org/) using the following DOI (doi:10.5061/dryad.j3f08d2).

### PHYLOGENETIC RECONSTRUCTION

Maximum likelihood (ML) phylogenetic analyses were carried out for each single gene using the ‘AUTO’ parameter in RAxML v.8.0.26 (Stamatakis, 2006) to establish the evolutionary model with the best fit. The general time reversible (GTR) was the preferred model for the three rRNA genes (18S, 12S and 16S), and MtZoa for the three mitochondrial protein-coding genes (COX1, COX3 and CytB). The topology with the highest likelihood score of 100 heuristic searches was chosen. Bootstrap values were calculated from 500 pseudoreplicates. Taxa with unstable phylogenetic affinities were identified and removed (following previously outlined sampling rules) using RogueNaRok (Aberer et al., 2013) based on evaluation of a 70% majority rule (MR) consensus tree.

The concatenated dataset, divided into six gene partitions, each with a separate gamma distribution, was analysed using RAxML, as outlined above. Bayesian inference (BI) was performed using a modified version of MrBayes v.3.2 (Huelsenbeck &
Ronquist, 2001) incorporating the MtZoa evolutionary model (https://github.com/astanabe/mrbayes5d).

The dataset was executed, as before, with six genes partitions each under a separate gamma distribution. Two independent runs, each with three heated and one cold Markov Chain Monte Carlo (MCMC) chain,

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Table 1. Species, sequence method and accessions numbers of genes used in this study. Names in bold represent species for which molecular data were generated during this study. SEMs for each species are provided in Supporting Information, Figure S8, except Microporella sp. from Qingdao (China) for which we do not have a voucher of the same colony we sequenced. *The accepted name for this species is Oshurkovia littoralis (Hastings, 1944): http://www.marinespecies.org/aphia.php?p=taxdetails&id=146830

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<th>Species (samples)</th>
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were started from a random starting tree. The MCMC chains were run for 40 000 000 generations with trees sampled every 1000th generation. The posterior probabilities and mean marginal likelihood values of the trees were calculated after the burn-in phase, which was determined from the marginal likelihood scores of the initially sampled trees. The average split frequencies of the two runs were <0.01, indicating the convergence of the MCMC chains.

RESULTS AND DISCUSSION

We here present 103 newly sequenced gene copies from 21 cheilostome bryozoan species (Table 1). Their sequences are deposited in GenBank with the accession numbers MG977029-MG977131. Newly generated and previously published orthologous sequences (Table 1) were used to infer the concatenated six-gene phylogeny shown in Figure 2. While we present a ML topology in Figure 2, the Bayesian tree topology is topologically comparable (Supporting Information, Fig. S1). For completeness, we also present ML single-gene trees (Supporting Information, Figs S2–S7).

BROAD PHYLOGENETIC RELATIONSHIPS AMONG CHEILOSTOME BRYOZOANS

The separation of outgroup taxa (Ctenostomata) from ingroup taxa (Cheilostomata) (Fig. 2) was highly supported with 98 nodal bootstrap support (BS) and 1.00 posterior probability (PP). The earliest diverging cheilostome clade (100BS/1.00PP) in Figure 2 constituted four anascan-grade genera (in which the primary cuticular frontal wall is typically unprotected by a calcified frontal shield and there is no compensation sac) – Scruparia, Aetea, Electra and Membranipora, currently classified in three suborders – Scrupariina, Aeteina and Membraniporina (Cook et al., 2018). The remainder of the tree comprises neocheilostomes in the suborder Flustrina, all of which have lecithotrophic larvae. The first Flustrina clade comprises a mixture of anascan- (Callopora, Flustra and Otonellina) and ascoporan-grade genera (with a compensation sac and a protective frontal shield; Celloporella), with support values of 78BS/0.99PP; a comparable topology has been recovered previously (Waeschnebach et al., 2012). The next lineage to diverge is represented by two species from the family Catenicellidae (100BS/1.00PP), typified by erect jointed colonies and gymnocystal frontal shields with (Costaticella) or without (Orthoscuticella) costae and having large pseudopores. This family, the representatives of which are here sequenced for the first time, has a highly supported placement in the tree. It is sister to the main clade, which contains only ascoporan-grade genera, which contains species with lepralioid and umbonuloid frontal shields. The polyphyletic nature of anascan- and ascoporan-grade cheilostomes in our tree corroborates similar findings of the non-validity of such traditional groupings of cheilostome bryozoans based on key morphological characters (Dick et al., 2009; Fuchs et al., 2009; Knight et al., 2011; Waeschnebach et al., 2012).

THE SEPARATION OF FENESTRULINA AND MICROPORELLA

In the World Register of Marine Species (Bock & Gordon, 2018), the family Microporellidae consists of two species-rich genera, Fenestrulina and Microporella, and six relatively species-poor genera: Flustramorpha Gray, 1872, Diporula Hincks, 1879, Callopora Neviani, 1896, Adelascopora Hayward & Thorpe, 1988, Tenthrenulina Gordon, 1984 and Chronocerastes Gordon, 1989. An additional genus, Fenestruloides Soule, Soule & Chaney, 1995, was established to include not only one Fenestrulina-like species with an avicularium, but other similar non-aviculiferous species with a dense distribution of pseudopores on their frontal shields. This is a variable character, however, and the World Register of Marine Species (Bock & Gordon, 2018) currently treats Fenestruloides as a junior subjective synonym of Fenestrulina.

We have increased the amount of sequence data substantially among species of these two genera and demonstrate for the first time that Fenestrulina (99BS/1.00PP) and Microporella (100BS/1.00PP) are each a separate monophyletic lineage within a larger strongly supported monophyletic group (96BS/1.00PP). However, and in contradiction to apparent morphological similarities (Fig. 1), these genera have a polyphyletic relationship, separated by three well-supported nodes (indicated with parentheses and bold font in Fig. 2). They are polyphyletic and, hence, should not be placed in the same family, Microporellidae.

In Figure 2, Fenestrulina forms a monophyletic group together with Hippomennella and Escharoides with medium nodal support (67BS/0.98PP), the latter two genera belonging to the family Romancheinidae. This clade is further extended to include Chiastosella (Escharinidae) with moderate ML and full BI nodal support (73BS/1.00PP). All these genera, however, share almost no clearly synapomorphic characters with Fenestrulina and it is clear that further taxonomic and gene sampling is necessary to clarify these relationships.

The sister clade (i.e. Microporella + Clade A in Fig. 2.) to the above-mentioned clade is supported by moderate ML and full BI support values (72BS/1.00PP). The well-supported sister group to
Microporella (Clade A; 85BS/1.00), consists of members of several families (Umbonulidae, Cryptosulidae, Watersiporidae, Bitectiporidae, Celleporidae, Phidoloporidae and Schizoporellidae), including the previously unsequenced genera Bitectipora, Galeopsis and Rhynchozoon.

As described by Ostrovsky (2013), Fenestrulina and Microporella differ in ooecial structure. The endooecium in Microporella is fully calcified, typically with small, blind pits (‘pseudopores’), the whole layer separated from the cuticular ectooecium by a very narrow coelomic cavity that is confluent with the hypostegal coelom of the distal zooid. In Microporella, ovicell closure is either acleithral or cleithral. In Fenestrulina, the ectooecium is likewise cuticular but the calcified entooecium lacks pseudopores or pits. Owing to a raised peripheral strip of gymnocrystal calcification to which the ectooecium attaches, the ooecial coelom in Fenestrulina is not confluent with the hypostegal coelom of the distal zooid. Here, the ovicell is subcleithral (for definitions see: Ostrovsky, 2013).

Given the overwhelming lack of support for Fenestrulina and Microporella being confamilial, we herein resurrect the family name Fenestrulinidae Jullien, 1888. The separation of these two long-associated genera underscores the necessity to clarify morphology-based phylogenies by using sequence data. In passing, we note that Jullien did not segregate Fenestrulinidae from Microporellidae because of perceived morphological differences; rather he argued that, because a British colleague included the gymnocrystal-shielded genus Chorizopora in Microporellidae, he deemed this family to be then too heterogeneous to have continuing validity; hence, Fenestrulinidae was a replacement name. This action in itself was invalid, as family names are based on the type genus, not a constituent suite of genera; however, since Fenestrulinidae is based on its own valid type genus, it can stand.

Figure 1. A comparison of Microporella and Fenestrulina. A, Microporella ordo Brown from Spirits Bay, New Zealand (pdt6245) and, B, Fenestrulina from Korea (pdt19310) for comparison. The white scale bars are 100 microns.
THE EVOLUTION OF MICROPORELLIDAE (SENSU LATO) AND INFERRED RELATIVES

Notwithstanding the lack of sequence data for other microporellid (sensu lato) genera, our results reflect the distinctions in frontal-shield morphology, ooeica and polymorphs that were already evident between Microporella and Fenestrulina. Accordingly, we hypothesize that further sequence data will show Adelascopora and Tenthrenulina to be confamilial with Fenestrulina, owing to some shared features of frontal-shield and ooeicial morphology. For example, species of Adelascopora have zooidal frontal shields that are nearly identical to those of some species of Fenestrulina, but their colonies are erect and bilamellate, their frontal-shield pores are non-radiate, and they have large, multiporous mural septula instead of basal pore chambers in the lateral and transverse walls. Like Fenestrulina, the sole species of Tenthrenulina, lacks avicularia and has basal pore-chambers, but the pseudopores are deep, the ascopore is immediately subjacent to the orificial rim and the ooeical surface is flush with the zooidal surface and has strong frontal ribbing. Similarly, we hypothesize that Diporula and Flustramorpha will ally with Microporella in molecular phylogenies. Frontal shields in all three genera are alike, but Diporula and Flustramorpha have distinctive erect colony forms, with only small, multiporous septula in lateral walls in Diporula (not yet described in Flustramorpha).

On the other hand, Calloporina and Chronicerastes may be wholly unrelated to either Microporellidae or Fenestrulinidae. Both genera have mostly or wholly non-pseudoporous frontal shields that differ from those in both Microporella and Fenestrulina and have ooeica that structurally resemble those in Chiastosella Canu & Bassler in Bassler, 1934 (family Escharinidae), i.e. with a distal crescentic pseudoporous area on the ooeicum (Bassler, 1934). Brown noted this similarity and suggested that Calloporina and Chiastosella were closely related to each other and unrelated.
to *Microporella* (Brown, 1952). Two years later, he described a new species from New Zealand, *Chiastosella enigma*, with a narrow orificial sinus that seemed to bridge the morphological gap to *Calloporina* species with a narrow slit-like ascopore, and he argued that *Calloporina* and *Chiastosella* ought to be considered congeneric (Brown, 1954). DNA sequencing may yet bear this out, as well as supporting the long-held hypothesis that the ascopore in Microporellidae evolved by the ontogenetic separation of the orificial sinus (i.e. the ascus opening) from the orifice (Hamer, 1902; Levinsen, 1909). These authors suggested a schizoporellid (*sensu lato*) ancestor. The genus morphologically closest to *Microporella* that lacks an ascopore, but which has a similar frontal shield, aperture and ooecium, plus paired avicularia with pivot bars and a narrow orificial sinus, is *Taylorius* Gordon, 2014, currently in Escharinidae (Gordon, 2014). Escharinidae itself is morphologically heterogeneous and is likely to be split pending molecular data. Lastly, a family that is morphologically close to Fenestrulinidae, but which has zooids in erect, cylindrical branches, is Calwelliidae. Their zooids have an ascopore, but no pseudopores or avicularia. Calwelliidae and Fenestrulinidae might be sister clades.

All of these hypothesized additional relationships among genera of Microporellidae *sensu lato* and putative relatives can be tested once enough sequence data become available. Importantly, molecular data can be combined with morphological data from abundant bryozoan fossils (especially where fine-grained sequence stratigraphy is available) to achieve a more robust phylogeny.

We also note that a couple of *Fenestrulina* and *Microporella* species that we sequenced may be new species that require full taxonomic treatment (see *Microporella* species that we sequenced may be new for discussions). A more robust phylogeny.

**CONCLUSIONS**

The Cenozoic diversification of cheilostome bryozoans has given rise to clades that are impressively rich in both morphological and species diversity. It remains unclear how many times, when or in which clades several key morphological innovations appeared *de novo* or convergently, and these include ovicells, frontal shields and hydrostatic compensation spaces, avicularia (Lidgard et al., 2012). In this study, we have substantially increased the taxon sampling in the most derived part of the bryozoan tree and established with strong nodal support that *Fenestrulina* and *Microporella* are not confamilial. This study further highlights the importance of increasing taxon sampling for molecular phylogenies to resolve evolutionary relationships within Bryozoa, while integrating morphological insights.

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**REFERENCES**


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

**Table S1.** Metadata.

**Table S2.** Primers.

**Table S3.** PCR cycling conditions.

**Figure S1.** A concatenated six-gene Bayesian tree with posterior probability support values.

**Figure S2.** 18S ML single gene tree with bootstrap.

**Figure S3.** 16S ML single gene tree with bootstrap.

**Figure S4.** 12S ML single gene tree with bootstrap.

**Figure S5.** COX1 ML single gene tree with bootstrap.

**Figure S6.** COX3 ML single gene tree with bootstrap.

**Figure S7.** Cytb ML single gene tree with bootstrap.

**Figure S8.** A powerpoint file of the SEM plates of voucher specimens with brief descriptions.