Boring systematics: a genome skimmed phylogeny of ctenostome bryozoans and their endolithic family Penetrantiidae with the description of one new species

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Abstract

Ctenostomes are a group of gymnolaemate bryozoans with an uncalcified chitinous body wall having few external, skeletal characters. Hence, species identification is challenging and their systematics remain poorly understood, even more so when they exhibit an endolithic (boring) lifestyle. Currently, there are four Recent families of endolithic bryozoans that live inside in mineralized substrates like mollusk shells. In particular, Penetrantiidae Silén, 1946 has received considerable attention and its systematic affinity to either cheilostomes or ctenostomes has been debated. Species delimitation of penetrantiids remains difficult, owing to a high degree of colonial and zooidal plasticity. Consequently, an additional molecular approach is essential to unravel the systematics of penetrantiids, their phylogenetic placement and their species diversity. We therefore sequenced the mitochondrial (mt) genomes and two nuclear markers of 27 ctenostome species including nine penetrantiids. Our phylogeny supports the Penetrantiidae as a monophyletic group placed as sister taxa to the remaining ctenostomes alongside paludicellids and arachnoidids. Our results also suggest that the endolithic lifestyle evolved at least twice independently within ctenostomes, since the boring families Terebriporidae d'Orbigny, 1847 and Penetrantiidae are well separated. Ctenostome paraphyly is supported by our data, as the cheilostomes nest within them. A Multiporata clade is also well supported, including the former victorelloid genus Sundanella. Altogether, this study provides new insights into ctenostome systematics, assists with species delimitation and contributes to our understanding of the bryozoan tree of life.

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Abstract

Ctenostomes are a group of gymnolaemate bryozoans with an uncalcified chitinous body wall having few external, skeletal characters. Hence, species identification is challenging and their systematics remain poorly understood, even more so when they exhibit an endolithic (boring) lifestyle. Currently, there are four Recent families of endolithic bryozoans that live inside in mineralized substrates like mollusk shells. In particular, Penetrantiidae Silen, 1946 has received considerable attention and its systematic affinity to either cheilostomes or ctenostomes has been debated. Species delimitation of penetrantiids remains difficult, owing to a high degree of colonial and zooidal plasticity. Consequently, an additional molecular approach is essential to unravel the systematics of penetrantiids, their phylogenetic placement and their species diversity. We therefore sequenced the mitochondrial (mt) genomes and two nuclear markers of 27 ctenostome species including nine penetrantiids. Our phylogeny supports the Penetrantiidae as a monophyletic group placed as sister taxa to the remaining ctenostomes alongside paludicellids and arachnoidids. Our results also suggest that the endolithic lifestyle evolved at least twice independently within ctenostomes, since the boring families Terebriporidae d'Orbigny, 1847 and Penetrantiidae are well separated. Ctenostome paraphyly is supported by our data, as the cheilostomes nest within them. A Multiporata clade is also well supported, including the former victorelloid genus *Sundanella* . Altogether, this study provides new insights into ctenostome systematics, assists with species delimitation and contributes to our understanding of the bryozoan tree of life.

Keywords: *Penetrantia* , *Terebripora* , cryptic, Multiporata, skimming, mitogenomes

Introduction

Bryozoa is a clade of sessile and filter feeding metazoans that occurs in marine and freshwater habitats. As epibenthic organisms, they colonize various hard substrates and can create colonies up to several centimeters in size, which are composed of individual units (zooids) (Ryland, 1970; Schwaha, 2020a). Zooids that contain a tentacle crown (lophophore and digestive system) and actively feed are called autozooids, while polymorphic zooids specialized for reproduction, defense or colonial connectivity are called heterozooids (Mukai et al., 1997; Schack et al., 2019). The majority of the currently known extant ~6,000 bryozoan species thrive in marine environments from intertidal to subtidal regimes and to depths of more than 7,000 meters (Bock and Gordon, 2013; Ryland, 1970; Grischenko et al., 2019). The largest and most diverse taxon, Gymnolaemata, is subdivided into Cheilostomata and "Ctenostomata". Cheilostomes comprise about 5,000 known Recent species, while ctenostomes account for approximately 350 described species (Bock and Gordon, 2013). Cheilostomes have an (at least) partially calcified body wall that exhibits many skeletal features suitable for species identification, while ctenostomes lack such features (Schwaha, 2020b, d). Ctenostomes remain widely understudied, although they are important for our understanding of the interrelationships and evolution of gymnolaemates. It is presumed that cheilostomes originated from ctenostome-like ancestors, rendering the latter paraphyletic (Jebram, 1973; Todd, 2000; Waeschenbach et al., 2012; Schwaha, 2020c). Traditionally, ctenostomes were classified into eight superfamilies (Benedeniporoidea, Alcyonidioidea, Arachnidioidea, Hislopioidea, Paludicelloidea, Vesicularioidea, Victorelloidea and Walkerioidea) (Jebram, 1973, 1986; Todd, 2000), but the monophyly of some superfamilies remains uncertain. Ctenostomes have a wide ecological diversity living in marine, brackish and freshwater habitats and evolved specialized lifestyles, e.g., endolithic or solitary (Ryland, 1970; Schwaha, 2020c). Endolithic (boring) ctenostomes have received a considerable amount of attention thanks to their long fossil record, which dates back to the Ordovician (Pohowsky, 1978) However, their ecology and phylogeny remain poorly understood (Schwaha, 2020c). Endolithic bryozoans live immersed in calcareous substrates such as mollusk shells. They produce their cavities by means of chemical dissolution with only minute boring traces of about 50 – 100 μm visible from the outside (Pohowsky, 1978). There are four Recent endolithic ctenostome families currently recognized: Terebriporidae d'Orbigny, 1847, Spathiporidae Pohowsky, 1978, Immergentiidae Silén, 1946 and Penetrantiidae Silén, 1946 (Pohowsky, 1978; Schwaha, 2020c). Of special interest is the boring family Penetrantiidae, with its sole genus *Penetrantia* including ten Recent species (Silén, 1946, 1947; Pohowsky, 1978). Penetrantiids feature an operculum and brood chambers similar to cheilostomes, but have an uncalcified body wall and stolonate colonies commonly found in ctenostomes (Silén, 1947; Soule and Soule, 1969; Pohowsky, 1978). They were judged to be associated with cheilostomes or ctenostomes, the subject of considerable debate (Soule and Soule, 1969; Pohowsky, 1978; Smyth 1988). Several morphological investigations suggested convergent evolution of these cheilostome-like features in penetrantiids, placing them firmly among ctenostomes (Pohowsky, 1978; Schwaha, 2020c; Decker et al., 2023). However, uncertainty remains concerning their classification and phylogenetic position. Based on the presence of polymorphic stolons, a close relationship with the two other ctenostome superfamilies that feature true stolons, Vesicularioidea and Walkerioidea, was suggested (Pohowsky, 1978; Hayward, 1985; Schwaha, 2020c).

Because only a few external characters can be detected, correct species identification of penetrantiids remains challenging and some species were described based on their boring traces alone. Therefore, thorough histological investigations are necessary to successfully delimitate penetrantiid species (Decker et al., 2023).

Although the number of molecular phylogenetic studies has increased in recent years, most of them focused on cheilostome bryozoans, with only a few studies included ctenostome representatives (Fuchs et al., 2009; Waeschenbach et al., 2012, 2015; Orr et al., 2019, 2021, 2022). The most comprehensive ctenostome molecular phylogenies are based on a handful of genes but play a crucial role in our understanding of ctenostome systematics and support their paraphyly (Waeschenbach et al., 2012, 2015). Consequently, this study seeks for a larger phylogenetic analysis based on data from mitochondrial (mt) genomes and nuclear ribosomal RNA (rRNA) genes 18S and 28S. Our analysis includes 27 ctenostome species representing seven of their eight superfamilies. The molecular phylogenetic framework is combined with known morphological characters to shed light on 1) general ctenostome phylogeny, 2) the systematic position of the Penetrantiidae

and 3) the interrelationships of Penetrantiidae. Additionally, this study aims to unravel potential cryptic species complexes in the genus *Penetrantia* by including specimens from ten different geographical regions. Furthermore, we include one species of the boring ctenostome family Terebriporidae to evaluate whether an endolithic lifestyle evolved independently within ctenostomes. As this is one of the first studies comprising such a large molecular dataset of ctenostome bryozoans, it will also contribute to future analysis on the systematics of Gymnolaemata and might help to resolve the origin of cheilostomes and the paraphyly of ctenostomes.

Material and methods

Sample collection and imaging

27 specimens from eleven different localities were collected for genome skimming including nine different *Penetrantia* and 18 additional ctenostomes specimens (Table 1). One additional penetrantiid specimen (*Penetrantia* sp.) was collected in Helgoland, Germany (54°08.339'N 7° 52.298'E) for sanger sequencing of the cytochrome c oxidase subunit I (cox1) gene (OR632352) and genetic distance analysis only (see below). Samples were either collected in the intertidal zone by hand or in shallow subtidal areas by dredging. All samples were fixed either in 96% or absolute ethanol and stored at 4°C until further investigation. Stereomicroscopic pictures were taken with a Nikon SMZ25 stereomicroscope (Nikon, Tokyo, Japan) equipped with a DsRi2 microscope camera, or with a Hirox RH-2000 3D digital microscope (Hirox Co., Ltd., Tokyo, Japan). Scanning electron microscopic images were generated using a JEOL IT 300 (JEOL, Akishima, Tokyo, Japan) with a secondary detector at 10-25 KeV.

DNA extraction

Genomic DNA (gDNA) of all samples was extracted using the QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer's guidelines. Specimens of the endolithic genera *Penetrantia* and *Terebripora* were removed from their calcareous substrate either by mechanical breakage or by dissolving the substrate with 20% ethylenediaminetetraacetic acid (EDTA).

PCR amplification, sequencing and cox1 gene sequence analysis

Prior to genome skimming, the cox1 gene was sequenced for each specimen using PCR and Sanger sequencing. PCR amplification used universal (Folmer et al., 1994) or specific bryozoan primers (Table A1). PCR reactions were performed in 30 µl reaction volumes with 1 µl of 20 µM of each primer, 1-3 µl of gDNA and 15 µl of Red HS Taq Master Mix (Biozym, Oldendorf, Germany). PCR products were cleaned using an enzymatic cleanup reagent A'SAP (ArcticZymes Technologies ASA, Tromsø, Norway) and sent to Microsynth Austria GmbH for sequencing. Chromatograms were edited with SeaView v5.0.5 (Gouy et al. 2010) and aligned with MAFFT v7.520 using the model L-INS-i (Katoh et al., 2002, 2005).

Illumina sequencing, assembly and annotation

Library preparation and sequencing were conducted by the Next Generation Sequencing Facility at the Vienna BioCenter Core Facilities (VBCF). Genomic DNA libraries were constructed using NEBNext® Ultra II FS DNA Library Prep Kit for Illumina, with inputs > 100ng (# E7805). Multiplexing was done using the NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600). Libraries were sequenced on an Illumina NextSeq 550 platform using the 300 Cycle Mid Output mode.

Prior to assembly, raw Illumina reads were quality-checked with FastQC v0.11.8 (www.bioinformatics.babraham.ac.uk/projects/fastqc; last accessed April 08, 2022) and trimmed of adapters and low-quality sequences using Trim Galore v0.6.5 (https://github.com/FelixKrueger/TrimGalore; last accessed April 08, 2022) with default setting. The clean reads were *de novo*assembled using SPAdes v3.15.3 (Bankevich et al., 2012) with k-mers of 21, 33, 55, 77, 99 and 127. Mt genome contigs were identified using BLASTN (Altschul et al., 1990) and annotated with the MITOS2 web server (Donath et al., 2019) using the metazoan reference database RefSeq 63 and the invertebrate genetic code. Circularized mitochondrial genome maps (Figure A1) were generated with OrganellarGenome-DRAW (OGDRAW) online server v 1.3.1

(Greiner et al., 2019). Manual curation of the mitogenomes was undertaken using previously published mitogenomes of bryozoans available on NCBI as references. In cases incomplete mitogenome contigs were not recovered, Exonerate v2.4.0 (Slater and Birney, 2005) with the affine: local model and maximum intron length set to 40kb was used to scan the remaining contigs in the assemblies to identify any missing mt genes (13 protein-coding genes [PCG] and 12S and 16S rRNA genes; transfer RNAs were not scanned). 18S and 28S rRNA genes were annotated using RNAmmer (Lagesen et al., 2007).

Phylogenetic analysis

For phylogenetic inference we used twelve PCGs (cox1, cox2, cox3, cob, nad1, nad2, nad3, nad4, nad4l, nad5, nad6 and atp6), two mt rRNA genes (12S and 16S) and two nuclear rRNA genes (18S, 28S) (Table A2). The PCGs were translated into amino acids and aligned with MAFFT v7.310 (Katoh et al. 2002, 2013) with the parameters: auto, localpair, maxiterate 1000. Ambiguously aligned amino acids were removed using BMGE v. 1.12.2 (Criscuolo and Gribaldo, 2010). The rRNA genes were aligned with MAFFT using the same settings as above. Ambiguously aligned nucleotide positions were removed with trimAl v1.4. rev15 (Capella-Gutierrez et al., 2009) using the parameters gt 0.6 and some manual adjustments. Finally, the single gene alignments were concatenated into a supermatrix using AMAS (Borowiec, 2016).

Phylogenetic trees were constructed using Bayesian Inference (BI) and Maximum Likelihood (ML) on a mixed partitioned data matrix including 16 partitions (12 PCGs, two mt rRNA genes (12S and 16S) and two nuclear rRNA genes (18S and 28S)). Mt PCGs were processed as amino acids while mt rRNA and nuclear rRNA genes as nucleotides. The best-fitting evolutionary model for each partition was estimated using ModelTest-NG v0.1.7 (Darriba et al., 2020) based on the corrected Akaike Information Criterion. The GTR+I+G4 was the best-fitting model for the rRNA genes and the MtZoa+G4+F was the best-fitting model for the PCGs. The ML tree was inferred using RAxML-NG v. 1. 0. 2 (Kozlov et al., 2019) using the best-fitting model for each partition as determined by ModelTest-NG. Topological support was assessed with 1000 bootstrapping replicates. BI analysis was conducted with MrBayes5d 3.2.6 (*https://github.com/astanabe/mrbayes5d*: last accessed on 26.02.2023), a modified version of MrBayes 3.1.2 incorporating the MtZoa evolutionary model (Ronquist and Huelsenbeck, 2003). Analyses were composed of two independent runs with four Markov Chain Monte Carlo (MCMC) chains, each. Chains were run for five million generations. Tree and parameter sampling were every 100th generation. The GTR+I+G4 model was used to correct for multiple substitutions of the nuclear and mt rRNA gene partitions and the MtZoa+G4 model was used for mt PCGs gene partitions. Convergence of the MCMC chains was assessed by inspection of the tracefile outputs in Tracer (Nascimento et al., 2017). The convergence was also assed based on the average standard deviation of split frequencies (ASDOSF) and was <0.01 (0.000034)" The first 25% of samples were discarded as burn-in and the remaining trees were used to calculate posterior probability values and to build the consensus tree. The final ML and BI trees were visualized and adjusted in Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Genetic distance

ML-corrected substitutions per site were calculated in MEGA 7 using the maximum composite likelihood parameter with a gamma parameter of 1.0 (Tamura et al., 2004, 2021; Kumar et al., 2008).

Alignment

We generated sequences of 27 specimens that belong to 25 morphospecies and successfully assembled and annotated all PCGs, two rRNAs and two nuclear rRNA genes of 20 specimens while the atp8 gene was not recovered in seven samples (Table A2). As a result, the atp8 gene was excluded from our final data matrix. The remaining sequences of these 27 samples were combined with published sequences of the ctenostome*Monobryozoon ambulans* (Schwaha et al., submitted), nine cheilostome species (Orr et al., 2021) and the phylactolaemate*Pectinatella magnifica* (Leidy, 1851) as outgroup (Fuchs et al., 2009; Waeschenbach et al., 2009; Gim et al., 2018) (Table A2).

Our data matrix included 16 genes (12 mt PCGs, two mt rRNA and two nuclear rRNA genes) totaling 9702 characters (2834 amino acids and 6868 nucleotide sites).

Results

Phylogenetic analysis

Ctenostome phylogeny and placement of *Penetrantia*

The ML tree, which is based on the complete data matrix, is shown in Figure 1. Highly consistent tree topologies were observed from both phylogeny reconstruction methods (ML, Figure 1 and BI, Figure A2). The phylogeny is robust and most nodes are either fully supported (100 bootstrap (BS) / 1.00 Posterior Probability (PP)) or highly supported (>90 BS / >0.99 PP); while only four nodes have moderate support (<80 BS). Hereafter only supports below 100BS and 1.00PP will be mentioned as all remaining branches are fully supported.

Gymnolaemata includes ctenostomes and the monophyletic cheilostomes. Ctenostomes form three main clades: **A** , **B** and**C** . Clade **A** is highly supported (99 BS/1.00 PP) and includes the monophyletic Penetrantiidae as sister group to a highly supported clade (94 BS/1.00 PP) comprising *Paludicella articulata*and *Arachnidium* sp. (Figure 1).

Clade **B** represents the superfamily Alcyonidioidea and is divided into two clades, one comprising *Alcyonidium* and*Monobryozoon* and the other including *Pherusella* ,*Flustrellidra* and *Sundanella* , the latter representing Multiporata. *Monobryozoon ambulans* is the sister taxon to the monophyletic Alcyonidiidae represented here by two species of the genus*Alcyonidium* . Within Multiporata, *Sundanella sibogae* forms the sister taxon to a clade comprising *Pherusella liowae* and*Flustrellidra hispida* . The two representatives of *S. sibogae* from Brazil and Singapore display a genetic divergence of only 0.5% (cox1) and are therefore considered to represent the same species (Figure 1, "Ctenostomata" B).

Clade **C** is the sister taxon to cheilostomes, thus confirming the paraphyletic status of ctenostomes, and includes all remaining ctenostomes in this study: Vesicularioidea, Victorellidae, as well as the walkerioid *Aeverrillia setigera* and the hislopioid*Hislopia malayensis* . The representative of the Walkerioidea superfamily *A. setigera* represents the sister taxon to all other members of Clade **C** . *Hislopia malayensis* is the sister taxon to victorelloids and vesicularioids with high support (96 BS/1.00 PP). Victorellidae and Vesicularioidea each are monophyletic, although the monophyly of Victorellidae is only supported moderately (73 BS/1.00 PP). Both taxa form a highly supported sister-group relationship (90 BS/1.00 PP). Within Victorellidae, *Tanganella muelleri* is the sister taxon to *Bulbella abscondita* and *Amphibiobeania epiphylla* . Amongst Vesicularioidea, the endolithic bryozoan*Terebripora* sp. is the sister taxon to all remaining species of this superfamily (*Amathia* and *Vesicularia*). *Amathia gracilis* is the sister taxon to the paraphyletic assemblage of*Amathia* with the inclusion of *Vesicularia* . With moderate support (82 BS/1.00 PP), *Amathia ernsti* and *Vesicularia spinosa* cluster together with *Amathia distans* as sister taxon (Figure 1, "Ctenosotmata" C).

Interrelationships of *Penetrantia* and their genetic distances

Penetrantia japonica sp. nov. is the sister taxon to all other*Penetrantia* species in our study. The next branch is formed of*Penetrantia irregularis* from New Zealand and is well separated from the other New Zealand penetrantiids of the *parva* clade. With moderate support (74 BS/1.00 PP), *Penetrantia* sp. from France (Roscoff) is the sister taxon to a clade composed of *Penetrantia concharum* from Sweden and France (Roscoff), *Penetrantia clionoides* from Guam and representatives of the *parva* complex from Chile and New Zealand. *Penetrantia clionoides* is the sister taxon to the *P. parva* complex (Figure 2). Both species from France possess *concharum* -like borehole apertures, which are typically kidney-shaped, however the cox1 genetic divergence is 21.9 %, confirming them to be different species. Contrary, *P. concharum*specimens from Sweden and France exhibit a genetic distance of 0.3 % (Figure 2; Table A3).

Penetrantia sp. from France (Roscoff) is also confirmed in Germany (Helgoland) with a genetic divergence of 2.3% based on the barcoding region of the cox1 gene (Table AA4).

The *parva* complex forms a monophyletic clade with high support (91 BS/1.00 PP) and all three representatives exhibit the species-specific aperture outline with prominent apertural notches (Figure 2). *Penetrantia*

parva from the northern Island of New Zealand is the sister taxon to a clade represented by *P.* cf.*parva* from the southern Island of New Zealand and *P.* cf.*parva* from Chile with moderate support (89 BS/1.00 PP) (Figure 2). The cox 1 genetic distances between representatives of this complex are: northern and southern Islands of New Zealand - 12.1 %; *P. parva* from northern New Zealand and *P.* cf. *parva* from Chile - 12.9 %; southern New Zealand and Chile - 9.8% (Table A3).

Systematic account/Species description of *Penetrantia japonica* sp. nov.

Phylum Bryozoa Ehrenberg, 1831

Class Myolaemata Schwaha et al., 2020

Subclass Gymnolaemata Allman, 1856

Order *Ctenostomata* Busk, 1852 asterisk indicating the paraphyletic status

Family Penetrantiidae Silen, 1946

Genus Penetrantia Silen, 1946

Penetrantia japonica sp. nov.

Penetrantia sp. Decker et al. 2023, Figures 3, 5, 8, 9, 13, 14, 17, 21 and 22

Type material . *Holotype:* NSMT-Te1270, National Museum of Nature and Science, Tokyo, Japan. Collected at Tenjin-Jima Island, Sagami Bay, Japan (35deg13.336'N 139deg36.152'E), intertidal, 29th October2020, by Masato Hirose. In shell of hermited *Tegula rugata* (A. Gould, 1861) (Figure 3a). All paratypes were collected at the same location as the holotype.*Paratype1:* NSMT-Te1271, collected 29th October 2020, hermited *Tegula rugata. Paratype2:* NSMT-Te1272, collected 4th November 2020, hermited *Reishia clavigera* (Kuster, 1860). *Paratype3:* NSMT-Te1273, collected 19th October 2020, live *Japeuthria ferrea* (Reeve, 1847).

Diagnosis. Boring traces commonly found in live or hermited intertidal gastropod shells (*Tegula rugata* , *Japeuthria ferrea* , *Reishia clavigera*). Young colonies often close to aperture of gastropod shell, larger colonies all over shell, commonly close to apex. Typical feather-shaped colony with leading principal stolon and secondary stolons branching of orthogonally. Older colonies strongly ramified with different stolons intercrossing, generating mesh-like pattern (Figure. 3a). True kenozooidal stolons separated by distinct septa/pore plates. Zooids pedunculate, placed along both lateral sides of stolons (Figure. 3a, b). Borehole apertures 80-100 µm in width, circular to keyhole-shaped, sometimes with small apertural notches on oral side, rarely with calcareous apertural rim (Figure. 3b, c). Tubulet pores often visible, small holes along stolons in intervals of 180-200 µm, 8-12 µm in width (Figure 3b). **Autozooids** tubular with slightly pointed basal tip, vertically in substrate, 380-430 µm in length, 120 µm in width (Figure 3d). Always 12 tentacles. Prominent exterior cuticle, extending far frontally of operculum (Figure 3f). Operculum about 100 µm in width, dome-shaped in cross section, rough crescent area on frontal-oral side, partially composed of calcium carbonate (Figure 3f, g). Multiple brown bodies common (Figure 3d).**Gonozooid** same length as autozooid, brood chamber on anal side about 230 µm long, pear-shaped in longitudinal section. Gonozooidal tube longer than brood chamber with slandered basal tip, bending in anal direction. Operculum same as for autozooid. Polypide reduced, no tentacles (Figure 3e).

Etymology . Japonica refers to the type locality of this possibly endemic species.

Distribution . Yoshihama Bay, Iwate Prefecture, Japan (39°6.984'N 141° 52.355'E) and along the coast of Sagami Bay, Japan (35°13.336'N 139°36.152'E).

Remarks . The operculum morphology is very similar to*Penetrantia clionoides* Smyth, 1988 and *Penetrantia bellardiellae* Schwaha, 2019 with a rough crescent-shaped area on its frontal side but *P. japonica* sp. nov. has the largest tubulet intervals and unique gonozooids with a slandered basal tip.

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Discussion

Interrelationships of "Ctenostomata" and their paraphyly

Gymnolaemata is a widely accepted monophyletic class of bryozoans which is the sister taxon to Stenolaemata (Todd, 2000; Waeschenbach et al., 2012; Schwaha, 2020a, d.). Within Gymnolaemata, ctenostomes form a paraphyletic assemblage that includes the monophyletic cheilostomes (Fuchs et al., 2009; Waeschenbach et al., 2012; Schwaha, 2020a). However, the taxon sampling of ctenostomes for phylogenetic analyses was rather poor until now. Our study represents the broadest taxon sampling to date and resulted in three main clades of ctenostomes. The first main clade (A) includes representatives of three different families of ctenostome bryozoans (Arachnidiidae, Paludicellidae and Penetrantiidae). Although a close relationship between the superfamilies Arachnidioidea and Paludicelloidea was previously proposed (Jebram, 1973; Todd, 2000; Waeschenbach et al., 2012; Schwaha 2020c), all three groups (including penetrantiids) possess distinct morphological traits that make their close relationship unexpected. Most arachnidioids are characterized by cystid appendages that can be anastomosing and create "pseudostolonal" connections between zooids (Jebram, 1973; Schwaha and De Blauwe, 2020), though some species in this group lack such appendages (see Jebram, 1986). *Paludicella articulata* lacks such cystid appendages, has a unique cruciform branching pattern and is restricted to freshwater habitats. These major differences led to the placement of*Paludicella* into the separate superfamily Paludicelloidea (Jebram, 1973; Todd, 2000; Schwaha, 2020c). A denser taxon sampling that includes more members of the Arachnidioidea, e.g., Immergentiidae and Nolellidae, might resolve the unexpected sister-group relationship of arachnidiids and paludicellids. The third family within clade A, Penetrantiidae, possess many distinct characters (e.g., operculum and kenozooidal stolons) that are not present in Paludicellidae or Arachnidioidea and will discussed in more detail later (see below) (Jebram, 1973; Schwaha, 2020c).

The second main clade (B) represents the superfamily Alcyonidioidea, a taxon characterized by tightly arranged zooids that are always in close contact with the body wall of neighboring zooids and never by stolon-like connections (Schwaha, 2020c). Unlike other studies (Jebram, 1986; Todd, 2000; Waeschenbach et al., 2012), our phylogenetic analysis does not support alcyonidioids as sister taxon to all remaining ctenostomes but of clade **A** instead (Arachnidiids, Paludicellids and Penetrantiids). Consequently, our study indicates that the serial arrangement of zooids as found in paludicellids, arachnidioids and penetrantiids could represent the ancestral colony structure of ctenostomes rather than simple encrusting sheet-like colonies of alcyonidioideans as previously suggested (Jebram, 1973; Schwaha, 2020c). Certainly, a larger taxon sampling within both clades might alter the phylogeny, since arachnidioids are only represented by one species in this study.

The only superfamily not included in the current study is Benedeniporoidea and was previously considered the sister taxon to all remaining ctenostomes. This led to the establishment of the "Protoctenostomata" - "Euctenostomata" concept, with Benedeniporoidea as early protoctenostome and all remaining Recent ctenostomes belonging to euctenostomes (Jebram, 1973; Todd, 2000). However, this phylogenetic hypothesis would imply that a ctenostome-like ancestor possessed serially erect colonies, which is a rare state among Recent ctenostomes. Additionally, species of this superfamily were only rarely found and detailed information on their morphology as well as sequence data is missing (Schwaha, 2020c).

Multiporata, a recently erected taxon of alcyonidioid bryozoans that is characterized by multiporous pore plates, is monophyletic and nests within Alcyonidioidea. These distinct pore-plates are usually known from cheilostomes and not found in other ctenostome bryozoans (Schwaha et al., 2022a). The multiporate genera *Flustrellidra* and*Pherusella* are sister taxa in our analysis and share some specific characters, e.g., a rectangular to bilateral shaped orifice and pseudocyphonautes larvae. The latter is only present in these two families and resembles an apomorphy of this group (Reed, 1991; Decker et al., 2020; Decker et al., 2021). This close relationship was also shown by a recent phylogenomic study (Saadi et al., 2022). Our study supports the affiliation of sundanellids to Multiporata and not to Victorelloidea. This affiliation is supported by several morphological characters e.g., multiporous pore plates, large bilateral lophophores with high tentacle numbers (more than 30) and a vestibular collar (Schwaha et al., 2022a). A close relationship of *Sundanella sibogae* to the multiporate *Flustrellidra hispida* was recently indicated by a phylogenetic analysis based on

the nuclear marker 18S gene (Schwaha et al., 2022b). Remarkably, *S. sibogae* is confirmed in Singapore as well as in Brazil by our study and thereby underlines its vast distribution. *Sundanella sibogae* was reported from Indonesia, Singapore, the eastern and western coast of Africa and the Western Atlantic before (Marcus, 1937, 1941; Harmer, 1951; Schwaha et al., 2022a). The only multiporate genus not included in our study is*Elzerina* , which is currently placed in the family Flustrellidridae. However, the presence of pseudocyphonautes larvae in*Elzerina* (like in *F. hispida*) is not confirmed but internal brooding of lecithotrophic larvae seems possible. Since an intertentacular organ is only present in *Elzerina* and neither in*Flustrellidra* nor in *Pherusella* , the latter two genera may share a closer relationship (Schwaha et al., 2021). Consequently, future studies should include sequence data of the genus *Elzerina* to confirm this idea.

Our study also suggests a sister-group relationship between the genus*Alcyonidium* and *Monobryozoon ambulans* . The latter is a solitary bryozoan species living in sandy marine sediments as part of the meiofauna and was just recently rediscovered (Remane, 1936; Schwaha et al., submitted). Monobryozoidae were traditionally placed either among arachnoidids, primarily due to the presence of non-kenozooidal cystid appendages (Jebram, 1986), or as incertae sedis (d'Hondt, 1983). More recent investigations suggest an affinity of monobryozoids with alcyonidioids (Schwaha, 2020c; Schwaha et al., submitted), which is also confirmed in our study. This affinity is reflected by alcyonidioid-like characters such as a circular orifice, the presence of a prominent orifical sphincter and a vestibular anus (Schwaha, 2020c; Schwaha et al., submitted). However, the genus *Alcyonidium* was considered to be paraphyletic (Waeschenbach et al., 2012), which is not the case in our analysis. With only two species, however, the taxon sampling of this particular genus is unrepresentative and might not reflect the actual phylogeny. With an assemblage of more than 70 species,*Alcyonidium* represents one of the largest ctenostome genera (Schwaha, 2020c). Since only little information on soft body characters as well as molecular data is available, this genus urgently needs future revision.

The third main clade (C) includes species of four different ctenostome superfamilies, two of them are characterized by kenozooidal stolons as found in penetrantiids. The origin of cheilostomes within ctenostomes is the most accepted scenario and supported by morphological and molecular data and thereby renders ctenostomes paraphyletic (see Waeschenbach et al., 2012; Orr et al., 2022). However, it was still unclear which of the Recent ctenostome clades is the closest relative to cheilostomes. Former investigations suggested a close relationship and potential ancestry of cheilostomes with *Arachnidium* -like ctenostomes (Banta, 1975; Taylor, 1986, 1990). More recent studies favor a sister group relationship of cheilostomes to the ctenostome superfamilies Hislopioidea and Vesicularioidea (Waeschenbach et al., 2012). Our study suggests a similar sister-group relationship of cheilostomes, however, additionally includes representatives of Walkerioidea and Victorellidae, which were not included in Waeschenbach et al. (2012). Thus, it seems reasonable that cheilostomes and the superfamilies in clade **C**(Walkerioidea, Victorellidae, Hislopioidea, Vesicularioidea) share a most recent common ancestor. Future studies should continue to tackle this question by increasing taxon sampling especially including more representatives of walkerioid and hislopioid bryozoans.

Regarding the sister-group relationship of Victorelloidea and Vesicularioidea, it is evident that they share a well-developed funicular system and a cardiac constrictor often with a gizzard (Schwaha, 2020c). However, while vesicularioid bryozoans are characterized by zooids that always are connected by true kenozooidal stolons, victorelloids lack stolons and are restricted to brackish and freshwater habitats (excluding sundanellids) (Schwaha, 2020c). Based on morphological characters, the superfamily Victorelloidea was previously considered to be polyphyletic, which is supported in our analysis by the placement of *Sundanella* within Multiporata (see also Schwaha et al., 2022a, b). Consequently, a morphological revision of Victorelloidea, with the exclusion of *Sundanella* , may reveal additional shared characters. In our analysis, *Amphibiobeania epiphylla* clusters together with the remaining two victorelloid species, with *Bulbella abscondita* as sister taxon and*Tanganella muelleri* being the sister taxon to both aforementioned. Formerly, *A. epiphylla* was regarded as cheilostome bryozoan due to the presence of an opercular-like structure (Metcalfe et al., 2007). Recent morphological investigations proved typical ctenostome features (denticulate gizzard, low tentacle numbers (eight), a large number of interzooidal pore plate cells and the lack of duplicature bands) and indicate a potential affinity with vesicularioids and victorelloids (Schwaha et al., 2022b). An operculum was not confirmed in *A. epiphylla* and therefore assumed to be absent (Schwaha et al., 2022b). Furthermore, a

phylogenetic analysis based on the 18S gene revealed its ctenostome affinity (Schwaha et al., 2022b), which is also confirmed in our analysis. Since this species was only reported from mangroves it may be adapted to brackish environments with changeable salinities, which again might cohere with a victorelloid affiliation of *A. epiphylla* (Metcalfe et al., 2007; Schwaha, 2020c; Schwaha et al., 2022b).

In our analysis, *Terebripora* sp. from Chile is the sister taxon to a clade composed of representatives of *Amathia* and *Vesicularia spinosa* . The family Terebriporidae is one of four Recent endolithic ctenostome families already placed among vesicularioid ctenostomes, owing to true stolonate colonies and the presence of a gizzard (see below) (Soule and Soule, 1969; Schwaha, 2020c).

Within Vesicularioidea, *Amathia gracilis* is the sister taxon to all remaining *Amathia* species as well as to *Vesicularia spinosa* . *Amathia gracilis* was previously placed in the genus*Bowerbankia* and just recently re-assigned to *Amathia*(Waeschenbach et al. 2015).

A ctenostome affiliation of Penetrantiidae and their closest relatives

Our analysis confirms a ctenostome affiliation of Penetrantiidae as suggested by several morphological studies previously (Silén, 1946, 1947; Pohowsky, 1978; Schwaha, 2020c; Decker et al., 2023), and contradicts other studies that favored a cheilostome affinity (Soule and Soule, 1969; Smyth, 1988). Especially, the presence of cheilostome-like features such as the operculum and the brood chamber started a long-lasting discussion on the placement of penetrantiids. However, these structures appear to have evolved convergently in Penetrantiidae and Cheilostomata, since there are major morphological differences, particularly in the underlying musculature (see Decker et al., 2023).

Additionally, the absence of opercula and brood chambers in the closely related taxa (paludicellids and arachnidioids) points to apomorphic characters of Penetrantiidae. *Paludicella pentagonalis* differs in its colony pattern from *P. articulata* , in contrast to the*P. articulata* , *P. pentagonalis* has a linear series of zooids with no lateral branches (Annandale, 1916). *Paludicella pentagonalis* is also reported to sometimes possess "stolon-like" connections between zooids (see Rogick and Brown, 1942) that might support a potential relationship of *P. pentagonalis* with arachnidioids or penetrantiids. Therefore, it would be essential to investigate whether these stolon-like tubes feature pore plates, because no information is currently available about the kenozooidal status of these tubes limiting their phylogenetic value.

The presence of true polymorphic stolons in penetrantiids traditionally favored a close relationship with the other stolon-bearing groups vesicularioids or walkerioids (Schwaha, 2020c and Decker et al., 2023). The presence of a gizzard also supports a vesicularioid affinity (Silèn, 1946, 1947; Pohowsky, 1978; Schwaha, 2020c). However, the other two stolonate groups are not considered closely related to penetrantiids and also do not form a monophyletic group. Consequently, our study suggests that kenozooidal stolons have evolved at least three times independently within ctenostomes (vesicularioids, walkerioids and penetrantiids). The polyphyly of the artificial construct of "Stolonifera" was already suggested (Jebram, 1973 and Schwaha, 2020c) and is also supported by recent molecular studies (Waeschenbach et al., 2012, 2015). This hypothesis is also based on several morphological and ontogenetical differences in the stolons of these two taxa (see Jebram, 1973 and Schwaha, 2020c). Additionally, the presence of a true gizzard in penetrantiids was questioned, as the gizzard-like structure is indistinct, does not feature denticles and thereby resembles a proventriculus (Decker et al., 2023). Overall, a closer relationship of penetrantiids and vesicularioids is unlikely.

A close relationship of Penetrantiidae with the other three endolithic ctenostome families is also unlikely. Terebriporidae is represented by one species in our analysis and is the sister taxon to all other vesicularioids. Based on morphological characters, Spathiporidae are also considered belonging to Vesicularioidea as they likewise possess kenozooidal stolons and a distinct gizzard (Soule and Soule, 1975; Pohowsky, 1978; Schwaha, 2020c). On the other hand, species of the boring family Immergentiidae are considered closely related to arachnidioids possessing a network of "pseudostolonal" cystid appendages and no gizzard (Silèn, 1947; Pohowsky, 1978; Schwaha, 2020c). Consequently, the boring endolithic lifestyle has evolved several times independently within the major clades of "Ctenostomata" (see below).

Interrelationship of Penetrantiidae

The sequences of nine different penetrantiid specimens correspond to eight genetically diverged species in our analysis. However, there are two cryptic species complexes present, which can be hardly differentiated based on morphological characters. Cryptic speciation is a common phenomenon known from many different groups of bryozoans becoming more evident with the increase of molecular investigations (Thorpe and Ryland, 1979; Chimenz Gusso et al., 2004; Fehlauer-Ale et al., 2014; Waeschenbach et al., 2015). Particularly, the soft bodied ctenostomes, without any distinct skeletal characters, are prone to this taxonomic issue (Thorpe et al., 1978; Waeschenbach et al., 2015).

We unraveled two cryptic species complexes within the genus*Penetrantia* : 1) a species complex in the North Sea and the Northern Atlantic; 2) *parva* complex in the Southern Pacific. The species assembly in the Northern Atlantic is intriguing since at least two similar species do co-occur in the same region (Roscoff, France),*P. concharum* and *Penetrantia* sp.. *Penetrantia concharum* from Roscoff is genetically identical to *P. concharum*from Sweden while *Penetrantia* sp. from Roscoff is genetically very different from *P. concharum* and most likely represents an undescribed species. Although *P. concharum* and *Penetrantia*sp. do not form a monophyletic clade in our analysis, their morphology is very similar and they form almost identical borehole apertures and colonies. A recent study found minor soft body differences between*Penetrantia* sp. and *P. concharum* . For instance,*Penetrantia* sp. features a collar, has a thinner operculum and on average smaller autozooids than *P. concharum* from Sweden (see Decker et al., 2023). However, since these morphologically investigated specimens were not sequenced it is not possible to assign these characters to one species with certainty. Furthermore, there are reports of *Penetrantia* along the Iberian coast that were not assigned to one of the known European penetrantiid species (*P. concharum* or*Penetrantia brevis*) and might represent the undescribed species in Roscoff (Reverter-Gil et al., 1995; Reverter-Gil and Souto, 2014; Reverter-Gil et al., 2016; Decker et al., 2023). The picture becomes even more complex as the undescribed *Penetrantia* sp. from Roscoff (France) is also confirmed in Helgoland (Germany), which is geographically much closer to Sweden than France, and suggests an overlapping distribution of both species in the North Sea and the Northern Atlantic. Consequently, a much more detailed analysis at population level is required to delineate *Penetrantia* species occurring in the Northern Atlantic that should also include specimens from Norway, United Kingdom, Belgium, Spain and Portugal.

The second cryptic species complex is the *parva* complex distributed throughout the Southern Pacific and represented by three specimens in our study (northern and southern Islands of New Zealand and Chile). *Penetrantia parva* was also reported from New Caledonia and Hawaii and has one of the largest distributions of penetrantiids (see Table 2 in Decker et al., 2023). This species complex is morphologically characterized by unique borehole apertures with prominent apertural notches, heavy cuticularized opercula, and gonozooids where the brood chamber is half as long as the gonozooid itself (Silén, 1946, 1947; Decker et al., 2023). Interestingly,*P.* cf. *parva* from southern New Zealand is more closely related to the Chilean one than *P. parva* from northern New Zealand. As zooid dimensions are very similar, the only considerable difference is the presence of a shallow pit in the frontal side of the operculum in some specimens of *P. parva* from northern New Zealand. Since this pit was never observed in specimens of the remaining two representatives of the parva complex, it might indicate a more distant relationship between *P. parva* from northern New Zealand to both *P* . cf. *parva* from southern New Zealand and Chile (Decker et al., 2023). However, the genetic distances between specimens from all three localities are sufficient (>10%) to consider each of them as a separate species, when applying a cox1 genetic distance of more than 3% as the threshold for species delimitation (see Baptista et al., 2022). The threshold of genetic distance for species delimitation is, however, still debated and depends on the marker gene and the group of animals investigated, but a threshold of about 3% is considered to have the lowest error rate with an optimum of 2.6% for cowrie gastropods (Meyer and Paulay, 2005).

Similar cryptic speciation was observed in the cheilostome *Bugula neritina* , which was considered to have a cosmopolitan distribution, yet only one of the three cryptic species in this complex is distributed globally (Fehlauer-Ale et al., 2014). On an even smaller geographical scale, cryptic bryozoan species were discovered

in a recent study focusing on *Reteporella* species from the Azores and Mediterranean Sea (Baptista et al., 2022). Accordingly, cryptic speciation in bryozoans seems to be unexplored with many cryptic species complexes awaiting discovery. Considering that most bryozoans have short-living lecithotrophic larvae, including penetrantiids, the gene flow between populations might be rather restricted and consequently, speciation may occur on smaller geographical scales (Reed, 1991; Todd et al., 1998; Gruhl, 2020; Decker et al., 2023). However, level of gene flow and genetic structure between populations are not solely explained by pelagic larvae duration (PLD), since there are many examples of species that have a restricted distribution despite having a long PLD and vice versa (Todd et al., 1998).

Similar to the cryptic species complex in the Northern Atlantic, future work should include more specimens from different locations and combine molecular results with thorough morphological investigations. Additionally, it might be important to apply different genetic markers and a larger dataset to better resolve cryptic speciation in*Penetrantia* and to better understand intra- and interspecific genetic diversity (see Fehlauer-Ale et al., 2014 and Baptista et al., 2022). Despite large genetic distances, potential new cryptic penetrantiid species should be validated with mating trials to confirm whether they are truly different biological species or not (see Gomez et al., 2007). Nevertheless, there are three penetrantiid species in our study (*P. clionoides* , *P. irregularis* and *P. japonica* sp. nov.) that do not form species complexes and are well separated from other penetrantiids on molecular and morphological basis.

Penetrantia clionoides from Guam is the sister taxon of the *P. parva* clade and differs in the morphology of its operculum and gonozooid from the latter. The operculum of *P. clionoides* has a rough crescent-shaped area on its frontal side and is partially composed of calcium carbonate, which is otherwise only known from*P. japonica* sp. nov. from Japan and *P. bellardiellae* from Papua New Guinea (Smyth, 1988; Schwaha et al., 2019; Decker et al., 2023). Although, the latter three species (*P. clionoides, P. bellardiellae* and *P. japonica* sp. nov.) exhibit similar opercula, they clearly differ in terms of gonozooid shape and/or interval length between tubulets (Decker et al., 2023). The geographically closest species to Japan is *Penetrantia taeanata*from South Korea (Seo et al., 2018). This species is much smaller than*P. japonica* sp. nov. and with an average autozooid length of 160 µm by far the smallest penetrantiid (see Table 3 in Decker et al., 2023). Consequently, we propose *P. japonica* sp. nov. as a new species here due to distinct morphological differences and discrete phylogenetic placement.

Terebriporidae and the convergent evolution of the boring life style

In our analysis, the family Terebriporidae is represented by a sole species from Chile and is placed among vesicularioid ctenostomes. As mentioned before, a vesicularioid affiliation seems very likely especially based on morphological characters (kenozooidal stolons and gizzard) (Schwaha, 2020c). The original type specimen of the family and genus, *Terebripora ramosa,* was collected in Peru with additional type material from Chile (d'Orbigny, 1847; Pohowsky, 1978). Characteristic tubulets arising from the zooids were reported for*T. ramosa* along with very symmetrical feeder-shaped colonies, such that our specimens closely resemble *T. ramosa* (Pohowsky, 1978). However, as there is no information regarding soft body morphology of the latter species, we cannot assign these specimens to*T. ramosa* with certainty. In fact, there is a lot of confusion in the literature about the correct affiliation of many terebriporid species, particularly of fossils. The enantiomorphic apertures of boring traces of *Immergentia* and *Terebripora* can appear very alike and probably led to the wrong assignment of species (Pohowsky, 1978), e.g., *Spathipora comma* (Soule, 1950a) was previously assigned to *Terebripora* and there is still confusion whether*Immergentia philippinensis* Soule, 1950b is a terebriporid or immergentiid species (Soule, 1950a, b; Bobin and Prenant, 1954; Pohowsky, 1978). Accordingly, it is not easy to assign boring traces to a family without information on stolon and gut morphology. In general, an affiliation of boring traces to a family, genus or even a species should be treated carefully as these traces resemble the boring activity of an animal and not true morphological characters. Therefore, such assignments should be considered separate ichnotaxa instead of a true biological species (see Bertling et al., 2006; Rosso, 2008; Wisshak et al., 2019; Decker et al., 2023). The problem becomes even more apparent as the family Terebriporidae was erected based on boring traces and colony patterns alone without any soft body information, rendering the entire family an ichnotaxon (d'Orbigny, 1847; Bertling et

al., 2006; Wisshak et al., 2019). Accordingly, a histological reinvestigation of the type material would be necessary to provide soft body information and confirm the taxonomic integrity of the family Terebriporidae.

Our study suggests that an endolithic and boring lifestyle within calcareous substrates has evolved at least twice convergently among ctenostome bryozoans, since *Penetrantia* and *Terebripora*are well-separated taxa. As boring bryozoans have a long fossil record dating back to the Ordovician, an early radiation within different ctenostome taxa seems plausible (Pohowsky, 1978). Most likely such an adaptation also occurred within the Arachnidioidea, which includes the family Immergentiidae (Silèn, 1947; Pohowsky, 1978; Schwaha, 2020c). The fourth Recent endolithic bryozoan family, Spathiporidae, is commonly assigned to vesicularioids and probably closely related to terebriporids and might be sister taxa, since they share unique tubulets arising from autozooids, which the other two boring families Penetrantiidae and Immergentiidae are lacking. The most distinct difference between spathiporids and terebriporids is the connection of the zooids to their stolonal network. While spathiporids have pedunculate zooids, terebriporids have their zooids placed along the stolons and lack a peduncle (Soule and Soule, 1975; Pohowsky, 1978; Schwaha, 2020c).

There seems to be a tendency within ctenostomes towards a boring or burrowing lifestyle as it has also evolved for other substrates, e.g., in wood, parchment-like polychaete tubes (*Bulbella abscondita* and*Hypophorella expansa* Ehlers, 1876) or inside cheilostome skeletons (*Harmeriella terebrans* Borg, 1940) (Borg, 1940; Pohowsky, 1978). In contrast to cyclostomes and cheilostomes, ctenostomes lack a calcified body wall (Schwaha, 2020b), thus placing the delicate zooids into substrates probably gains additional protection. Since *H. expansa* and *H. terebrans* apply a mechanical boring method versus the chemical boring method found in endolithic taxa, their burrowing lifestyle probably evolved independently as well (Borg, 1940; Pröts et al., 2019; Schwaha, 2020c). Colonies of *B. abscondita* live inside degraded wood, which is yet another different substrate, and its burrowing lifestyle most likely evolved independently too, reflected in the separated placement and affiliation of *B. abscondita* to victorelloids (Braem, 1951; Schwaha, 2020c). Overall, such lifestyles have evolved at least five times independently within ctenostomes and probably even more often when taking all the different boring ctenostome taxa into account that are only known from the fossil records (Pohowsky, 1978).

Conclusion

This study provides the most comprehensive up-to-date phylogeny of ctenostome bryozoans, including representatives of all commonly accepted ctenostome superfamilies. It corroborates the paraphyletic status of "Ctenostomata" by the inclusion of cheilostomes as the sister taxon of a clade comprising Walkerioidea, Victorellidae, Hislopioidea and Vesicularioidea. Furthermore, this study gives the first molecular support for a ctenostome affiliation of Penetrantiidae and reveals a potential sister-group relationship to a clade containing*Paludicella articulata* and *Arachnidium* sp. It also unravels two cryptic species complexes, a species complex in the North Sea and Northern Atlantic, and the *parva* complex in the Southern Pacific. Additionally, it confirms and describes *Penetrantia japonica* sp. nov. as a new species from Japan. The boring family Terebriporidae is confirmed to belong to Vesicularioidea and thereby distantly related to penetrantiids, suggesting the endolithic lifestyle has evolved at least two times independently within ctenostomes.

Moreover, our study proposes monophyletic Alcyonidioidea, with the Multiporata nesting firmly within the latter and is monophyletic itself including the family Sundanellidae. Since this study provides the first complete mt genomes of 27 different ctenostomes it contributes to recent and future studies on this cryptic group of bryozoans.

Data Availability Statement

All sequence data used in this study can be found on GenBank (NCBI), the corresponding accession numbers are listed in Table 1 and Table A2.

Conflict of Interest Statement

All authors declare no conflict of interest.

Author Contributions

The study conception was designed by SHD and TS. Samples were collected by TS, MH, SL, AS, FA, LMV, AMS and SHD. Sample preparation and DNA extraction was done by SHD and CB. Sequence analysis and phylogenetic reconstructions were done by AJS and SHD. The first draft of the manuscript was written by SHD with the help of AW and AJS, and all authors contributed to the final version of the manuscript. The final version of the manuscript was read and approved by all authors.

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Figure legends

Table 1 Sample details and accession numbers of specimens used for genome skimming in this study.

Figure 1 Maximum Likelihood phylogenetic tree based on a data matrix of 16 genes comprising 28 ctenostomes, 9 cheilostomes from Orr et al., 2021 (branch collapsed) and the phylactolaemate bryozoan*Pectinatella magnifica* (from Fuchs et al., 2009; Waeschenbach et al., 2009; Gim et al., 2018) as an outgroup to root the phylogenetic tree (see Table A2). Values on internal nodes correspond to ML bootstrap support (1000 replicates) and posterior probabilities for BI (based on the last 75% of trees) respectively. Values are only shown for nodes that are not fully supported by both phylogeny reconstruction methods. Different colored boxes represent different clades - orange: Gymnolaemata, blue: *Penetrantia* , green: Multiporata, pink: Victorellidae, yellow: Vesicularioidea. Grey: three main clades A, B and C. Clade B reflects the superfamily Alcyonidioidea. Cheilostomata has been collapsed to allow better visualization. The scale bar represents 1 substitutional change per 100 character positions.

Figure 2 Interrelationships of nine penetrantiids from seven different regions. Subtree of the genus *Penetrantia* derived from the phylogeny shown in Figure 1. Values on internal nodes correspond to ML bootstrap support (1000 replicates) and posterior probabilities for BI (based on the last 75% of trees) respectively. Values are only shown for the nodes that are not fully supported by both phylogeny reconstruction methods. Images show borehole aperture and/or operculum of respective species. The image of the borehole apertures of *P. clionoides* is modified from Smyth, 1988. Drawings are generalized outlines of the borehole apertures. Two cryptic species complexes - green: North Sea-complex, blue: *parva* -complex. The scale bar represents 1 substitutional change per 100 character positions.

Figure 3 *Penetrantia japonica* sp. nov. from Japan. Stereomicroscopic images of the holotype (NSMT-Te1270) in the shell of the gastropod *Tegula rugata* (A. Gould, 1861) **(a)** and**(d)** . Borehole apertures with apertural notches in the gastropod *Japeuthria ferrea* (Reeve, 1847) **(b)** .Scanning electron microscopic images of a borehole aperture with apertural rim **(c)** . Microscopic image of a wholemount showing a gonozooid with its unique basal extension **(e)** . Scanning electron microscopic images of the operculum in **(f)** and**(g)** with its peculiar crescent-shaped and rough area on the frontal side (arrows).

ap – aperture, apn – apertural notches, apr – apertural rim, bb - brown body, bc – body cavity, bch – brood chamber, e – embryo, exc - exterior cuticle, op – operculum, st – stolon, tu - tubulet

Appendix

Figure A1 Circularized mitochondrial genome map of*Penetrantia parva* from northern New Zealand (a) and*Penetrantia clionoides* from Guam (b). Arrows show direction of transcription with outer strand corresponding to the forward and the inner to the reverse strand.

Figure A2 Bayesian Inference phylogenetic tree based on a data matrix of 16 genes comprising 28 ctenostomes, 9 cheilostomes from Orr et al., 2021 (branch collapsed) and the phylactolaemate bryozoan*Pectinatella magnifica* (from Fuchs et al., 2009; Waeschenbach et al., 2009; Gim et al., 2018) as an outgroup to root the phylogenetic tree (see Table A2). Values on internal nodes correspond to BI posterior probabilities.

Table A1 PCR primers used for PCR amplification.

Table A2 Species and genes of the data matrix used in the phylogenetic reconstruction shown in Figures 1, 2 and A2.

Table A3 Estimates of evolutionary divergence between nine*Penetrantia* specimens based on the cox1 gene full length. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1506 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

Table A4 Estimates of evolutionary divergence between ten*Penetrantia* specimens including Helgoland, Germany based on the cox1 gene barcoding region. The number of base substitutions per site between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 640 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

Supplementary File

S1 – Images of investigated specimens in this study.

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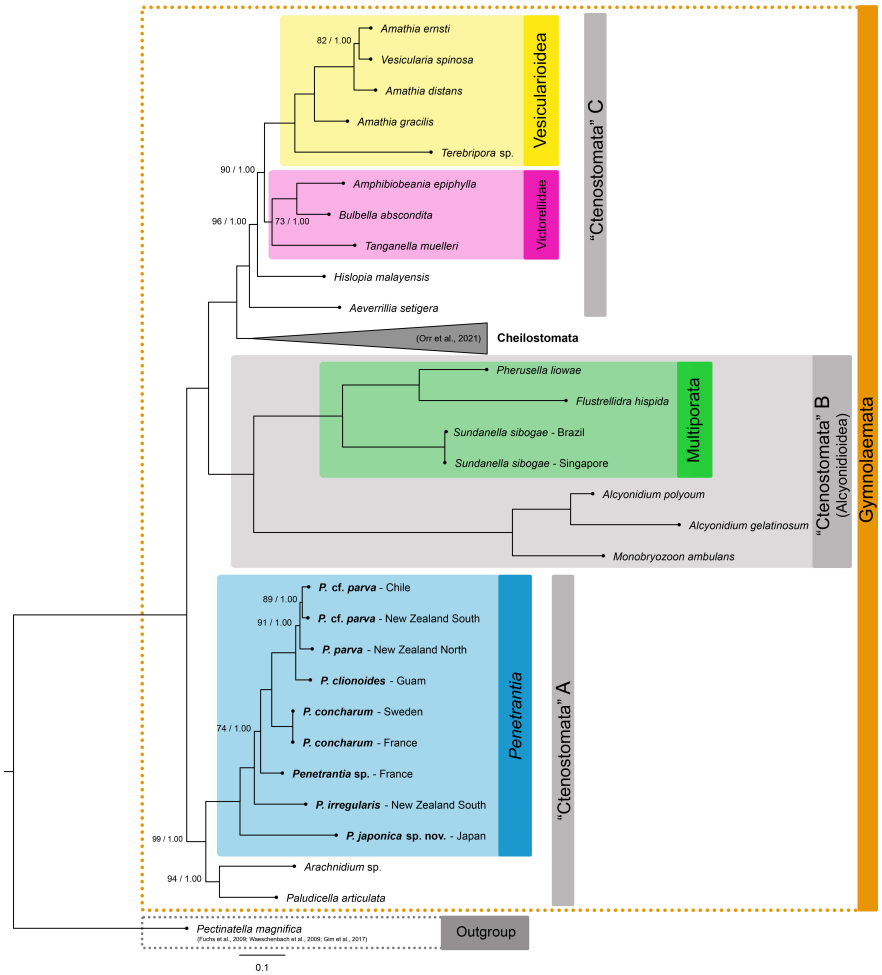
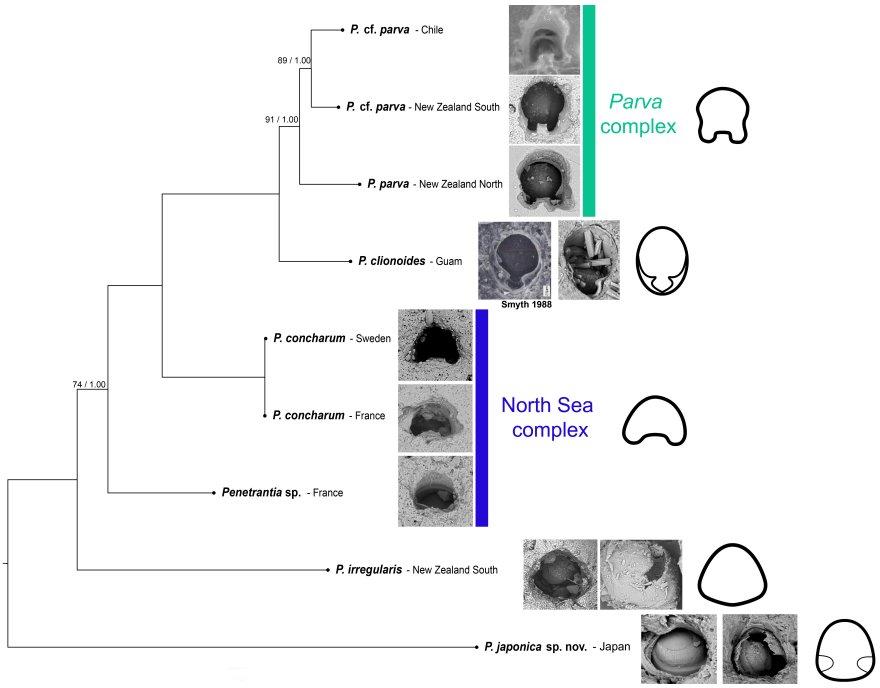
`Table 1_Sample details.xlsx` available at https://authorea.com/users/671941/articles/671244-boring-systematics-a-genome-skimmed-phylogeny-of-ctenostome-bryozoans-and-their-endolithic-family-penetrantiidae-with-the-description-of-one-new-species





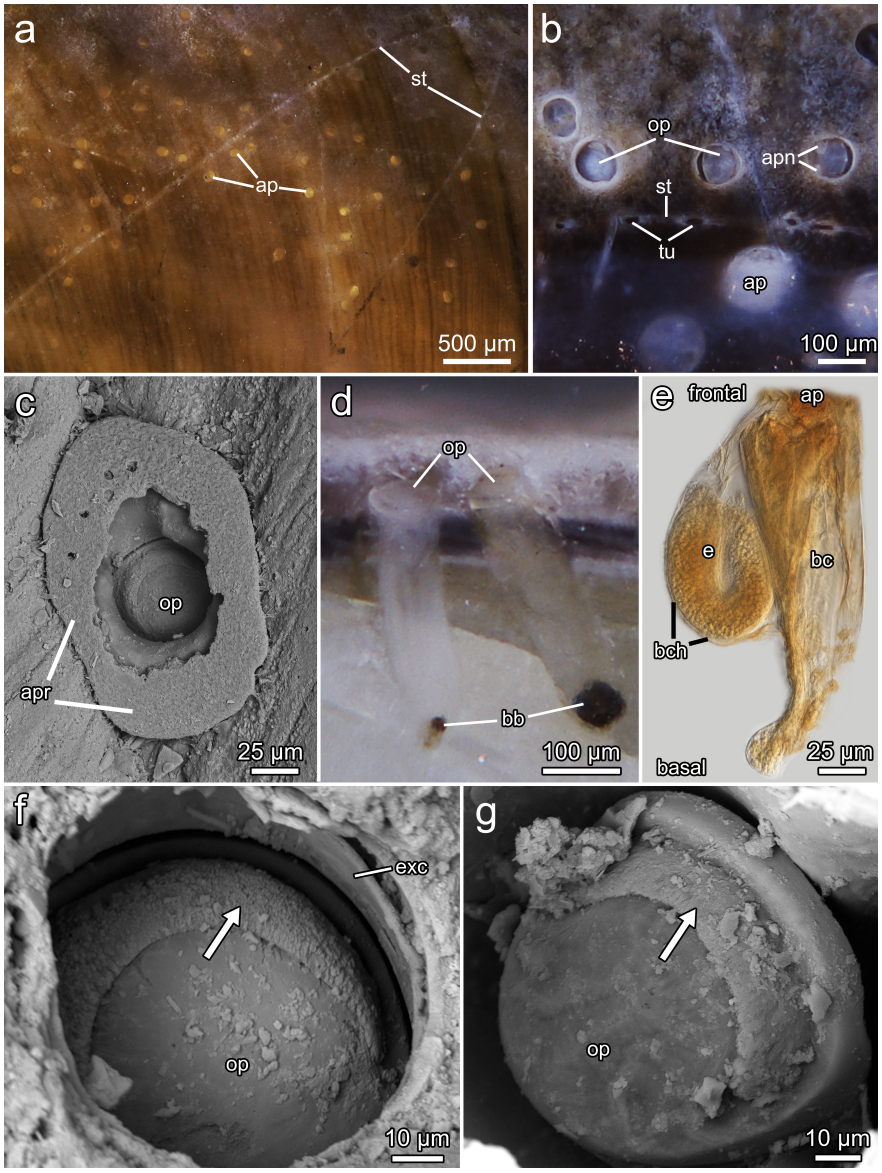


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Table A1_primer.xlsx available at https://authorea.com/users/671941/articles/671244-boring-systematics-a-genome-skimmed-phylogeny-of-ctenostome-bryozoans-and-their-endolithic-family-penetrantiidae-with-the-description-of-one-new-species

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Table A2_data matrix.xlsx available at https://authorea.com/users/671941/articles/671244-boring-systematics-a-genome-skimmed-phylogeny-of-ctenostome-bryozoans-and-their-endolithic-family-penetrantiidae-with-the-description-of-one-new-species

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Table A3_genetic divergence.xlsx available at https://authorea.com/users/671941/articles/671244-boring-systematics-a-genome-skimmed-phylogeny-of-ctenostome-bryozoans-and-their-

endolithic-family-penetrantiidae-with-the-description-of-one-new-species

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Table A4_genetic divergence.xlsx available at https://authorea.com/users/671941/articles/671244-boring-systematics-a-genome-skimmed-phylogeny-of-ctenostome-bryozoans-and-their-endolithic-family-penetrantiidae-with-the-description-of-one-new-species