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Corallinapetrales and Corallinapetraceae: A new order and family of coralline red algae including *Corallinapetra gabrielii* comb. nov.[1]

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ABSTRACT

The coralline algal genus *Corallinapetra* is currently monospecific, and was established on the species *Corallinapetra novaezelandiae,* known from a single collection from north-eastern New Zealand. On the basis of multi-gene phylogenetic analyses, *Corallinapetra* has been resolved apart from all currently recognized families and orders within the Corallinophycidae. We analysed DNA sequence data from the holotype of *Lithothamnion gabrieli*, which has been considered a heterotypic synonym of *L. muelleri*, and an unidentified sample collected from Stewart Island in New Zealand, using *psb*A, *rbc*L, and COI-5P genes. We also observed detailed morphological characters with light and scanning electron microscopy. Our phylogenetic analyses showed that *L. gabrieli* and the sample from New Zealand belonged to the same clade as *Corallinapetra*, distinct from other families and orders in the Corallinophycidae. Members of this clade are distinguishable from other families and orders in the Corallinophycidae by possessing sporangia that are surrounded by remnant sterile filaments that are weakly calcified in mature multiporate sporangial conceptacles that produce zonately divided tetrasporangia. Therefore, we propose that *Corallinapetra* be placed in its own family, Corallinapetraceae and order, Corallinapetrales, and that *L. gabrieli* should be assigned to *Corallinapetra*, as *C. gabrielii*, to reflect their phylogenetic relationships. We also obtained a partial *rbc*L sequence data from the lectotype of *L. muelleri*, the generitype of *Lithothamnion*. Comparison of the *L. muelleri* type sequence with *L. gabrieli* unambiguously demonstrated that these two species are not conspecific, and confirm the placement of *L. muelleri* within the Hapalidiales.

Key index words: COI-5P; Corallinophycidae; *Corallinapetra gabrielii*; Corallinapetrales; Corallinapetraceae; phylogeny; *psb*A; *rbc*L; taxonomy

Abbreviations: BI, Bayesian inference; BP, bootstrap; COI-5P; mitochondrial cytochrome *c* oxidase subunit I; CN, Herbier, Université de Caen, Caen, France; G, Gamma distribution; GTR, General Time Reversible model; I, proportion of invariable sites; ML, maximum likelihood; *psb*A, plastid-encoded photosystem II reaction center D1 protein gene; TRH, Norwegian University of Science and Technology, Trondheim, Norway

INTRODUCTION

Molecular systematics of coralline algae is revolutionizing our understanding of this important and ubiquitous group of benthic marine rhodophytes at all taxonomic ranks. DNA sequence data and phylogenetic analyses have become an essential tool in coralline studies and have led to major insights in the diversity and evolutionary history of this group (e.g., Hind et al. 2016, Rösler et al. 2016, Pezzolesi et al. 2019, Twist et al. 2019). Significant findings based on multi-gene analyses already have resulted in revisions at species level and at higher taxonomic ranks such as circumscription of a new subclass (Corallinophycidae; Le Gall and Saunders 2007) and of new orders (Sporolithales, Le Gall et al. 2010; Hapalidiales, Nelson et al. 2015). Despite active molecular phylogenetic studies, the taxonomy of coralline algae is not yet fully resolved and numerous taxonomic changes, even at higher taxonomic ranks, are still expected (Hind et al. 2016).

The Corallinophycidae, with about 730 species currently recognized (Guiry and Guiry 2020), is a large subclass regarded as encompassing four orders (Rhodogorgonales, Sporolithales, Hapalidiales, and Corallinales). This subclass is morphologically defined by pit plugs with a domed outer cap layer and thalli that are mineralized due to calcite precipitation (Le Gall and Saunders 2007, Nelson et al. 2015). With the exception of the Rhodogorgonales, all orders of Corallinophycidae are commonly referred to as 'coralline algae' (Townsend and Huisman 2018) as they play similar functional roles in the ecosystem and as they are the main builders of a typical algal reef with their hard texture. Each of the orders is distinguished based on specific reproductive and anatomical features, particularly the arrangement and division of tetrasporangia (cruciate/zonate), position of sporangia (sori/conceptacles), and the presence or absence of apical plugs in conceptacles (Le Gall et al. 2010, Bahia et al. 2015, Nelson et al. 2015). The Sporolithales encompasses those non-geniculate coralline algae with cruciately divided tetrasporangia each of which bears an apical plug, produced singly in a calcified compartment or grouped in a fertile area (sorus) and interspersed by calcified paraphyses (Woelkerling 1988, Verheij 1993, Le Gall et al. 2010, Bahia et al. 2015). The Hapalidiales encompasses only non-geniculate coralline algae with zonately divided tetrasporangia, each bearing an apical plug, and arranged in multiporate sporangial conceptacles (Nelson et al. 2015). The Corallinales is the only order possessing both non-geniculate and geniculate coralline algae; members produce zonately divided tetrasporangia without apical plugs that are arranged in uniporate sporangial conceptacles (Le Gall et al. 2010, Nelson et al. 2015). In regards to the sporophytic phase, the Sporolithales and the Hapalidiales

have a similar pattern in forming apical plugs above each sporangium, except that in the latter the vegetative filaments involved in conceptacle development tend to degenerate after sporangia mature (reviewed in Johansen 1981, Wilks and Woelkerling 1995 as 'sterile filaments').

Recently, the genus *Corallinapetra* was described by Nelson et al. (2015), including only one species, *C. novaezelandiae*. In the phylogenetic analyses, *Corallinapetra* was not resolved within any of the currently recognised families and orders, and was shown to have a sister relationship with the combined clade of Hapalidiaceae and Corallinaceae (Nelson et al. 2015). Although Nelson et al. (2015) observed detailed morpho-anatomical structures in an effort to clarify relationships between *Corallinapetra* and other members of the Corallinophycidae, they did not erect higher ranks for the genus *Corallinapetra* because of insufficient material for a detailed developmental and anatomical study. *Corallinapetra* was characterized as possessing non-geniculate crustose thalli, with smooth to slightly textured surfaces, flared epithallial cells, cell fusions between contiguous cells, a sporophytic phase with individual compartments grouped in shallow depressions small pores visible on the thallus surface giving appearance of multiporate conceptacles, and stalk cells of tetrasporangia (Nelson et al. 2015). The sporangial features of *Corallinapetra* were interpreted as "intermediate" between Sporolithales (individual sporangial compartments grouped) and Hapalidiales (multiporate sporangial conceptacles). In addition, the occurrence of taxa with flared epithallial cells is reported in both orders (Woelkerling 1988).

Of the coralline algae that develop zonate tetrasporangia within multiporate sporangial conceptacles, the genus *Lithothamnion* is the only genus forming flared epithallial cells (Harvey et al. 2003). Currently, 83 *Lithothamnion* species are recognized worldwide, mostly based solely on morphological analyses (Guiry and Guiry 2020). In order to resolve the relationship between *Lithothamnion* and *Corallinapetra*, based on the generitype of *Lithothamnion*, *L. muelleri* (type locality: Western Port Bay, Victoria, Australia; Wilks and Woelkerling 1995), and a species currently regarded as a heterotypic synonym, *L. gabrieli* (type locality: Ocean Beach, Philip Island, Victoria, Australia; Womersley 1996: 183), we sequenced type material, and included these data in the dataset downloaded from GenBank. We also included sequence data from recent collections from Stewart Island (southern New Zealand). In addition, detailed morpho-anatomical observations were made using both decalcified and non-decalcified material to better understand of the anatomy of these specimens. The goals of the present study were to (i) re-evaluate characters for delimitation of the genus *Corallinapetra*, (ii) determine higher-level taxonomic

relationships of the genus *Corallinapetra*, and (iii) reassess the taxonomic relationship between *Corallinapetra* and *Lithothamnion* using the type material of *Lithothamnion muelleri*.

MATERIAL AND METHODS

Specimen collections.

Fresh material of non-geniculate corallines was collected from Port Adventure, Stewart Island, New Zealand, at a depth of 30-35 m, by a ship-operated grab on March 30, 2017. Samples were placed in silica gel for DNA sequencing and for morpho-anatomical examination, and the voucher specimen was deposited in CUK (Herbarium, Chosun University, Gwangju, Korea). Subsamples of the holotype of *Lithothamnion gabrieli* (TRH B15-2362) and of the lectotype of *Lithothamnion muelleri* (CN unnumbered) were loaned from TRH and CN. Herbarium acronyms follow Thiers (2020) continuously updated.

DNA extractions, PCR amplification and sequencing.

For DNA extraction and PCR amplification, samples were cleaned with autoclaved seawater using a dissecting microscope. Genomic DNA was extracted using a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Genomic DNA from type materials of *Lithothamnion muelleri* and *L. gabrieli* was extracted using a QIAamp® DNA Micro Kit (Qiagen S.A.S., Les Ulis, France) following the manufacturer's protocol. We sequenced three genes: *psb*A, *rbc*L, and COI-5P. Each polymerase chain reaction (PCR) tube contained a 30 μL mixture of 4 μL genomic DNA, 1 μL of 10 pmol forward + 1 μL of reverse primers, 8.6 μL distilled water, and 15.4 μL the HelixAmp Ready–2x–Go Series (NanoHelix, Daejeon, Korea). The plastid *psb*A gene was amplified and sequenced using primers *psb*AF1 and *psb*AR2 (Yoon et al. 2002) following the protocol of Kim et al. (2012), and the plastid *rbc*L gene was amplified and sequenced in two reactions using primer pairs F57/R1150 and F993/R*rbc*Start following the protocol of Freshwater and Rueness (1994). The mitochondrial COI-5P region was amplified and sequenced using primers GazF1 and GazR1 (Saunders 2005) or SueF1 and SueR1 (Clayden and Saunders 2010) following the protocol of Saunders (2005). Amplified DNA was purified using the PCRquick-spin PCR product purification kit (iNtRON Biotechnology, Seongnam, Korea). The same primers were also used in cycle sequencing. Sequencing was performed by Macrogen (Seoul,

South Korea). Partial fragment of *rbc*L sequences (226 bp) were obtained from the lectotype material of *Lithothamnion muelleri* using primers F1150Cor and R*rbc*Start (Sissini et al. 2014); these PCR products were purified and sequenced by Eurofins (Eurofins Scientific, Nantes, France) and were assembled and aligned with the assistance of CodonCode Aligner® (CodonCode Corporation, USA) and adjusted by eye using SeaView version 4 (Gouy et al. 2010). Sequences were deposited in GenBank (Table S1 in the Supporting Information).

Sequence and phylogenetic analyses.

We analysed a dataset of concatenated *psb*A, *rbc*L, and COI-5P sequence data including our sequences, and sequences from *Corallinapetra* and members of all four extant orders within the Corallinophycidae to resolve phylogenetic relationships within the subclass. We included sequences from *Palmaria palmata* (Palmariales, Rhodophyta) and *Thorea violacea* (Thoreales, Rhodophyta) as outgroups. We also analysed a dataset of *rbc*L sequences only, including a larger number of sequences from the Corallinophycidae, in order to test the phylogenetic position of *Lithothamnion muelleri.* Nomenclature for New Zealand sequences follows that of Twist et al. (2019), omitting the '-Twist-2019' extension. Sequence datasets were aligned using ClustalW (Thompson et al. 1994) and manually corrected using MEGA7 (Kumar et al. 2016). PartitionFinder 2 (Lanfear et al. 2016) was used to determine the best partition scheme and model of evolution as implemented by RAxML. Maximum likelihood analysis was conducted under the GTR+G+I model of sequence evolution with 1,000 bootstrap replicates using RAxMLGUI v1.5 (Stamatakis 2006, Stamatakis et al. 2008, Silvestro and Michalak 2012). Bayesian analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Markov chain Monte Carlo runs were carried out for two million generations, each with one cold chain and three heated chains, using the GTR+Γ+I evolutionary model, with sampling and printing occurring every 1,000 generations. Summary trees were generated using a burn-in of 25%.

Morphological observations.

All the materials observed for morpho-anatomical studies were confirmed by DNA sequences. For light microscopy, samples were previously decalcified in 0.6M nitric acid, then rinsed with distilled water and stained using a 3:7 mixture of 1% aqueous aniline blue and glycerin solution (10% glycerol: 90% absolute ethanol) for 30-120 min (Jeong et al. 2019). Thallus sections, 5–20

μm thick, were prepared using an embedding matrix (O.C.T.; CellPath, Newtown, Wales, UK) and a freezing microtome (Shandon Cryotome FSE; Thermo Shandon, Loughborough, UK). Sections were then treated with Mayer's hematoxylin (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Micrographs were taken using an Olympus microscope (BX51TRF; Olympus, Tokyo, Japan) equipped with an Olympus DP71 digital camera. Permanent slides were mounted in 70% Karo corn syrup.

For scanning electron microscopy (SEM), selected fragments of samples were mounted on aluminium stubs using double-sided adhesive carbon tape (Nisshin EM, Tokyo, Japan) and coated with 18–20 nm of gold using a digital ion sputter coater (SPR-20, COXEM, Korea). Samples were examined in a COXEM EM-30 PLUS (Mini SEM; COXEM, Korea) SEM at an accelerating voltage of 15 kV.

To confirm the degree of calcification of the remnant sterile filaments within the tetrasporangial conceptacles and vegetative cell walls, the type material of *Lithothamnion gabrieli* was analysed by SEM-EDS at the Centre for Scientific Instruments of Chosun University, Korea. This method was used for spot analyses to qualify the elemental composition of representative parts of the remnant sterile filaments within the tetrasporangial conceptacles and vegetative cell walls of *L. gabrieli*. This analysis was carried out with the help of computer controlled cold-cathode field emission scanning electron microscope SEM (Hitachi S-4800) equipped with an energy-dispersive spectrometer system (EDS, Horiba, ISIS310). The samples were mounted on aluminium stubs using double-sided adhesive carbon tape (Nisshin EM Co. Ltd, Tokyo, Japan) and were not coated. The working distance was 14-16 mm and the accelerating voltage was at 15 kV. The live time for each measurement was 60s. The system was calibrated using magnesium oxide (MgO) and wollastonite ($CaSiO_3$) as standards for magnesium and calcium. Three measurements were taken of the remnant sterile filaments located within tetrasporangial conceptacles andvegetative cell walls.

RESULTS

Sequence data.

We obtained sequence data (Fig. 1) from the *psb*A (819 bp), *rbc*L (1323 bp), and COI-5P (603 bp) loci from the sample newly collected from Stewart Island, southern New Zealand. Target DNA sequences (*psb*A, 819 bp; *rbc*L, 783 bp; COI-5P, 603 bp) were obtained from the holotype of

Lithothamnion gabrieli (TRH B15-2362) and also *rbc*L sequence data (226 bp) from the lectotype of *Lithothamnion muelleri* (CN Herb. Lenormand, unnumbered) (Table S1). Although the *rbc*L fragment from the lectotype specimen of *Lithothamnion muelleri* was short, it differed by more than 32 bp (16.7%) from the sequence data obtained from holotype specimen of *L. gabrieli*. On this basis, we concluded that *Lithothamnion muelleri* and *L. gabrieli* are not conspecific. Furthermore, the *rbc*L sequence from the type specimen of *Lithothamnion muelleri* revealed that sequences available in GenBank attributed to *Lithothamnion* species, although not based on type material, such as "*L*". *crispatum*, "*L*". *lemoineae*, and "*L*". *glaciale*, are not resolved with *L. muelleri* and represent a different genus (Fig. 2). Sequence divergences between *Lithothamnion muelleri* and sequences other "*Lithothamnion*" for *rbc*L gene species were 13.0–14.2%.

Sequence divergences between the holotypes of *Lithothamnion gabrieli* and *Corallinapetra novaezelandiae* (NZC2381) were 0.7–1.0% for *psb*A, 1.5–1.6% for *rbc*L, and 5.4% for COI-5P genes. Sequence divergences between holotype material of *C. novaezelandiae* (NZC2381) and a sample newly collected from Stewart Island were 0.5% for *psb*A and 0.4% for *rbc*L genes.

Phylogenetic analyses.

A total of 7 sequences were newly generated in the present study: 2 *psb*A, 3 *rbc*L, and 2 COI-5P (Table S1). The concatenated dataset consisted of 36 taxa and 2745 nucleotides; the individual *rbc*L dataset consisted of 54 taxa and 1269 nucleotides. The result of the concatenated analysis is shown in Figure 1. *Corallinapetra* sequences were resolved with full support as a separate clade from remaining orders. Full support was also obtained for Rhodogorgonales; support for the Sporolithales (1.00/99 for Bayesian posterior probability and ML BP, respectively), Hapalidiales (1.00/98) and Corallinales (1.00/91) was also strong. The result of the *rbc*L analysis is shown in Figure 2. In this analysis, all four extant orders within Corallinophycidae were resolved as monophyletic with good support. *Corallinapetra* sequences were again determined as a separate clade with full support; *Lithothamnion muelleri* was resolved within the Hapalidiales. However, interestingly, in both analyses *L. gabrieli* was resolved in a strongly supported monophyletic clade with *C. novaezelandiae*. These analyses demonstrate that *Corallinapetra* is resolved as a distinct clade within the subclass Corallinophycidae and *L. gabrieli* is resolved within the *Corallinapetra* clade.

SEM-EDS.

Characteristic X-ray peaks of elemental composition detected with EDS focused on the remnant sterile filaments within the tetrasporangial conceptacles and vegetative cell walls shown in Table 1. Three measurements were taken from holotype material of *Lithothamnion gabrieli*. The SEM-EDS microanalysis showed low X-ray peaks of calcium and magnesium in the remnant sterile filaments within the tetrasporangial conceptacles, indicating low calcification. On the other hand, the higher peaks of calcium and magnesium in the vegetative cell walls, indicating the presence of calcium carbonate.

Taxonomic results.

On the basis of the morpho-anatomical observations and molecular phylogenetic data, we propose a new combination for *Lithothamnion gabrieli*, following the International Code of Nomenclature for Algae, Fungi, and Plants (ICNAFP; Turland et al. 2018), and emend the description of the genus *Corallinapetra*.

Corallinapetra gabrielii (Foslie) S.Y.Jeong, W.A.Nelson, B.Y.Won & T.O.Cho **comb. nov.** (Figs. 3–5)

Basionym: Lithothamnion gabrieli Foslie, K. norske Vidensk. Selsk. Skr. (5): 3 (1905).

Holotypus: TRH B15-2362 (Fig. 3A), collected on April, no date, 1905 by J. Gabriel from Ocean Beach, Philip Island, Victoria, Australia, deposited at TRH (Woelkerling et al. 2005: 316).

Description: Non-geniculate, pink to light red in colour, encrusting to slightly lumpy, firmly adherent to the substrate. Texture glossy, smooth to uneven-surfaced (Fig. 3A). Thalli 632–1750 μm thick, pseudoparenchymatous, dorsiventrally organized, with monomerous construction. Haustoria absent. Medulla plumose, non-coaxial, 57–240 μm thick, comprised of approximately eight to twelve cell layers, usually less than 9–17% of mature thallus thickness (Fig. 3C). Protuberances low domed, up to 0.1–0.5 mm long and up to 0.5–0.7 mm wide at their rounded distal end. Medullary cells rectangular to elongated with rounded corners, two to three times as long as wide, 5–40 μm long, 4–7 μm wide. Medullary filaments curved toward thallus surface to form a zoned cortex (cortical region). Cortex 557–1220 μm thick, usually comprising over 83–91% of mature thallus thickness. Cortical cells rectangular to elongated, 9–11 μm long, 4–6 μm wide. Sub-epithallial initials ovoid to elongated with rounded corners, as long as or longer than the cells immediately subtending them, 8–11 μm in length, 4–6 μm in diameter (Fig. 3D). Epithallus

composed of a single layer of flared and flattened cells, 2–3 μm long, 4–6 μm wide (Fig. 3D). Epithallial cells with thickened calcified cell walls that surround central concavities (Fig. 3B). Cell fusions narrow, abundant, frequently occupying most of the walls of adjoining medullary and cortical filaments (Fig. 3E). Secondary pit connections and trichocytes not observed.

No gametangial structures were observed. Tetrasporangial conceptacles multiporate, raised to mound-like, 510–720 μm in external diameter, 65–88 μm in external height above the surrounding thallus surface (Fig. 4, A and B). Pore plates slightly sunken in depression, 360–550 μm in diameter, have 80–90 pores (Fig. 4, A and B). Each pore surrounded by 7–10 rosette cells (Fig. 4C). Conceptacle primordia developed adventitiously below, more than 10 cell layers from the thallus surface (Fig. 4E). Conceptacle chambers transversely elliptical (Fig. 4D), measuring 405–604 μm in diameter and 147–170 μm in height, separated by 4–5 remnant sterile filaments comprised of 6–8 elongate cells each that are narrower and more elongated than surrounding vegetative filaments (Fig. 5, A and B). Sporangia individually separated from one another by weakly calcified remnant sterile filaments in mature tetrasporangial conceptacle chamber (Fig. 5C). Tetrasporangia zonately divided, scattered across the chamber floor, 45–172 μm in length, 12–83 μm in diameter (Fig. 5D), each one subtended on a single stalk cell measuring 4–13 μm in length, 7–18 μm in diameter (Fig. 5E). Bisporangia not observed. Conceptacle roofs 39–45 μm thick. Pore canals blocked by apical plugs 11–17 μm in diameter (Fig. 5F). Pore canals lined by filaments composed of 4–5 elongate cells (Fig. 5G). Chambers possess a basal layer of elongate cells (Figs. 5H and 5I). Buried, senescent tetrasporangial conceptacles not observed.

Distribution: Confirmed by DNA sequence data to be distributed in Victoria, Australia.

Comments: Corallinapetra gabrielii can be distinguished from *C. novaezelandiae* by the raised tetrasporangial conceptacles with slightly sunken pore plates and the presence of elongated cells on the bottom of tetrasporangial conceptacle chambers.

Nelson et al. (2015) originally described *Corallinapetra* with the following features: non-geniculate, crustose, smooth to slightly textured surface, flared epithallial cells, cell fusions, gametophytic phase with uniporate conceptacles, sporophytic phase with individual compartments grouped in shallow depressions, small pores visible on thallus surface giving appearance of multiporate conceptacles, and stalk cells.

Our detailed morpho-anatomical observations of *Corallinapetra gabrielii* confirmed the growth-form as crustose thalli, flared epithallial cells, cell fusions between contiguous cells, a sporophytic phase with weakly calcified remnant sterile filaments in multiporate conceptacles, and

stalk cells. However, *C. gabrielii* is distinguished from *C. novaezelandiae* based on its tetrasporangial conceptacles: raised tetrasporangial conceptacles with a concave pore plate and the presence of elongated basal cells on the bottom of tetrasporangial conceptacles in the former, whereas depressed sporangial conceptacles below the thallus surface and the absence of elongated basal cells on the bottom in the latter. While the characteristics of *C. gabrielii* found in the present study support the concept of *Corallinapetra* established by Nelson et al. (2015), the original generic concept of *Corallinapetra* requires emending:

Corallinapetra **T.J.Farr, W.A.Nelson & J.E.Sutherland, 2015; emendavit S.Y.Jeong, W.A.Nelson, B.Y.Won, & T.O.Cho**

As emended here, zonately divided tetrasporangia, produced singly, with apical plugs, within weakly calcified remnant sterile filaments in multiporate sporangial conceptacles, developing conceptacle primordia (more than 10 cells) adventiously, cell fusions, and lacking secondary pit-connections.

DISCUSSION

Morpho-anatomical similarities among members of the Corallinophycidae may mask significant developmental differences, resulting in taxonomic confusion. Molecular-based phylogenies of Corallinophycidae have challenged the traditional systems of classification based solely on morphology. Recent multi-gene based molecular investigations have explored the phylogenetic relationships within the red algal subclass Corallinophycidae (Le Gall and Saunders 2007, Le Gall et al. 2010, Nelson et al. 2015, Rösler et al. 2016, Caragnano et al. 2018, Twist et al. 2019). Moreover, techniques have improved for obtaining sequence data from older specimens and are now being applied to type specimens, enabling the resolution of taxonomic problems for some species of coralline algae (Gabrielson et al. 2011, Hind et al. 2014, 2015, Sissini et al. 2014, Adey et al. 2015, Basso et al. 2015, Hernandez-Kantun et al. 2015, Gabrielson et al. 2018, Richards et al. 2017).

Corallinapetra gabrielii was assigned to *Lithothamnion* by Foslie (1905) because it resembled members of *Lithothamnion* anatomically by forming flared epithallial cells and apical plugs in sporangial conceptacles ("small warty thallus with irregular excrescences, relatively thin medulla, immersed or slightly raised sporangial conceptacles with shallowly concave pore plate, 60–80 pores, and absence of buried senescent sporangial conceptacles"). Adey (1970), transferred the

species to *Mesophyllum* without comment, but likely due to the hypothallus which was described by Foslie (1907) as coaxial. Wilks and Woelkerling (1995) made a detailed study of southern Australian *Lithothamnion* species, and they concluded that *Lithothamnion gabrieli* was morphologically and anatomically concordant with *L. muelleri* and thus treated it as a heterotypic synonym. Wilks and Woelkerling (1995) provided morpho-anatomical descriptions using type material of both *Lithothamnion muelleri* and *L. gabrieli*. However, the molecular evidence presented here does not support the previous synonymy of "*Lithothamnion gabrieli*" (= *C. gabrielii*) with *Lithothamnion muelleri* as accepted by Wilks and Woelkerling (1995). The poor condition of the conserved type material of *Lithothamnion muelleri* prevents any definite generic assignment for this specimen (Fig. S1 in the Supporting Information). Although Wilks and Woelkerling (1995) referred to a fertile plant when providing detailed accounts of *L. muelleri*, the sporangial conceptacle images provided were not all taken from the type material. The image presented as figure 4A in the paper of Wilks and Woelkerling (1995) was taken using the specimen LTB-14121 collected in Western Australia. This image clearly shows the remnant sterile filaments interspersed among sporangia (Wilks and Woelkerling 1995) like *Corallinapetra*, and thus the identification of specimen LTB-14121 needs to be confirmed by molecular analysis. Further research to clarify *L. muelleri* is required.

Phylogenetic analyses of *rbc*L sequence data revealed three taxa attributed as *Lithothamnion* species, "*L.*" *crispatum*, "*L.*" *glaciale*, and "*L.*" *lemoineae,* did not belong in the *Lithothamnion* clade based on data from the lectotype of *Lithothamnion muelleri*. Sequence data from type material of these three species is required to confirm their generic placement. Morpho-anatomical analyses of a wide array of *Lithothamnion muelleri* samples are necessary to determine its generic characters and geographic distribution.

The genus *Corallinapetra* was monotypic when first described. Nelson et al. (2015) commented that, based on nSSU sequence data, *C. novaezelandiae* is closely related to an undescribed taxon collected in southern Australia by Bailey et al. (2004), and they considered this to be indication that *Corallinapetra* may not be monotypic. The nSSU sequence obtained from holotype material of *C. gabrielii* is identical with that obtained from the unidentified sample in the analysis of Bailey et al. (2004) (AY247408.1, as Corallinales sp. CB-2003 in GenBank; data not shown). The material sequenced by Bailey et al. (2004) would thus appear to be *C. gabrielii.*

Our recently collected material of *Corallinapetra novaezelandiae* sampled from Stewart Island in southern New Zealand corresponds in habit and vegetative structure to the description of

Nelson et al. (2015). Our multi-gene sequences revealed intraspecific differences in the *psb*A sequences (0.5%), and *rbc*L sequences (0.4%), respectively. As a comparison, cryptic species recognized in other genera of Corallinales differed by >0.7-0.8% in the *psb*A gene sequences and >0.5%-0.8% in the *rbc*L gene (Gabrielson et al. 2011, Hind et al. 2016, Pezzolesi et al. 2019). However, we were not able to find any morphological differences to distinguish the northern and southern populations of *Corallinapetra* based on material currently available, and we therefore are recognising a single species. Further material is needed for a more detailed reproductive study. The distributional range of *C. novaezealandiae* is extended from the North Island to the new record from Stewart Island, New Zealand. Our phylogenetic analyses of concatenated *psb*A, *rbc*L, and COI-5P gene sequences show that *Corallinapetra* forms a strongly supported monophyletic clade with a sister relationship with the combined clade of Hapalidiales and Corallinales. This agrees with the molecular phylogeny of the Corallinophycidae proposed by Nelson et al. (2015). While molecular data can provide compelling evidence for the erection of taxonomic hierarchies, in our view they should not be the sole basis for determining higher level classification to the exclusion of vegetative, reproductive, and ultrastructural information. It is desirable that new, higher level taxa such as families and orders, are based on additional factors (Saunders and Kraft 1994). We have therefore reviewed the anatomy of *C. novaezelandiae* and *C. gabrielii* in relation to the other families and orders in the Corallinophycidae.

The division of tetrasporangia and the structure of sporangial conceptacles have been used as key characters for distinguishing higher taxonomic ranks within Corallinophycidae (Nelson et al. 2015). The principal morphological character delineating *Corallinapetra* is that the weakly calcified groups of sterile filaments are interspersed amongst developing sporangia in multiporate sporangial conceptacles. Members of the Sporolithales can be distinguished by tetrasporangia that produce cruciately arranged spores that develop within calcified sporangial compartments in sporangial sori (Townsend et al. 1995, Le Gall et al. 2010). In Hapalidiales, zonately divided tetrasporangia are produced in multiporate sporangial conceptacles with apical plugs, whereas in Corallinales zonately divided tetrasporangia are produced in uniporate sporangial conceptacles without apical plugs (Townsend et al. 1995, Nelson et al. 2015). Morpho-anatomical observations of *C. gabrielii,* using both SEM and light microscope, revealed a distinctive tetrasporangial conceptacle structure that includes groups of sterile filaments interspersed amongst developing sporangia. Each tetrasporangium was enclosed by four to five weakly calcified remnant sterile filaments comprised of six to eight elongate cells (resembling individual compartments) in

multiporate tetrasporangial conceptacles. These 'individual compartments' were also observed in *C. novaezelandiae* by Nelson et al. (2015). Weakly calcified remnant sterile filaments were not recognized under the light microscope, but SEM images confirmed that the sterile filaments were weakly calcified, showing their structure as membranous in several layers. Thus morpho-anatomical observations using both light microscope and scanning electron microscope are essential for better understanding the morphology of coralline algae.

Coralline algae are composed of high-Mg calcite with calcification occurring in the cell wall guided by a polysaccharide matrix (Borowitzka 1977, Nash et al. 2019). In order to compare the degree of calcification between the remnant sterile filaments and the vegetative cells, qualitative microanalysis using SEM-EDS enabled identification of the characteristic X-ray peaks of the two main elements of calcification present in *Lithothamnion gabrieli* (Table 1). The peaks of calcium and magnesium were very low in the remnant sterile filaments within the tetrasporangial conceptacles, while higher peaks were detected in the vegetative cells. This suggests that sterile filaments are weakly calcified when compared to other cells in the thallus.

Sterile filaments (paraphyses sensu Verheij 1993) have been observed in all species within the Sporolithales (Henriques et al. 2014). Each sporangium is isolated by elongated and heavily calcified paraphyses in sporangial sori (Townsend et al. 1995, Harvey et al. 2002). Some species belonging to the Hapalidiales and Corallinales also have been reported to have 'sterile filaments' or 'remnants of elongate cells' among sporangia in mature sporangial chambers (e.g., *Lithothamnion muelleri* and *Lithothamnion indicum,* Wilks and Woelkerling 1995; *Melobesia membranacea*, Wilks and Woelkerling 1991; *Mesophyllum engelhartii*, Riosmena-Rodríguez and Vásquez-Elizondo 2011; *Phymatolithon masonianum* and *Phymatolithon repandum,* Wilks and Woelkerling 1994; and *Titanoderma pustulatum*, Harvey et al. 2009). However, it is presumed that these filaments degenerate to form the conceptacle chamber. No vegetative filaments are usually preserved within the conceptacle separating the sporangia (Tomás et al. 2007). However, the remnant sterile filaments in *Corallinapetra* were weakly calcified and persisted after spore development and release. The sporangia in the Rhodogorgonales are not known to occur in conceptacles or sori (calcified compartments; Townsend and Huisman 2018).

Our morpho-anatomical observations and phylogenetic analyses support monophyly of the *Corallinapetra* clade within the subclass Corallinophycidae, as a sister taxon to the combined clade of Hapalidiales and Corallinales. We consider that the *Corallinapetra* group is clearly distinct at the ordinal level and therefore propose to place *Corallinapetra* in a new family, the

Corallinapetraceae fam. nov. and a new order, the Corallinapetrales ord. nov. Nelson et al. (2015) similarly considered that *Corallinapetra* could represent a previously unknown order within the Corallinophycidae. Figure 6 summarizes the characters of the five orders of the Corallinophycidae.

Corallinapetrales S.Y.Jeong, W.A.Nelson, B.Y.Won & T.O.Cho **ord. nov.**

Description: Corallinapetrales, with the characteristics of the Corallinophycidae (Le Gall and Saunders 2007, Nelson et al. 2015); differs from other orders (Corallinales, Rhodogorgonales, Hapalidiales, Sporolithales) in producing zonately divided tetrasporangia, with sporangia borne in multiporate sporangial conceptacles, each sporangium separated by weakly calcified remnants of sterile filaments comprised of elongate cells, with apical plugs; without genicula; cells of adjacent filaments connected by cell fusions, and lacking secondary pit-connections.

Type family: **Corallinapetraceae** S.Y.Jeong, W.A.Nelson, B.Y.Won & T.O.Cho **fam. nov.**

Description: sporangial cleavage exclusively zonate, each sporangium separated by weakly calcified remnant sterile filaments, with apical plugs, and developing beneath multiporate plates; without genicula; cells of adjacent filaments connected by cell fusions, lacking secondary pit-connections.

Some speculation is now possible concerning the evolutionary relationships within the subclass Corallinophycidae. According to Tomás et al. (2007), multiporate sporangial conceptacles could have originated from the fusion of several sporangial cavities, suggesting *Sporolithon rude* as the precursor of the sporangial multiporate conceptacles of the Hapalidiales based on anatomical and phylogenetic evidence. In addition, from a vegetative anatomical point of view, *Sporolithon* (Sporolithales) and *Lithothamnion* (Hapalidiales) have been known as the only two genera within Corallinophycidae with flared epithallial cells (Tomás et al. 2007). However, the presence of calcified walls of individual sporangial chambers within the fused structures and sporangial cavities grouped in sori confirm that *S. rude* is closer to *Sporolithon* than hapalidialeans (Tomás et al. 2007).

In our study, we confirmed that *Corallinapetra* also develops flared epithallial cells like *Sporolithon* and *Lithothamnion*. On the other hand, the sporangial chamber of *Corallinapetra* forms multiple apical pore canals, as seen in the multiporate sporangial conceptacles of the Hapalidiales, although separated by elongated and weakly calcified remnants of sterile filaments. Thus, the *Corallinapetra* group shows anatomical characteristics of both Sporolithales and Hapalidiales. Likewise, in the molecular phylogenetic analyses Corallinapetrales was positioned between the Sporolithales and Hapalidiales clades. Further work revisiting the fossil record to

search for putative members of the Corallinapetrales is needed to better understand the evolution of the morphology of sporangial conceptacles, a task beyond the scope of the present study.

To date Corallinapetrales species are restricted to New Zealand and South Australia. Future studies based on more extensive collections are necessary to understand the geographic ranges of species in Corallinapetrales, and to further investigate the relationships of this intriguing and distinct group of coralline algae. Our study reinforces the importance of molecular sequence data in resolving the taxonomic relationships of coralline algae.

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FIG. 1. Phylogenetic tree based on Bayesian analysis of the concatenated sequences (*psb*A, *rbc*L, and COI-5P). Values above branches denote Bayesian posterior probabilities (BPP) > 0.75/ maximum-likelihood bootstrap values (BP, %) > 50. BPP values < 0.75 and BP values < 50% are indicated by hyphens (-). BPP values of 1.00 and BP values of 100% are indicated by asterisks (*). New sequences in bold.

FIG. 2. Phylogenetic tree based on Bayesian analysis of *rbc*L sequences. Values above branches denote Bayesian posterior probabilities (BPP) > 0.75/maximum likelihood bootstrap values (BP) in % > 50. BPP values <0.75 and BP values <50% are indicated by hyphens (-). BPP values of 1.00 and BP values of 100% are indicated by asterisks (*). New sequences in bold.

FIG. 3. Habit and vegetative anatomy of *Corallinapetra gabrielii* (TRH B15-2362). (A) Holotype specimen of *C. gabrielii*. Scale bar = 1 cm. (B) Surface view of epithallial cells. Scale bar = 10 μm. (C) Vertical fracture of thallus showing monomerous construction. Scale bar = 25 μm. (D) Vertical fracture of outer thallus showing flared epithallial cells (e) and subepithallial initials (i) that are long as or longer than the cells subtending them. Scale bar = 10 μm. (E) Vertical fracture through thallus showing cell fusions (arrowheads) between adjacent filaments. Scale bar = 5 μm.

FIG. 4. Tetrasporangial anatomy of *Corallinapetra gabrielii* (TRH B15-2362). (A) Surface view of raised tetrasporangial conceptacle showing concave multiporate pore plate. Scale bar = 200 μm. (B) Magnified surface view of tetrasporangial conceptacle pore plate. Scale bar = 100 μm. (C) Magnified surface view showing conceptacle pore bordered by ten rosette cells (labelled 1 to 10). Scale bar = 10 μm. (D) Vertical section showing non-buried tetrasporangial conceptacles. Scale bar = 1 mm. (E) Vertical section through immature tetrasporangial conceptacle showing primordium developing adventitiously from group of vegetative cells (arrows). Scale bar = 50 μm.

FIG. 5. Tetrasporangial anatomy of *Corallinapetra gabrielii*. (A) Vertical fracture through sporangial conceptacle showing elliptical-shaped chamber and sporangia (s) separated by weakly

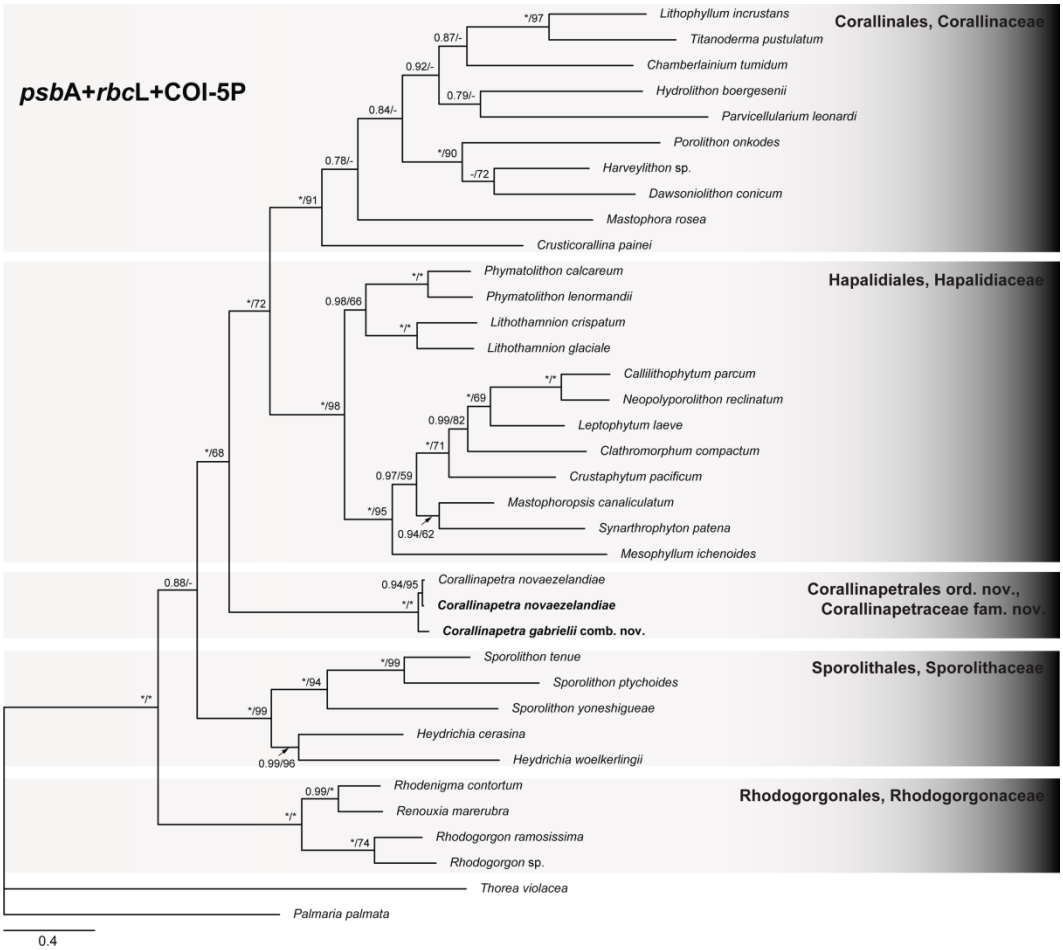
calcified remnant sterile filaments (arrows). Scale bar = 100 μm. (B) Vertical section through sporangial conceptacle showing sporangia (s) and remnant sterile filaments (arrows). Scale bar = 200 μm. (C) Vertical section through sporangial conceptacle showing four to five remnant sterile filaments (arrow). Scale bar = 50 μm. (D) Magnified view of tetrasporangia (t) in zonate division. Scale bar = 25 μm. (E) Vertical section through sporangial conceptacle showing tetrasporangium with basal stalk cell (arrow). Scale bar = 50 μm. (F) Vertical section through sporangial conceptacle showing apical plug (arrow). Scale bar = 20 μm. (G) Vertical fracture through sporangial conceptacle roof showing pore canal and cells (asterisks) lining pore canal. Scale bar = 10 μm. (H) Vertical section through old sporangial conceptacle bottom showing elongated cells lining bottom of conceptacle (arrow). Scale bar = 50 μm. (I) Magnified view of elongated cells on sporangial conceptacle bottom. Scale bar = 10 μm.

FIG. 6. Characters of the five orders of Corallinophycidae.

FIG. S1. Lectotype material of *Lithothamnion muelleri* in CN.

Table. 1. Mineral data of SEM-EDS of remnant of sterile filaments within tetrasporangial conceptacle and vegetative cell walls of *Lithothamnion gabrieli*. The present study concentrated on Ca and Mg.

Element		**Remnant of sterile filaments within tetrasporangial conceptacle**		**Vegetative cell walls**	
		Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
Ca K	No. 1	3.31	1.16	24.65	11.01
	No. 2	0.32	0.11	62.72	40.07
	No. 3	1.12	0.41	29.22	13.64
Mg K	No. 1	0.40	0.23	3.38	2.49
	No. 2	0.80	0.46	3.69	3.88



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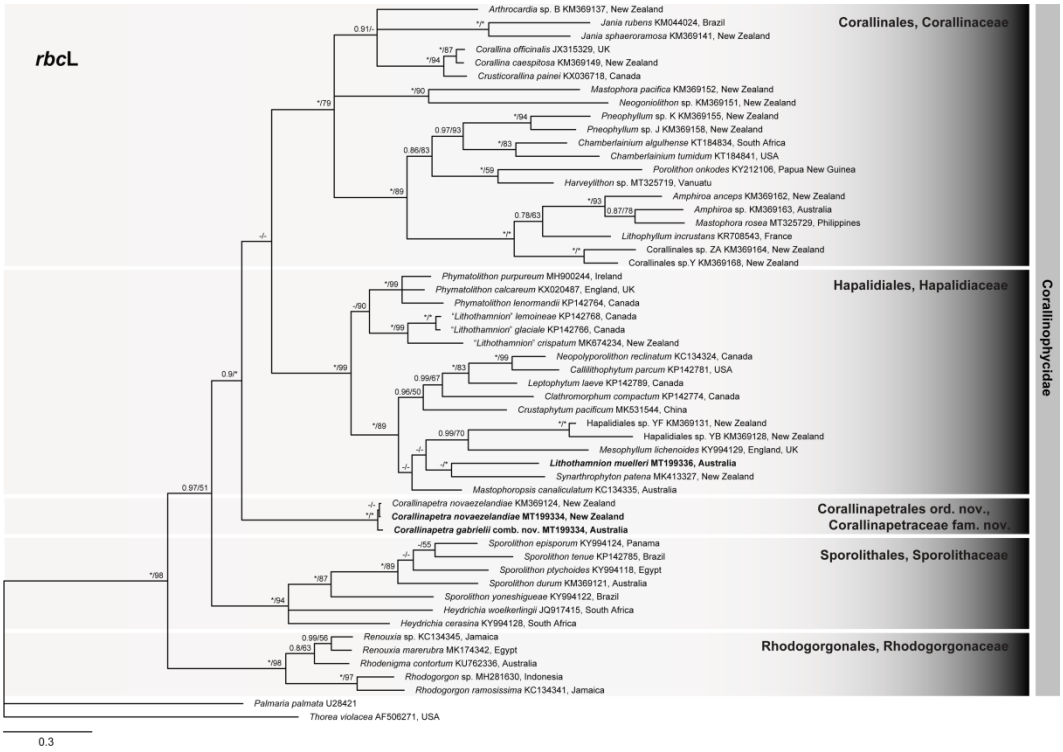

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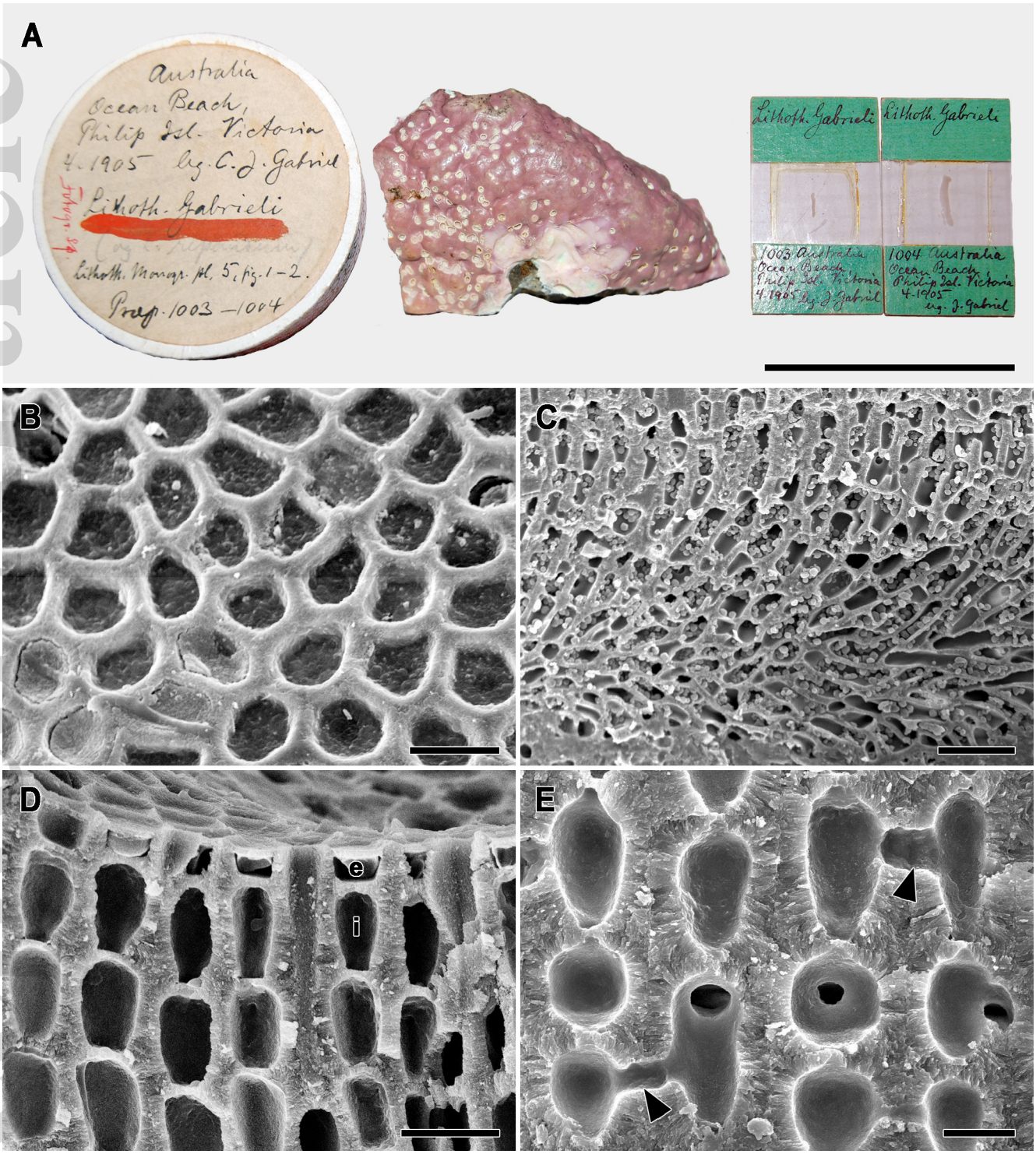

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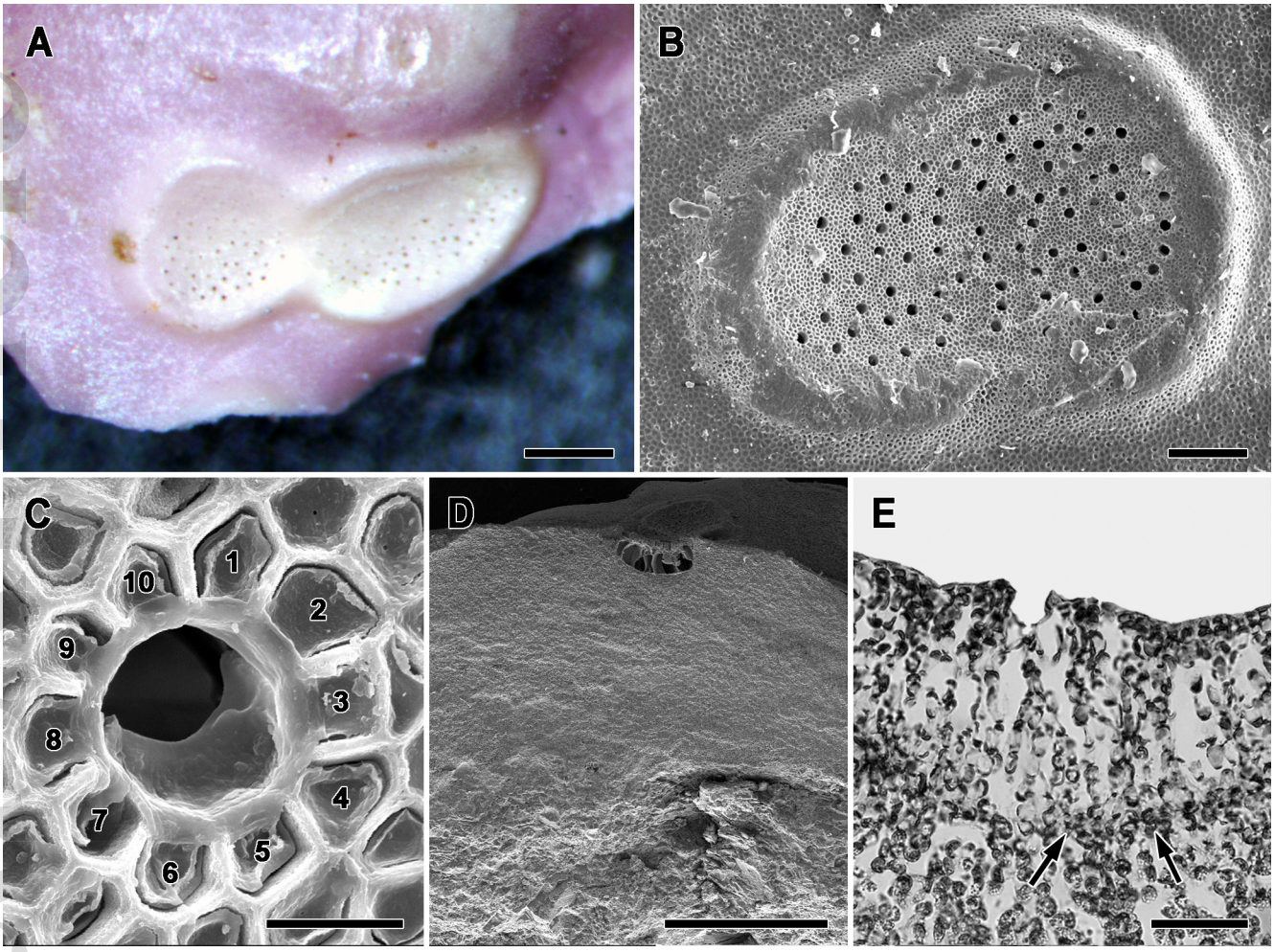

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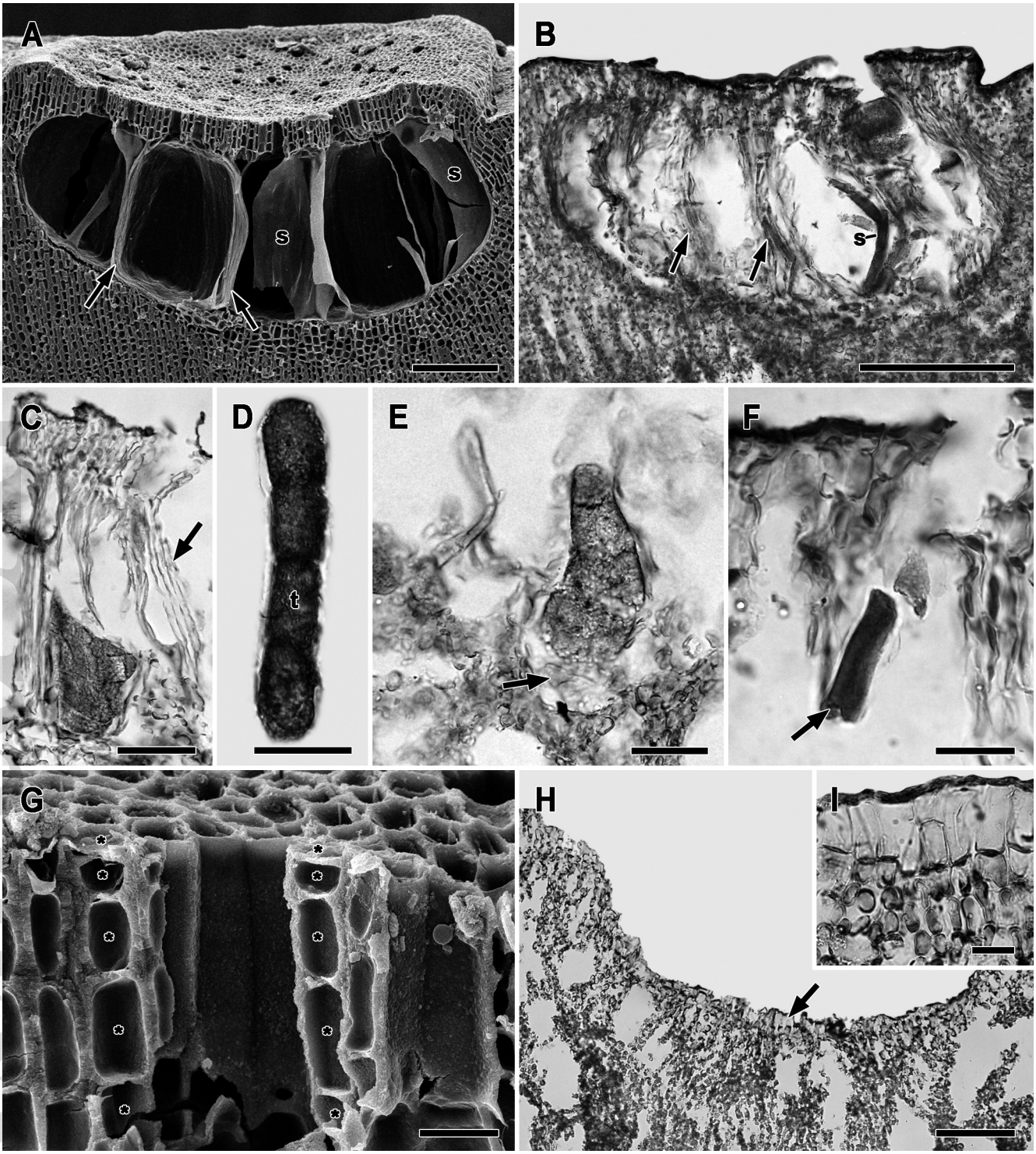

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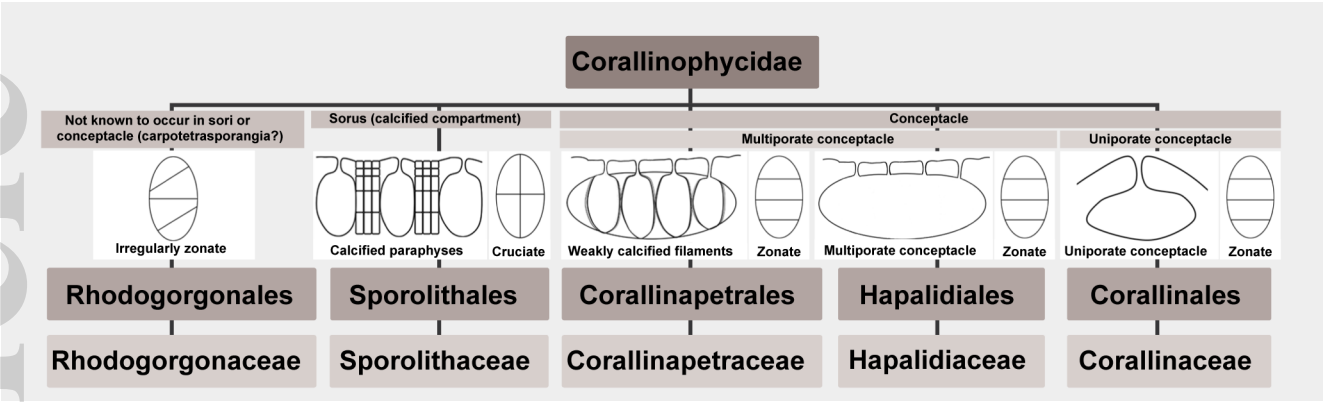

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