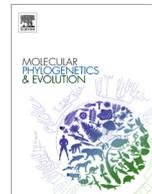




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A novel phylogeny of the Gelidiales (Rhodophyta) based on five genes including the nuclear *CesA*, with descriptions of *Orthogonacladia* gen. nov. and Orthogonacladiaceae fam. nov.



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ABSTRACT

Although the Gelidiales are economically important marine red algae producing agar and agarose, the phylogeny of this order remains poorly resolved. The present study provides a molecular phylogeny based on a novel marker, nuclear-encoded *CesA*, plus plastid-encoded *psaA*, *psbA*, *rbcL*, and mitochondria-encoded *cox1* from subsets of 107 species from all ten genera within the Gelidiales. Analyses of individual and combined datasets support the monophyly of three currently recognized families, and reveal a new clade. On the basis of these results, the new family Orthogonacladiaceae is described to accommodate *Aphanta* and a new genus *Orthogonacladia* that includes species previously classified as *Gelidium madagascariense* and *Pterocladia rectangularis*. *Acanthopeltis* is merged with *Gelidium*, which has nomenclatural priority. Nuclear-encoded *CesA* was found to be useful for improving the resolution of phylogenetic relationships within the Gelidiales and is likely to be valuable for the inference of phylogenetic relationship among other red algal taxa.

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1. Introduction

The goal of this study was to clarify the phylogenetic relationships among species in the Gelidiales (Kylin, 1923), an order economically important for the agarophytes commonly found on temperate and tropical coastlines of both hemispheres (Womersley and Guiry, 1994; Freshwater et al., 1995; Boo et al., 2014a, 2015a). Gelidiales are distinguished by thick-walled refractive rhizines (=internal rhizoidal filaments) in the cortex and/or medulla, transversely divided apical cells, pit plugs with a single cap layer, a 'Gelidium-type' spore germination pattern, transversely divided spermatangia, intercalary carpogonia that after fertilization produce gonimoblasts that connect to nutritive cells, and a triphasic life history (Feldmann and Hamel, 1936; Fan, 1961; Santelices, 1977; Akatsuka, 1986a; Hommersand and Fredericq,

1988; Norris, 1992; Womersley and Guiry, 1994). The three families in the Gelidiales, the Gelidiaceae, Gelidiellaceae, and Pterocladaceae, comprise ten genera and about 188 species (Perrone et al., 2006; Tronchin and Freshwater, 2007; Boo et al., 2013, 2015a; Guiry and Guiry, 2015).

The family Gelidiaceae (Kützing, 1843) is characterized by internal thick-walled refractive rhizines, the endogenous production of rhizoidal filaments from inner cortical cells that form brush-like haptera, a triphasic isomorphic life history, and bilocular cystocarps with an ostiole on each side (Norris, 1992; Womersley and Guiry, 1994; Perrone et al., 2006). It includes five genera; *Acanthopeltis* Okamura, *Capreolia* Guiry & Womersley, *Gelidiophycus* G.H. Boo, J. K. Park & S.M. Boo, *Gelidium* J.V. Lamouroux, and *Ptilophora* Kützing (Lamouroux, 1813; Kützing, 1847b; Yatabe, 1892; Guiry and Womersley, 1993; Boo et al., 2013). *Gelidium*, the most speciose genus with about 120 species (Guiry and Guiry, 2015), varies in size from about 1 cm in *G. minimum* K.M. Kim, I.K. Hwang, H.S. Yoon & S.

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M. Boo to more than 1 m in *G. robustum* (N.L. Gardner) Hollenberg & I.A. Abbott (Hollenberg and Abbott, 1965; Kim et al., 2012).

The family Gelidiellaceae (Fan, 1961) encompasses two tropical to warm temperate genera, *Gelidiella* Feldmann & Hamel and *Parviphycus* B. Santelices. The family is distinguished by the exogenous development of unicellular rhizoids arising from outer cortical cells of prostrate axes and the absence of rhizines, haptera and female reproductive structures (Feldmann and Hamel, 1934; Fan, 1961; Santelices, 2004; Perrone and Delle Foglie, 2006; Perrone et al., 2006; Bottalico et al., 2014; Boo et al., 2015a).

The Pterocliadiaceae (Perrone et al., 2006) includes the genera *Pterocladia* J. Agardh and *Pterocliadiella* Santelices & Hommersand (Agardh, 1851; Santelices and Hommersand, 1997; Perrone et al., 2006). This family is distinguished by internal thick-walled refractive rhizines, endogenously produced rhizoidal filaments coalesced in a thick sheath that form peg-like haptera, unilocular cystocarps, and gonimoblast and carposporangia developing on one side of the central plane of the blade, or surrounding the central axial cell filament. The genus *Pterocladia* J. Agardh, based on the generitype *P. lucida* (Brown ex Turner) J. Agardh, (basionym, *Fucus lucidus* Brown ex Turner), is characterized by having rhizines concentrated in the medullary layer, one or more cystocarpic ostioles on one surface of frond (Agardh, 1851, 1852; Okamura, 1934; Fan, 1961), and unilocular cystocarps (Santelices, 1991).

Aphanta is a monospecific genus represented by *A. pachyrrhiza* E.M. Tronchin & Freshwater, a species described from South Africa and Mozambique (Tronchin and Freshwater, 2007). *Aphanta* is characterized by its relatively prominent, robust prostrate system, the production of rhizoidal filaments endogenously and exogenously at initial developmental stages, and the presence in field collected material of both brush-like and peg-like haptera (Tronchin and Freshwater, 2007). Female, male and tetrasporangial structures are unknown. Because *A. pachyrrhiza* exhibits equivocal character states, or lacks characters used to distinguish families in the Gelidiales, its familial classification based on morphology is unknown. Molecular data have also been equivocal. Analyses of *rbcl* and SSU data did not resolve *Aphanta* within any of three Gelidiales families, but analyses of LSU sequences provided low to moderate support for its placement within the Pterocliadiaceae (Tronchin and Freshwater, 2007), where it has been tentatively included (Guiry and Guiry, 2015).

Molecular markers have greatly enhanced our understanding of species boundaries and phylogenetic relationships in the Gelidiales. Plastid-encoded *rbcl* has been the most frequently used locus in taxonomic studies (e.g. Freshwater and Rueness, 1994; Freshwater et al., 1995; Shimada et al., 1999; Millar and Freshwater, 2005; Nelson et al., 2006; Boo et al., 2013, 2014a). The plastid-encoded *psaA* (encoding the photosystem I P700 apoprotein A1) and *psbA* (encoding the photosystem II thylakoid protein D1), both intimately tied to the photosystem reaction centers, provide better resolution at deep branches of algal phylogenies (Yoon et al., 2002). These latter two genes have been used to generate phylogenies in the tribe Griffithsiaceae (Wrangeliaceae, Ceramiales) and the genus *Gelidium* (Yang and Boo, 2004; Kim et al., 2011b). The mitochondrial-encoded *cox1* is a DNA barcoding marker for red algae including gelidioid species (Freshwater et al., 2010; Kim et al., 2011b; Boo et al., 2013, 2014a). Internal transcribed spacer (ITS) and small and large subunits of the nuclear ribosomal RNA cistron (SSU, LSU) have also been used for taxonomic studies of the Gelidiales (Bailey and Freshwater, 1997; Freshwater and Bailey, 1998; Patwary et al., 1998; Shimada et al., 1999; Tronchin and Freshwater, 2007); however, these nuclear genes have rarely been used since the study by Shimada et al. (1999).

The assessment of diversity and phylogenetic relationships in the Gelidiales was inferred in this study from five molecular markers: plastid-encoded *psaA*, *psbA*, and *rbcl*; mitochondria-encoded *cox1*; and nuclear-encoded cellulose synthase catalytic subunit A (*CesA*). *CesA* encodes the cellulose synthase proteins in plasma-membrane rosettes of plant and algal cell walls (Lerouxel et al., 2006; Roberts and Roberts, 2009; Popper et al., 2011). This is the first five-gene phylogeny of the Gelidiales including sequences from all three genomes (plastid, mitochondrial, nuclear). The present taxon sampling included 107 species (of which seven were unidentified species) or 57% of about 188 species reported in the Gelidiales and represents the ten currently recognized genera. This dataset was generated to (i) test the utility of *CesA* for phylogenetic studies of the Gelidiales, (ii) test the monophyly of the currently recognized families and genera, (iii) reexamine the familial position of *Aphanta* and related taxa, (iv) assess the generic position of *Acanthopeltis*, and (v) characterize a new family and genus found in the present study. This will improve the current understanding of generic relationships and provide a phylogenetic framework for further studies of the morphology and biogeography of Gelidiales species.

2. Materials and methods

2.1. Taxon sampling and morphological observations

A total of 118 specimens representing 107 species were included in this study (Table S1). Fresh specimens were collected in Australia, Chile, Indonesia, Italy, Japan, Korea, Madagascar, New Zealand, the Philippines, Thailand, and USA. Herbarium specimens from the Herbarium of Cryptogamic Botany (PC) in Paris, France and the University Herbarium (UC) in Berkeley, USA (Thiers, continually updated) were also included. Tissues were sectioned using a freezing microtome (FX-801, Yamato Kohki Industrial Co., Ltd, Japan) and stained with 1% aqueous aniline blue for anatomical observations. Photographs were taken with a DP-71 camera (Olympus, Tokyo, Japan) attached to a BX-51 microscope (Olympus, Tokyo, Japan). Voucher specimens are deposited in the Department of Biology, Chungnam National University, Daejeon, Korea.

2.2. Molecular methods

Genomic DNA was extracted from ~5 mg of dried tissue ground in liquid nitrogen using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA extractions used in previous studies (Freshwater et al., 1995; Millar and Freshwater, 2005; Boo et al., 2013) were also used to amplify genes not previously sequenced from those specimens. Five gene regions were amplified: *CesA*, *rbcl*, *psaA*, *psbA*, and *cox1* (see Table 1 for primers). PCR reactions were carried out in a volume of 10 µl, containing 5 µl of 2X Quick Taq HS Dye-Mix (Toyobo, Osaka, Japan), 0.2 µl primer (each), 1 µl of genomic DNA, and sterilized deionized water. The cycle parameters were set as follows: a preliminary denaturation step 94 °C for 2 min, followed by 35–40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 68 °C. PCR products were purified by enzymatic treatment with Exonuclease (Exo) and Antarctic Phosphatase (AP) (Exo-AP PCR Clean-Up Mix, Doctor Protein, Korea). Sequencing of the forward and reverse strands of purified PCR products was performed by Genotech (Daejeon, Korea). The sequences were edited using Chromas v.1.45 (McCarthy, 1998) and rechecked manually for consistency.

Table 1
Primers used in the present study.

Gene	Primer name	Sequence (5'-3')	Annealing temp.	References
CesA	CesA3F	CTNGTYGARCAYTACCANRTC	57.9	Present study
	CesA915F	GAAGATRTYCTTCGTCCWGAYTG	59.8	Present study
	CesA3R	TTCATRATRGCRCCCTTRGC	57.3	Present study
	CesA1575R	CCAACGYGTCTTTGRAGCATRGC	63.6	Present study
rbcL	F7	AACTCTGTAGAACGNACAAG	54.2	Gavio and Fredericq (2002)
	F645	ATGCGTTGGAAAGAAAGATTCT	54.7	Lin et al. (2001)
	R753	GCTCTTTCATACATATCTTCC	54.0	Freshwater and Rueness (1994)
	RrbcS start	GTTCTTTGTGTTAATCTCAC	51.1	Freshwater and Rueness (1994)
psaA	psaA130F	AACWACWACTTGGATTGGAA	52.0	Yoon et al. (2002)
	psaA971F	ACTACWTCATGGCAYGCWCAACT	59.8	Yang and Boo (2004)
	psaA1110R	CCWATCCACATRTGATGTGT	54.2	Yang and Boo (2004)
	psaA1760R	CCTCTWCCWGGWCCATCRAWGG	65.1	Yoon et al. (2002)
psbA	psbA-F	ATGACTGCTACTTTAGAAAGACG	57.1	Yoon et al. (2002)
	psbA-R2	TCATGCATWACTTCCATACCTA	54.0	Yoon et al. (2002)
cox1	COXI43F	TCAACAAATCATAAAGATATTGGWACT	55.9	Geraldino et al. (2006)
	COXI1549R	AGGCATTCTTCAAANGTATGATA	55.0	Geraldino et al. (2006)

2.3. Phylogenetic analyses

Sequences were aligned with Se-*Al* v.2.0a11 (Rambaut, 2002). Phylogenetic trees of individual and concatenated datasets were reconstructed using Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian inference (BI). We determined the best-fitting combination of partitions among genes and substitution models for partitions using PartitionFinder v1.1.0 (Lanfear et al., 2012). The PartitionFinder analysis found that five partitions, as each gene, and GTR + Γ + I model based on Akaike Information Criterion (AIC) and the corrected Akaike Information Criterion (AICc). The ML analyses were performed using the Pthreads version of RAxML v8.0.X (Stamatakis, 2014) set as follows: a rapid bootstrap analysis and search for the best-scoring ML tree in one single program run with 1000 bootstrap replicates under the GTR + Γ + I substitution model.

The BI was performed for individual and concatenated datasets (CesA + cox1 + psaA + psbA + rbcL) with MrBayes v.3.2.1 (Ronquist et al., 2012) using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with the GTR + Γ + I model. For each matrix, two million generations of two independent runs were performed with four chains and sampling trees every 100 generations. The burn-in period was identified graphically by tracking the likelihoods at each generation to determine when they reached a plateau. Twenty-five percent of saved trees were removed, and the remaining trees used to calculate the Bayesian posterior probabilities.

The MP analyses were constructed with PAUP* 4.0b.10 (Swofford, 2003), using a heuristic search algorithm with the following settings: 1000 random sequence additions, tree bisection-reconnection (TBR) branch swapping, MulTrees, and unordered and unweighted characters and branches with a maximum length of zero collapsed to yield polytomies. Bootstrap values for the resulting nodes were assessed using 1000 bootstrapping replicates with 10 random sequences additions, TBR and MulTrees.

2.4. Phylogenetic informativeness

Phylogenetic informativeness (PI) profiles were used to determine a quantitative measure of signal and utility that individual markers contribute to phylogenetic inference (Townsend, 2007). We calculated PI profiles for each marker using PhyDesign (López-Giráldez and Townsend, 2011). Both net and per-site informativeness were computed and contrasted to assess cost effectiveness of five markers. The ultrametric tree was generated from the

concatenated data matrix of divergence time estimates analysis using MrBayes v.3.2.1 (Ronquist et al., 2012). HyPhy v2.1.1 (Pond et al., 2005) was used to calculate phylogenetic informativeness of nucleotide-based data using empirical base frequencies and the time-reversible model of substitution. Phylogenetic informativeness profiles for each individual marker were compared to the reference ultrametric tree.

3. Results

3.1. Concatenated five-gene phylogeny

Five gene sequences, *CesA*, *rbcL*, *psaA*, *psbA* and *cox1*, from 86 taxa including seven outgroups, were concatenated and analyzed to improve the resolution of phylogenetic relationships in the Gelidiales. The concatenated tree was highly concordant, with well supported relationships resolved in the individual gene trees (Fig. 1; Supplemental Figs. S1–S5), and provided better resolution of phylogenetic relationships at the family and genus levels (Figs. 1 and 2). Four major lineages were resolved in the Gelidiales. A monophyletic Gelidiaceae including the genera *Acanthopeltis*, *Capreolia*, *Gelidiophycus*, *Gelidium*, and *Ptilophora* was strongly supported (ML 100%, MP 94%, PP 1.0). *Gelidium* species were resolved in three separate clades, only two of which were included in the Gelidiaceae lineages. *Acanthopeltis* was nested within the large clade of *Gelidium* species that includes the genotype, *Gelidium corneum*. *Capreolia implexa* formed a clade with *Gelidium hommersandii* that was sister to *Gelidiophycus* and part of a fully supported clade that also included two species from Chile that represent an undescribed genus. The five species of *Ptilophora* formed a fully supported clade that was also resolved within the Gelidiaceae.

The Gelidiellaceae including genera *Gelidiella* and *Parviphycus* was fully supported, and the Pterocladaceae, including *Pterocladia* and *Pterocladia lucida*, was monophyletic with varying levels of support (ML 94%, MP 63%, PP 1.0). The 11 species of *Pterocladia* and *Pterocladia lucida* were resolved as separate, fully supported clades. The final major lineage of the Gelidiales was a fully supported clade that included the genus *Aphanta*, *Gelidium madagascariense* and *Pterocladia rectangularis*. *Aphanta*, including the genotype *A. pachyrrhiza* and an undescribed species from Thailand, was fully supported as monophyletic and sister to a fully supported monophyletic clade of *Gelidium madagascariense* and *Pterocladia rectangularis*, but this clade was independent of the Gelidiaceae, Gelidiellaceae and Pterocladaceae.

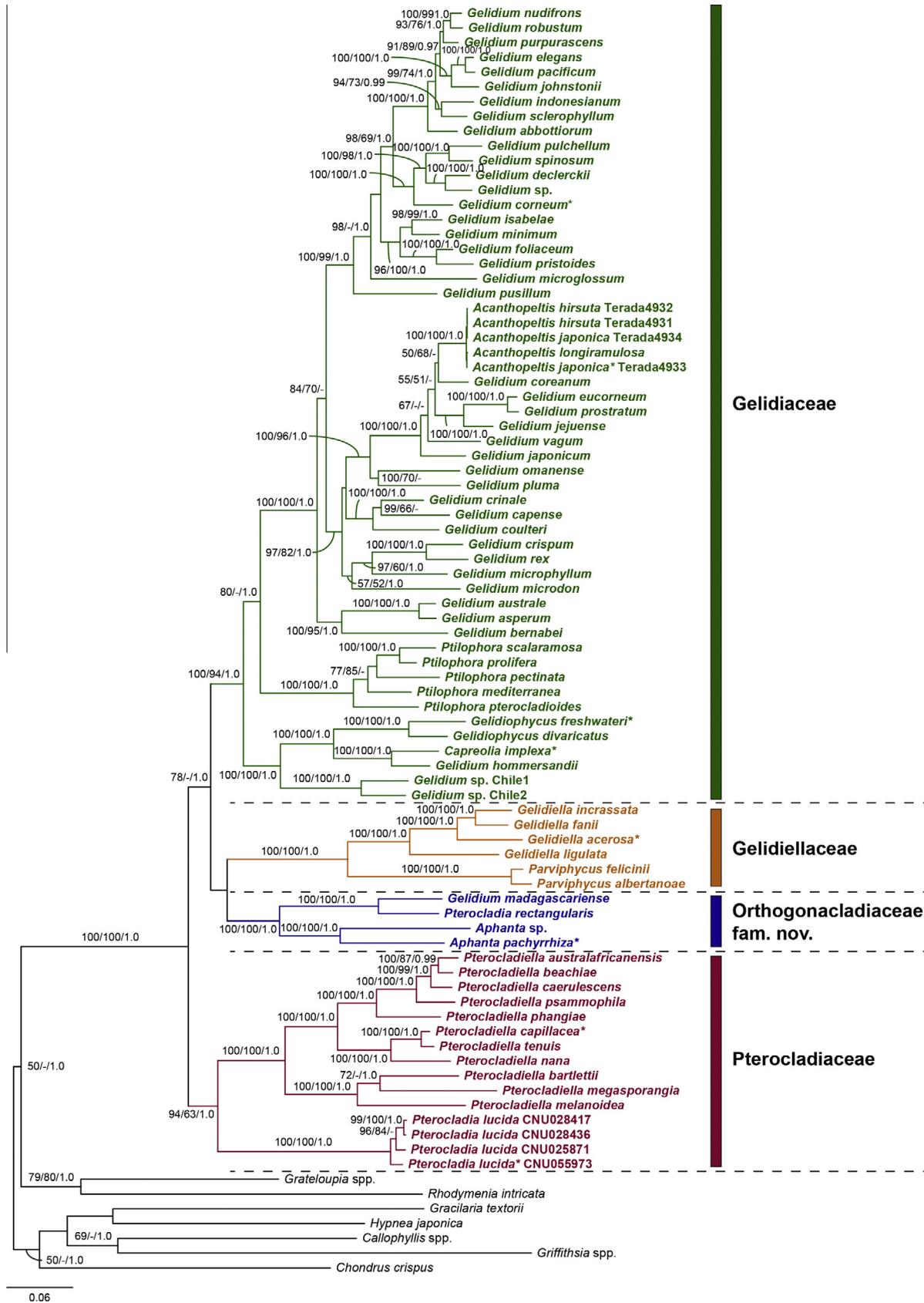


Fig. 1. Phylogenetic tree of the Gelidiales obtained by maximum-likelihood inference of the concatenated *CesA* + *psaA* + *psbA* + *rbcL* + *cox1* dataset (5363 bp). ML and MP bootstrap values ($\geq 50\%$) and Bayesian posterior probabilities (≥ 0.90) are indicated above and below branches, respectively. Asterisk (*) indicates the type species.

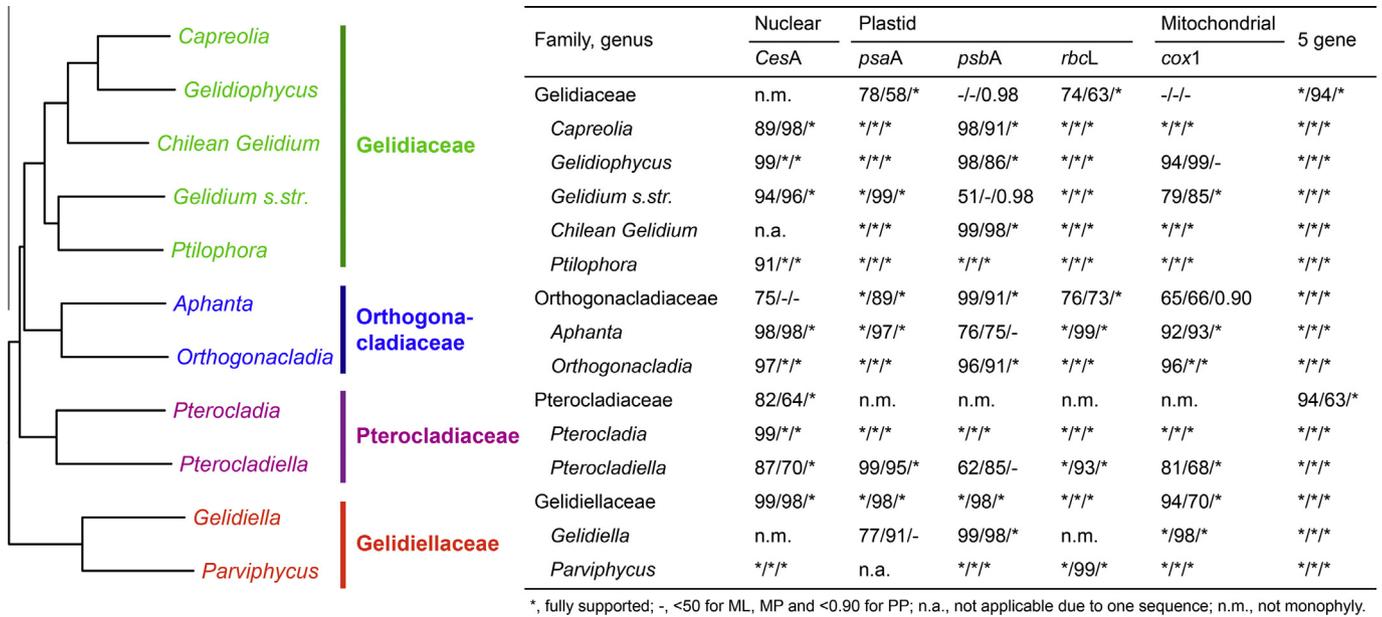


Fig. 2. Statistical support on familial and generic nodes in individual and concatenated datasets in the Gelidiales. The order for support values is ML/MP/PP.

3.2. Phylogenetic informativeness profiles

Phylogenetic informativeness profiles revealed that the *cox1*, *psaA*, and *rbcL* had the highest net informativeness (Fig. 3A; Table 2). When informativeness per site is considered, *CesA* shifted to second in rank behind *cox1*, similar to *psaA* (Fig. 3B; Table 2). The *psbA* region had the lowest net and per site informativeness among all markers. In net PI profiles, *CesA* and *psbA* had relatively gentle slopes, suggesting low but consistent phylogenetic signal (Fig. 3A). The steep sloping PI profiles of *cox1* indicated rapid accumulation of noise past the profile peak (indicated by colored dashed line) for Gelidiales (Fig. 3A and B). The *cox1* marker showed the highest level of informativeness for the most recent divergences in Gelidiales. However, the phylogenetic informativeness of both *psaA* and *rbcL* marker were relatively high at the branching point of the families in Gelidiales (deeper nodes indicated by black dashed lines).

3.3. Morphology of *Gelidium madagascariense*

Plants are dark red, cartilaginous, forming erect tufts up to 40 cm in length, arising from extensively branched prostrate system (Fig. 4A). Main axes are proximally terete and become compressed, and pinnately to bi-pinnately branched, with pinnae and pinnules arising in close, regular series almost at right angles to the parent branch (Fig. 4B). Apical cells are polygonal (Fig. 4C). Surface cortical cells are small and irregularly arranged (Fig. 4D). Cortical cells are irregular in shape and medullary cells are elongated and arranged in longitudinal files (Fig. 4E). Rhizines are congested in the inner cortical layers and absent or very rare in the medulla (Fig. 4F). The prostrate system consists of robust and irregularly branched terete stolons (Fig. 4G) with complex peg-like haptera composed of endogenous, thick-walled refractive rhizoidal filaments (Fig. 4H) protruding between the surface cells, and pigmented, multicellular uniseriate filaments originating from surface cortical cells (Fig. 4I). The rhizoidal filaments are pit-connected with their mother cells.

Branches bearing tetrasporangial sori are terete to compressed, becoming swollen centrally (Fig. 4J). Tetrasporangia originate from cortical cells, are arranged in a shallow v-shaped pattern, develop

acropetally, and are decussately and cruciately divided (Fig. 4K and L). Cystocarps and spermatangia were not observed in our collection or when Andriamampandry (1988) originally described the species.

3.4. Morphology of *Pterocladia rectangularis*

Plants are bright red, cartilaginous, forming erect tufts up to 25 cm in length, arising from extensively branched prostrate system (Fig. 5A). Main axes are proximally terete and become flattened, and pinnately to bi-pinnately branched, with pinnae and pinnules arising in close, regular series almost at right angles to the parent branch (Fig. 5B). Apical cells are polygonal (Fig. 5C). Surface cortical cells are small and irregularly arranged (Fig. 5D). Cortical cells are irregular in shape and medullary cells are thick-walled, elongated and round to irregular in cross section (Fig. 5E and F). Rhizines are congested in the inner cortical layers (Fig. 5E and F). The prostrate system consists of robust, irregularly branched terete stolons (Fig. 5G) attached by complex peg-like haptera (Fig. 5H) composed of endogenous, thick-walled refractive rhizoidal filaments protruding between the surface cells, and pigmented, multicellular uniseriate filaments originating from the surface cells (Fig. 5I and J). The rhizoidal filaments are pit-connected with their mother cells (Fig. 5J).

Branches bearing tetrasporangial sori are short-stalked and elliptical in shape (Fig. 6A). Tetrasporangia originate from cortical cells, are arranged irregularly, develop acropetally, and are decussately and cruciately divided (Fig. 6B and C). Spermatangial sori occur on small branchlets (Fig. 6D). Spermatangia are cut off from anticlinally elongated surface cortical cells by transverse divisions (Fig. 6E). Cystocarps were not observed in our collection but have been described by Womersley and Guiry (1994).

4. Discussion

The present study, including 107 species (among ca. 188 species in the order) and based on an analysis of five protein-coding genes including *CesA* from the nuclear genome, is the first taxon-rich molecular phylogeny of the Gelidiales. It includes the type species of all currently recognized genera except *Ptilophora spissa* (Suhr)

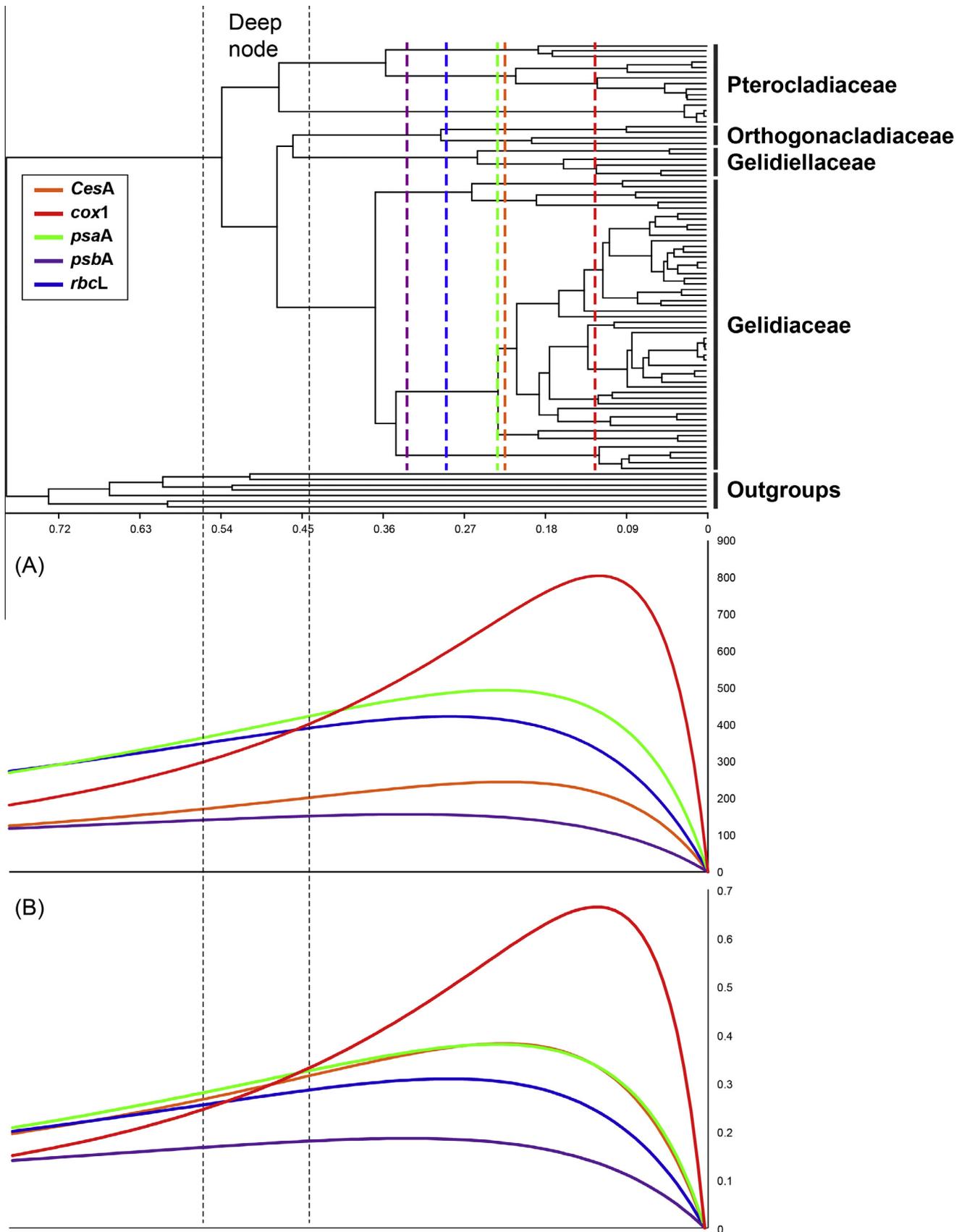


Fig. 3. Phylogenetic informativeness profiles of five markers with reference to the ultrametric tree: (A) Net informativeness profiles for *CesaA* (orange), *cox1* (red), *psaA* (green), *psbA* (purple), and *rbcL* (blue) gene partitions; (B) Per-site informativeness profiles for the same genes. The x-axis represents topologies using their nodes as epoch units, the y-axis represents the Phylogenetic Informativeness value for each molecular marker along the topologies. Colored dashed lines correspond to the region in the phylogeny where individual markers reach their peak informativeness level.

Table 2
Phylogenetic information, and net and per-site informativeness of five individual markers and combined dataset.

	<i>CesA</i>	<i>rbcl</i>	<i>psaA</i>	<i>psbA</i>	<i>cox1</i>	5 genes
No. of ingroup	64	118	59	61	79	79
Length	640	1371	1300	843	1212	5363
Constant sites	338	749	723	564	688	3081
Variable sites (%)	302 (47.2)	622 (45.4)	577 (44.4)	279 (33.1)	524 (43.2)	2282 (42.6)
Pi characters (%)	263 (41.1)	560 (40.8)	532 (40.9)	244 (28.9)	481 (39.7)	2064 (38.5)
Net informativeness (SD)	185.36 (47.42)	340.95 (80.53)	386.40 (91.43)	129.94 (31.01)	465.82 (208.36)	NA
Max net informativeness	244.07	421.70	493.50	155.96	804.17	NA
Per-site informativeness (SD)	0.290 (0.074)	0.249 (0.059)	0.297 (0.070)	0.154 (0.037)	0.384 (0.172)	NA
Max per-site informativeness	0.381	0.308	0.380	0.185	0.664	NA

Pi, Parsimoniously informative; NA, Not Available.

Kützing and *Parviphycus adnatus* (E.Y. Dawson) B. Santelices. A total of 414 DNA sequences, 214 of which were generated in the present study, were used for inferring the phylogeny of the Gelidiales. Our study supports phylogenies published in previous studies (Freshwater et al., 1995; Millar and Freshwater, 2005; Nelson et al., 2006; Tronchin and Freshwater, 2007; Boo et al., 2013, 2014a, 2015a), and greatly improves resolution at the generic and family level, revealing candidates for new genera and four major lineages that correspond to three previously recognized families as well as one newly described family.

4.1. Utility of *CesA* marker

Analyses of cellulose synthesis protein sequences have been used to explore Kingdom- and Division-level phylogenies because of their occurrence in algae, fungi and plants (Roberts and Roberts, 2009; Michel et al., 2010; Collén et al., 2013). Roberts and Roberts (2009) found that *CesA* in *Porphyra yezeensis* grouped with that of the oomycete *Phytophthora infestans*, suggesting that stramenopiles acquired the gene via lateral gene transfer from a red algal endosymbiont. We tested the utility of *CesA* nucleotide sequences for elucidating phylogenetic relationships in the Gelidiales. The 640 bp *CesA* fragment, though shorter than the *rbcl* (1371), *psaA* (1300), *psbA* (843) and *cox1* (1212) markers, has as large a percentage of variable and parsimony informative sites as these plastid and mitochondrial markers (Table 2).

CesA provides a highly informative signal in the concatenated dataset analyses, although *CesA* alone is not capable of fully resolving the relationships among the Gelidiales (Fig. 3A and B). *CesA* displayed high per-site PI profiles (Fig. 3B) indicating a high level of phylogenetic informativeness for the Gelidiales. The low signal in net PI profiles may be a limitation of *CesA*; however, the gentle slope profile of this nuclear marker indicates that it is less subject to homoplasy than plastid and mitochondrial markers, as reported in previous studies (Townsend, 2007; Townsend and Leuenberger, 2011). The short length, ease of amplification and sequencing of *CesA* make this marker attractive for phylogenetic studies. Independently, nuclear *CesA* has the same degree of per-site informativeness as *psaA* and *rbcl* (Fig. 3B), and is most powerful when combined with plastid and mitochondrial markers in a phylogenetic analysis. This study is the first to use nuclear *CesA* sequence data to infer phylogenetic relationships among marine algae.

4.2. The Gelidiaceae

The monophyly of the Gelidiaceae, including the *Capreolia/Gelidium caulacanthum/G. hommersandii* clade, *Gelidiophycus*, *Gelidium*, *Ptilophora*, and a clade of *Gelidium*-like Chilean species, is strongly supported in the five-gene dataset analyses. The five-gene tree resolves phylogenetic relationships that are largely congruent with the previous classification schemes, highlighting the

significance of the morphological features used to establish these classifications (Gardner, 1927; Feldmann and Hamel, 1934, 1936; Okamura, 1934; Loomis, 1960; Fan, 1961; Santelices, 1977, 2004; Akatsuka, 1986a; Norris, 1992; Womersley and Guiry, 1994; Santelices and Hommersand, 1997; Perrone et al., 2006; Boo et al., 2013). The genus *Gelidium* as currently constituted is not monophyletic; it is composed of three lineages; (i) the main *Gelidium* clade, including the genericity *Gelidium corneum*, (ii) the *Capreolia/Gelidium caulacanthum/G. hommersandii* clade, and (iii) the clade of Chilean species provisionally identified as *Gelidium* spp. which in fact belong to a novel genus. On the basis of this result, we refer to the species included in the main *Gelidium* clade as *Gelidium sensu stricto*. The inclusion of *Acanthopeltis* species in *Gelidium sensu stricto* is well supported by five markers from the three different genomes, confirming previous results using SSU and *rbcl* (Shimada et al., 1999). This suggests that sympodially arising leaf-like branchlets on subcylindrical fronds, a character that was used to distinguish the genus *Acanthopeltis*, may be plesiomorphic. We therefore conclude that species in the genus *Acanthopeltis* (*A. hirsuta*, *A. japonica*, and *A. longiramulosa*) should be transferred to the genus *Gelidium*, according to the rule of priority (article 11.3) of the International Code of Nomenclature for algae, fungi, and plants (McNeill et al., 2012).

Gelidium caulacanthum and *G. hommersandii* group consistently with *Capreolia*, as shown in previous studies (Freshwater et al., 1995; Bailey and Freshwater, 1997; Freshwater and Bailey, 1998; Millar and Freshwater, 2005; Nelson et al., 2006; Boo et al., 2013). All three species occur in Australasia (Guiry and Womersley, 1993; Millar and Freshwater, 2005; Nelson et al., 2006), although *Capreolia implexa* has been reported as a recent introduction to Chile from New Zealand (Boo et al., 2014b). *Capreolia* exhibits a biphasic life history in which the tetrasporophyte develops directly from the fertilized carposogonium, without a carposporophyte stage (Guiry and Womersley, 1993). This life history was the key character used to define the genus; however, cystocarps have been found in field-collected specimens of *Gelidium caulacanthum* and *G. hommersandii*, suggesting that these species have a triphasic life history (Chapman, 1969; Millar and Freshwater, 2005). The concept of *Capreolia* will need to be emended to include species with either biphasic or triphasic life histories if all species in this clade are to be transferred to this genus. We refrain from proposing a revision of the concept of *Capreolia* until further studies circumscribe the genus and distinguish its characters.

At least two more species of *Gelidium* from Chile deserve the generic rank, because their *rbcl* sequences resolved a distinct lineage and did not match those of previously assigned genera (Boo et al., 2013; Iha et al., 2015). These unidentified taxa from Chile are morphologically similar to *Gelidium chilense* (Montagne) Santelices & Montalva (basionym, *Acropeltis chilensis* Montagne, 1837) and *Gelidium lingulatum* Kützing. It is necessary to sequence the type specimens of these two species. If either is confirmed to be

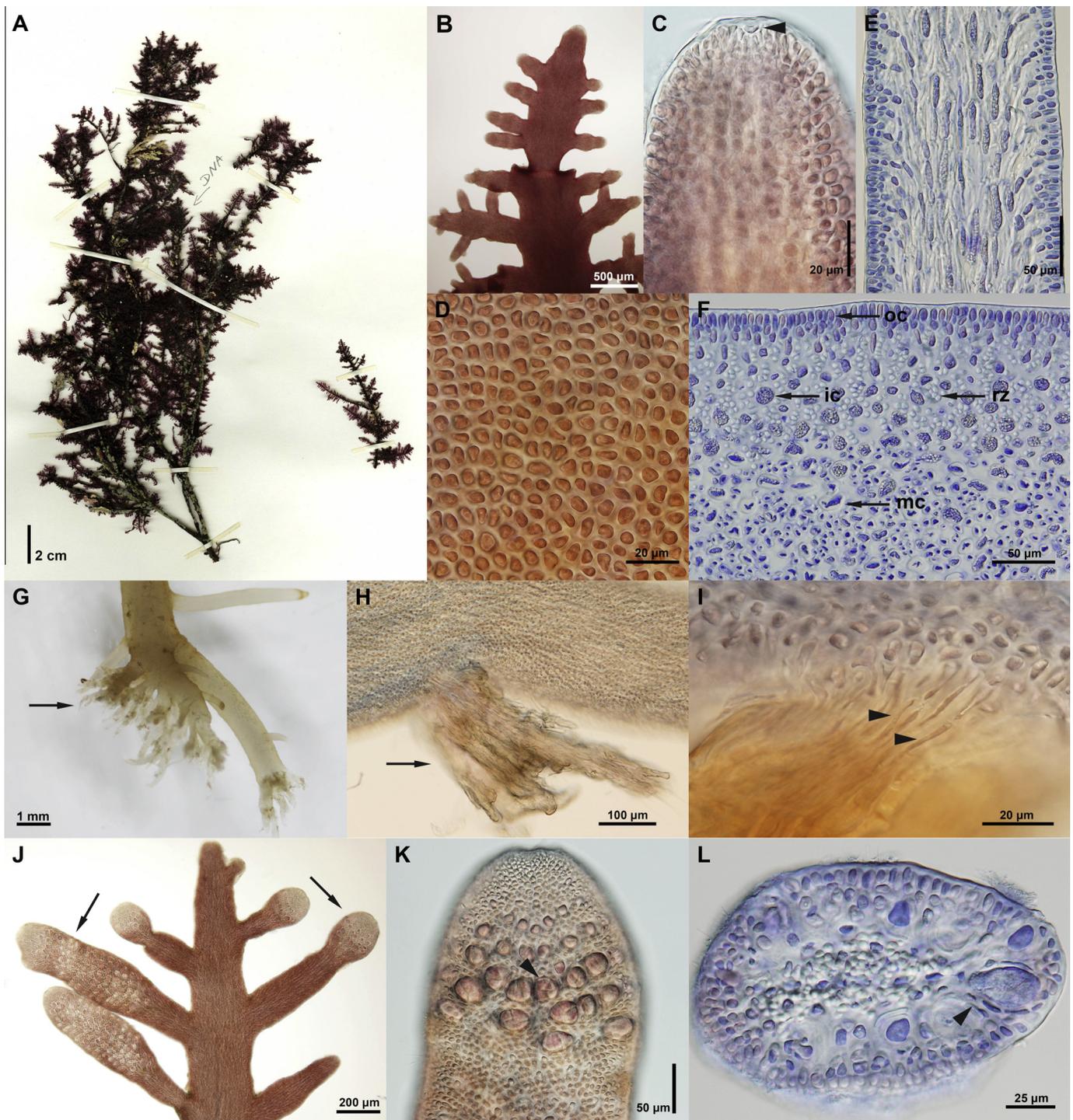


Fig. 4. *Orthogonacladia madagascariense* (A.V. Andriamampandry) G.H. Boo & L. Le Gall comb. nov.: (A) Image of specimens collected in Madagascar (PC0166457); (B) Pinnae arising at nearly perpendicular angles; (C) Apex of an upright branch showing polygonal apical cell (arrowhead); (D) Surface view of outer cortical cells; (E) Longitudinal section of branch axis; (F) Transverse section of branch axis, showing outermost cortical cells (oc), inner cortical cells (ic), rhizines (rz), and medullary cells (mc); (G) A robust, extensively branched prostrate system (arrow); (H) A peg-like hapteron (arrow); (I) Initial cortication of the basal part of the hapteron in surface view showing exogenous multicellular corticating filaments (arrowheads); (J) Thallus with tetrasporangial sori (arrows); (K) Apex of tetrasporangial sorus showing shallow v-shaped arrangement of tetrasporangia (arrowhead); (L) Transverse section of a sorus showing developing tetrasporangia (arrowhead) and centrally clustered rhizines.

the same as *G. chilense*, the genus *Acropeltis* should be reinstated following the ICN (McNeill et al., 2012). If not, a new genus can be described to accommodate both Chilean taxa.

Gelidiophycus was consistently monophyletic in the present multigene analyses, supporting its generic distinctiveness by Boo et al. (2013). *Gelidiophycus* was closely related to the *Capreolia*/

Gelidium caulacanthum/*G. hommersandii* clade. These two clades are geographically separated: *Gelidiophycus* occurs in eastern Asia, and the *Capreolia* clade occurs in Australasia (Boo et al., 2013, 2014b).

Ptilophora is consistently monophyletic in analyses of all five genes. This result supports previous studies that merged *Beckerella*

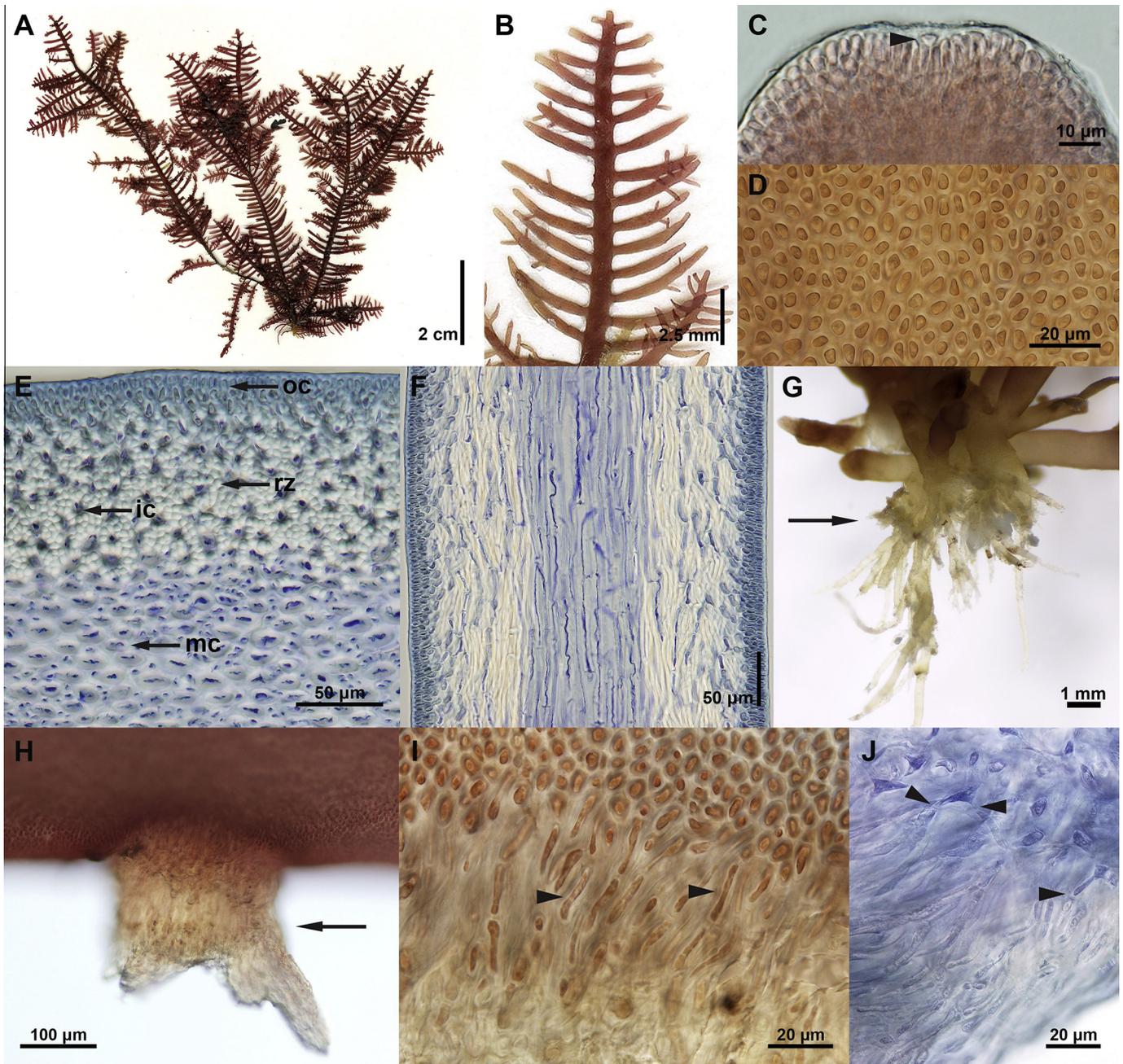


Fig. 5. *Orthogonacladia rectangularis* (Lucas) G.H. Boo & T. Wernberg comb. nov.: (A) Image of a specimen collected in western Australia (CNU041209). (B) Pinnae arising at nearly perpendicular angles. (C) Apex of an upright branch showing polygonal apical cell (arrowhead). (D) Surface view of outer cortical cells. (E) Transverse section of branch axis, showing outermost cortical cells (oc), inner cortical cells (ic), rhizines (rz), and medullary cells (mc). (F) Longitudinal section of branch axis; (G) A robust, extensively branched prostrate system (arrow). (H) A peg-like hapteron (arrow). (I) Initial cortication of the basal part of the hapteron in surface view showing exogenous multicellular corticating filaments (arrowheads). (J) Longitudinal section of hapteron, showing the origin of endogenous rhizoidal filaments from the inner cortical cells and pit-connections between rhizoids and the mother cells (arrowheads).

with the earlier-described *Ptilophora* (Norris, 1987; Tronchin et al., 2003, 2004).

The taxonomic proposal to merge *Acanthopeltis* with *Gelidium* is presented below according to the International Code of Nomenclature for algae, fungi and plants (McNeill et al., 2012).

4.2.1. Taxonomic conclusions

Gelidium J.V. Lamouroux, 1813, 128, nom. cons. Type: *Gelidium corneum* (Hudson) Lamouroux, 1813, 129, type cons.

Basionym: *Fucus corneus* Hudson, Fl. Angl.: 474, 1762.

Synonyms: *Acropeltis* Montagne, 1837, 355. Type: *Acropeltis chilensis* Montagne. Synonymized by Santelices and Montalva (1983).

Acanthopeltis Okamura in Yatabe, 1892, 157. Type: *Acanthopeltis japonica* Okamura in Yatabe. Synonymized in the present study according to article 11.3 (McNeill et al., 2012), which stipulates that the correct name is the earliest legitimate one with the same rank.

Onikusa Akatsuka, 1986b, 63. Type: *Onikusa pristoides* (Turner) Akatsuka. Synonymized by Tronchin et al. (2002).

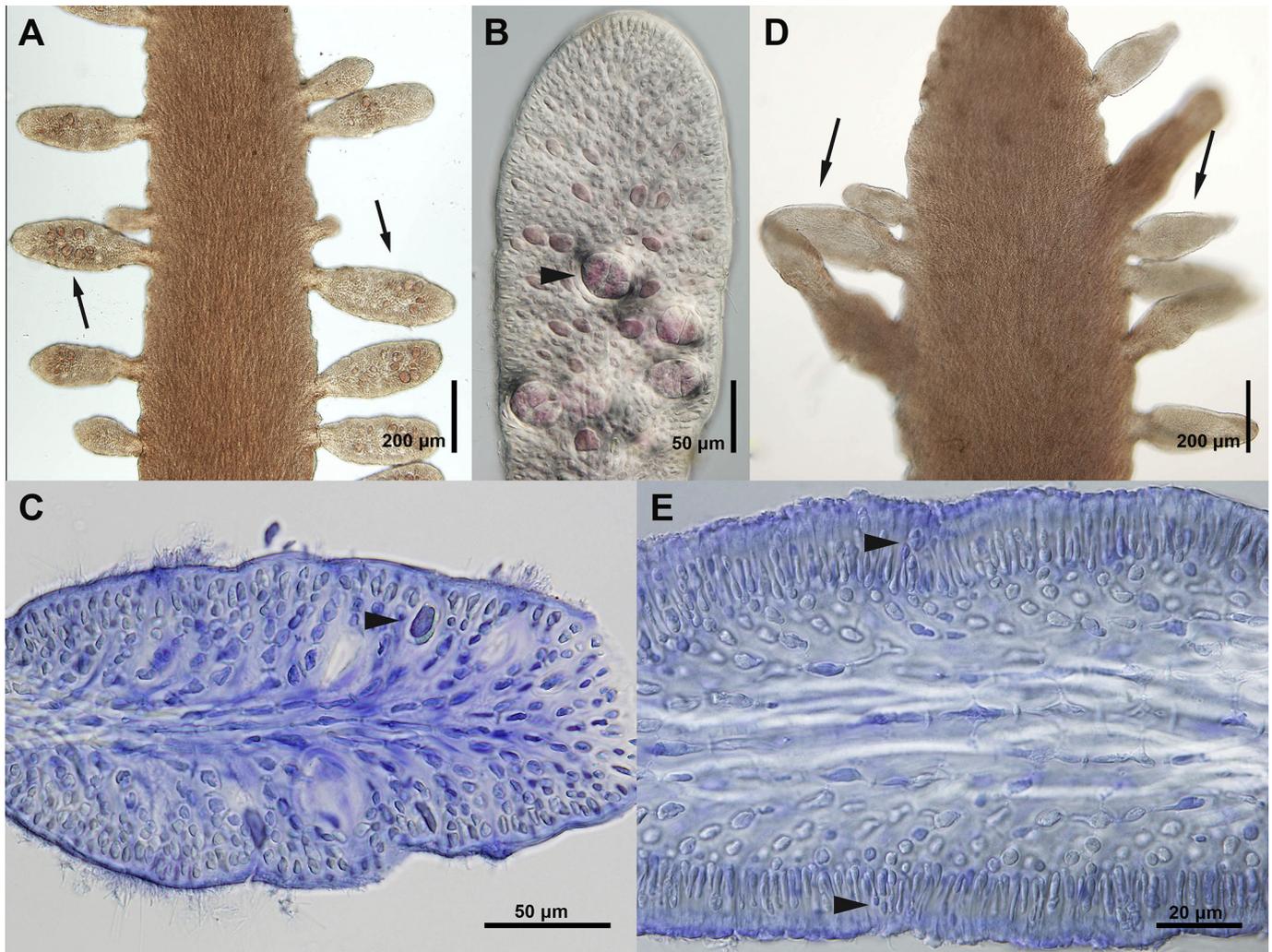


Fig. 6. *Orthogonacladia rectangularis* (Lucas) G.H. Boo & T. Wernberg comb. nov.: (A) Thallus with short-stalked, elliptical tetrasporangial sori (arrows). (B) Apex of tetrasporangial sorus showing cruciately divided tetrasporangia (arrowhead). (C) Longitudinal section of tetrasporangial sorus with developing tetrasporangia (arrowhead). (D) Pinnules with spermatangial sori (arrows). (E) Longitudinal section of spermatangial sorus with spermatangia cut off by transverse divisions from anticlinally elongated surface cortical cells (arrowheads).

Porphyroglossum Kützinger, 1847a, 775. Type: *Porphyroglossum zollingeri* Kützinger. Synonymized by Kim et al. (2011a).

Suhria J. Agardh ex Endlicher, 1843, 41. Type: *Suhria vittata* (Linnaeus) Endlicher. Synonymized by Tronchin et al. (2002).

Yatabella Okamura, 1900, p. 1. Type: *Yatabella hirsuta* Okamura. Synonymized in the present study according to article 11.3 (McNeill et al., 2012).

Gelidium yoshidae G.H. Boo & R. Terada nom. nov.

Basionym: *Acanthopeltis japonica* Okamura in Yatabe. Iconographia Florae Japonicae. Vol. I, No 2, pp. 157–158. Maruzen, Tokyo, 1892.

Remark: As a consequence of the merging of *Gelidium* and *Acanthopeltis* proposed in the present study, *Acanthopeltis japonica* should be transferred to the genus *Gelidium* (article 11.4); however, this would result in a later homonym of *Gelidium japonicum* (Harvey) Okamura (Okamura, 1934) (article 53. 1).

Etymology: A new name, *Gelidium yoshidae*, honors Prof. T. Yoshida from Hokkaido University, who has greatly contributed to the taxonomy of marine algae in Japan.

Gelidium hirsutum (Okamura) G.H. Boo & R. Terada comb. nov.

Basionym: *Yatabella hirsuta* Okamura, Illustrations of the Marine Algae of Japan 1: 1, pl. 1, 1900.

Synonym: *Acanthopeltis hirsuta* (Okamura) S. Shimada, T. Horiguchi & Masuda 1999.

Gelidium longiramulosum (Lee & Kim) G.H. Boo comb. nov.

Basionym: *Acanthopeltis longiramulosa* Lee & Kim, Phycol. Res. 51, 259–265, 2003.

4.3. The Gelidiellaceae

The family Gelidiellaceae was well resolved in the present analyses of single and concatenated datasets, and included *Gelidiella* and *Paviphycus*. *Gelidiella* is paraphyletic with respect to *Parviphycus* in the *CesA* and *rbcl* trees. Despite inclusion of more species in *rbcl* analyses (Fig. S2), the lack of monophyly in *Gelidiella* has resulted from the enigmatic position of *G. ramellosa*, which is weakly to strongly supported as basal to the *Parviphycus* clade, as seen in previous studies (Huisman et al., 2009; Bottalico et al., 2014; Boo et al., 2015a, 2015b; Iha et al., 2015). Further molecular and morphological studies are necessary to confirm the position of *G. ramellosa*. We note, as did Bottalico et al. (2015), that the Tun-

sian specimens identified as *G. ramellosa*, with a transversely regular arrangement of tetrasporangia (Feldmann and Hamel, 1934), was a misidentification of *Parviphycus albertanoae*.

The species of *Parviphycus* included in the present analyses were always resolved as a monophyletic clade, supporting their segregation from *Gelidiella* (Santelices, 2004; Bottalico et al., 2014). Because *Parviphycus* species are small and inconspicuous, more species may be discovered with intensive collections in warm temperate to tropical waters.

Although the absence of female structures and carposporophytes is considered characteristic of the Gelidiellaceae (Fan, 1961; Santelices, 2004; Lin and Freshwater, 2008), the presence of cystocarps in *Gelidiella acerosa* from Australia was reported without description or illustration by Huisman (2000), suggesting that *Gelidiella* may exhibit a triphasic life history like other members of the Gelidiales.

4.4. The Pterocladaceae

Our analyses of individual and combined datasets uphold the monophyly of *Pterocladia* and confirm that it is distinct from *Pterocladia*, supporting the morphology-based conclusion of Santelices and Hommersand (1997). *Pterocladia lucida* is a large (up to 40 cm) subtidal species found on exposed coasts in Australia and New Zealand. In their *rbcl* and *cox1* analyses, Boo et al. (2015c) identified four genetic groups in the *P. lucida* complex, interpreting them as four different species. Failure to recognize these cryptic species could lead to an underestimation of red algal diversity, especially in New Zealand. The *rbcl* and *cox1* datasets suggest natural dispersal events during the Pliocene between Australia and New Zealand. A taxonomic and nomenclatural revision of the *P. lucida* complex is clearly needed, but is beyond the scope of this paper and will appear elsewhere.

The seven to 13 species of *Pterocladia* included in the present analyses were consistently resolved as a generally well-supported monophyletic clade, supporting previous molecular and morphological distinctions of the genus (Freshwater et al., 1995; Santelices and Hommersand, 1997; Santelices, 1997, 1998; Shimada et al., 2000; Thomas and Freshwater, 2001; Tronchin and Freshwater, 2007; Sohrabipour et al., 2013). Although the present *rbcl*, *psaA*, *psbA* and *cox1* trees revealed that *Pterocladia* and *Pterocladia* are likely not monophyletic, these two genera cluster together in the *CesA* tree as well as in the five-gene datasets.

4.5. The new clade of *Aphanta*, *Gelidium madagascariense*, and *Pterocladia rectangularis*

The monophyly of the *Gelidium madagascariense*, *Pterocladia rectangularis* and the genus *Aphanta* is an unexpected, novel result that has not been revealed by previous morphological and molecular studies. *Gelidium madagascariense* and *Pterocladia rectangularis* are similar in vegetative structure, including pinnae and pinnules that arise almost at right angles to the parent branches in a regular series, and extensively branched prostrate systems with peg-like haptera composed of endogenously derived rhizoidal filaments. *Gelidium madagascariense* is endemic to Madagascar (Andriamampandry, 1988) while *Pterocladia rectangularis* is distributed from south of Perth, Western Australia to the Isles of St. Francis, South Australia (Lucas, 1931; Womersley and Guiry, 1994; Huisman, 2000).

The new genus *Orthogonacladia* is proposed on the basis of both individual and five-gene phylogenies (Figs. 1 and 2, S1–S5). *Orthogonacladia* is morphologically distinguished by a combination of large thalli, an extensively branched prostrate system with peg-like haptera of endogenously derived rhizoidal filaments, complanate axes with pinnae and pinnules arising at nearly perpendic-

ular angles, tetrasporangial sori developing on specialized branches, and unilocular cystocarps with single ostioles. *Orthogonacladia* is similar to *Gelidium abbottiorum* R.E. Norris, *G. profundum* Tronchin & Freshwater, *G. pteridofolium* R.E. Norris, Hommersand & Fredericq, and *G. serra* (S.G. Gmelin) T. Taskin & M.J. Wynne (as *G. bipectinatum* G. Furnari) in having orthogonal branching, but all the latter species are consistently nested in the *Gelidium s. strict.* lineage (Fig. S2, unpublished *rbcl* and *cox1* sequences for *G. serra* from Italy by the first author).

Aphanta, a monospecific genus, has been resolved as an independent lineage distinct from the three families of Gelidiales in the *rbcl* and SSU analyses and was positioned within the Pterocladaceae, but with marginal or no support, in the LSU analysis (Tronchin and Freshwater, 2007). A second *Aphanta* species was found in Thailand during the present study, and it was similar to *A. pachyrrhiza* in morphology and was also not reproductive. The two *Aphanta* species formed a clade in all analyses, and the monophyly of *Aphanta* was fully supported in the five-gene tree. *Aphanta* always clustered with *Orthogonacladia* in analyses of the individual and combined five-gene datasets.

The inclusion of *Orthogonacladia* in the present study provides a new perspective on the position of *Aphanta*, and a new family, Orthogonacladiaceae, is herein proposed to accommodate these two genera. The presence/absence of rhizines (=internal rhizoidal filaments of Perrone et al., 2006), cystocarp construction, and the type of prostrate system are the characteristics used to recognize the three current Gelidiales families, as summarized in Table 3 (Perrone et al., 2006; Santelices, 2007; Boo et al., 2015a). The new family Orthogonacladiaceae and the new genus *Orthogonacladia* are described below according to the International Code of Nomenclature for algae, fungi and plants (McNeill et al., 2012).

4.5.1. Taxonomic conclusions

Orthogonacladiaceae fam. nov. G.H. Boo, L. Le Gall, K.A. Miller & S.M. Boo

Plant consisting of prostrate and erect uniaxial axes; uprights compressed to flattened, sparsely, irregularly or pinnately branched; sometimes with pinnae and pinnules arising at nearly right angles to the parent branch; rhizines concentrated in the inner cortex and interspersed between medullary cells; prostrate system of robust, extensively branched stolons, with peg-like haptera of endogenously derived rhizoidal filaments and exogenously derived multicellular, or no, corticating filaments; tetrasporangia arranged irregularly or in shallow v-shaped parallel rows; cystocarps unilocular where known; distinguished from the three current families of the Gelidiales by individual and concatenate five-gene phylogenies.

Type genus: *Orthogonacladia* gen. nov. G.H. Boo & L. Le Gall.

The family occurs in Western and South Australia, Madagascar, Mozambique, South Africa, and Thailand.

Included genus: *Aphanta*.

***Orthogonacladia* gen. nov.** G.H. Boo & L. Le Gall

Description: Plant epilithic, erect axes up to 40 cm high; main axes proximally terete, becoming compressed to flattened, subdistichously branched with pinnae and pinnules arising at nearly perpendicular angles; prostrate system robust, extensively branched with complex peg-like haptera; haptera consisting of endogenously derived rhizoidal filaments from inner cortical cells protruding between surface cells and exogenous, pigmented multicellular, uniseriate corticating filaments. Both erect and prostrate axes growing from a polygonal-shaped apical cell undergoing transverse divisions; subapical cells dividing distichously. Outermost cortical cells isodiametric and irregularly arranged in surface view; inner cortical cells irregular in shape; medullary cells thick-

Table 3
A comparison of Orthogonadiaceae fam. nov. with other families in the order Gelidiales.

	Orthogonadiaceae	Gelidiaceae	Gelidiellaceae	Pterocladaceae
Authors	Present study	Kützing (1843)	Fan (1961)	Felicini & Perrone in Perrone et al., 2006
Number of genera	2	4	2	2
Rhizines	Present	Present	Absent	Present
Prostrate system	Robust, extensively branched stolons; peg-like haptera	Stoloniferous; brush-like haptera	Stoloniferous; independent exogenous unicellular rhizoidal filaments	Stoloniferous; peg-like haptera
Hapteron construction	Endogenous rhizoidal filaments coalesced within a thick sheath	Independent, endogenous rhizoidal filaments	Not Applicable	Endogenous rhizoidal filaments coalesced within a thick sheath
Hapteron cortication	Present	Present	Not Applicable	Present
Cystocarps	Unilocular	Bilocular	Not found	Unilocular
Distribution	Subtropical to tropical	Temperate to tropical	Warm temperate to tropical	Temperate to tropical
References	Womersley and Guiry (1994), Tronchin and Freshwater, 2007; Present study	Fan (1961), Perrone et al. (2006)	Fan (1961), Perrone et al. (2006)	Perrone et al. (2006)

walled, elongated in plane of blade; rhizines congested in the inner cortical layers and absent or very rare in the medulla. Tetrasporangial sori develop distally on main axes and laterals; tetrasporangia arranged irregularly or in shallow v-shaped parallel rows, subspherical, decussately and cruciately divided. Cystocarps unilocular where known; spermatangia forming colorless patches where known. Distinguished from other genera in the Gelidiales by individual and concatenate five-gene phylogenies.

Type: *Orthogonocladia madagascariense* (A.V. Andriamampandry) comb. nov.

Etymology: The genus name is a synthesis of 'orthogonus' (right angle) and 'clados' (branch).

Orthogonocladia madagascariense (A.V. Andriamampandry) G.H. Boo & L. Le Gall comb. nov.

Basionym: *Gelidium madagascariense* A.V. Andriamampandry, 1988, in Cryptogamie, Algologie 9, 243–259, Figs. 14–25. Type locality: Fort-Dauphin (plage de Libanona), Madagascar; September 1983; A.V. Andriamampandry No. 603 (PC).
Specimens examined: see Table S1.
Distribution: Madagascar.

Orthogonocladia rectangularis (Lucas) G.H. Boo & T. Wernberg comb. nov.

Basionym: *Gelidium rectangularis* Lucas, 1931, in Proc. Linn. Soc. N.S.W., 56, 407–411, pls 23–27. Lectotype locality: Flinders Bay, Western Australia (Womersley and Guiry, 1994); Lucas Herb. NSW, September 1928.
Homotypic synonym: *Pterocladia rectangularis* (Lucas) Womersley and Guiry, 1994
Specimens examined: see Table S1.
Distribution: Western Australia.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jympev.2016.05.018>.

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