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Utility of *psb*A and nSSU for phylogenetic reconstruction in the Corallinales based on New Zealand taxa

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Abstract

A number of molecular studies of the Corallinales, a calcified order of the red algae, have used the conservative nSSU gene to investigate relationships within the order. However interspecific variation at this locus is low for closely related species, limiting resolution of recently diverged groups. In this study, we obtained *psb*A sequence data from specimens of the order from New Zealand that had been identified according to current taxonomic criteria. We compared phylogenetic analyses based on *psb*A with those based on nSSU for the same dataset, and also analysed nSSU sequences of the New Zealand material with nSSU sequences of Corallinales taxa from other parts of the world. Our study shows that *psb*A has considerable potential as a marker for this group, being easily amplified and considerably more variable than nSSU. Combined analyses using both markers provide significant support for relationships at both distal and terminal nodes of the analysis. Our analysis supports the monophyly of all three families currently defined in Corallinales: the Sporolithaceae, Hapalidiaceae and Corallinaceae, and indicates cryptic speciation in *Mesophyllum* and *Spongites*.

Keywords: Corallinales; nSSU; *psb*A; Hapalidiaceae; Corallinaceae; Sporolithaceae; New Zealand; *Mesophyllum*; *Spongites*

1. Introduction

Members of the order Corallinales (Rhodophyta) are macroscopic calcareous algae, showing substantial calcification of the cell walls. These algae grow from the polar regions to the tropics, and are important components of coastal ecosystems in both intertidal and subtidal environments. Coralline algae can grow as either upright, geniculate forms with nodal decalcification that provides some flexibility to the thallus, or crustose, non-geniculate forms appressed to the substrate, which may be rock, animal, another alga or a marine plant. Some species have been shown to provide specific chemical cues which trigger settling of invertebrate larvae (e.g. Roberts, 2001). Members of the order are also important constituents of reefs (van den Hoek et al., 1995), where they are thought to provide structural reinforcement at wave-exposed sites. Unattached or free-living non-geniculate corallines, known as rhodoliths or maërl, form extensive beds on soft sediments, occurring worldwide over wide latitudinal and depth ranges (Foster, 2001; Steller et al., 2003). These beds are known to support a rich diversity of associated species including rare, unusual and endemic species. Recent studies have revealed that these benthic communities are vulnerable to damage from human activities. They are easily impacted by a number of different anthropogenic activities such as harvesting, trawling and anchoring, activities that reduce water quality, and the creation of coastal structures that influence current flow such as breakwaters and marinas (Barbera et al., 2003; Steller et al., 2003).

Taxonomy of non-geniculate coralline algae often rests on reproductive characters such as the structure of

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tetrasporangial compartments and the degree of branching of spermatangial filaments (Woelkerling, 1988; Harvey et al., 2005). Where this is the case, it logically limits identification to specimens that are fertile and bear adequate representatives of the appropriate structures; in particular intact tetrasporangial compartments are often required for identification to species level (*ibid*). A significant number of field collected specimens are non-reproductive and are therefore unidentifiable by current methods. Molecular characters provide an appealing alternative that could widen the range of identifiable specimens to include sterile and abraded material.

The first molecular studies of the Corallinales by Bailey and Chapman, 1996, 1998 using nSSU[1] sequences, supported the monophyly of the family Sporolithaceae, and the non-monophyly of geniculate corallines, which fell into two distinct clades. These were followed by several other studies applying the nSSU to investigate family and subfamily relationships within the order, addressing the phylogenetic position of specific taxa such as the parasitic genus *Choreonema* and assessing the utility of the gene for molecular identification (Harvey et al., 2003; Bailey et al., 2004; Vidal et al., 2003). The analysis of Harvey et al. (2003) recovered the families Corallinaceae and Hapalidiaceae as monophyletic groups. Bailey et al. (2004) included taxa in the subfamily Melobesioideae (family Hapalidiaceae—see Harvey et al., 2003), but did not recover a monophyletic subfamily and their study further suggested that the subfamily Mastophoroideae is not monophyletic, and did not find support for a monophyletic Hapalidiaceae. The use of an independent, more variable marker, or denser taxon sampling, or both these strategies combined, might throw light on these conflicting results. In this paper, we address both these strategies with the application of a more variable marker, and an extended analysis including more Corallinales and outgroup taxa.

The nSSU is known to be a conservative gene that shows little or no variation between species in some Rhodophyte groups (Bailey and Freshwater, 1997). Vidal et al. (2003) assessed the utility of the 5 prime region of the nSSU for species identification among the non-geniculate coralline algae of Chile. They found that some species in their dataset had identical sequences over this region of the nSSU, and noted that a more rapidly evolving marker was therefore required to separate some taxa. An obvious candidate for such a marker is the plastid encoded *rbc*L gene, which has been successfully applied to many Rhodophyte groups (Freshwater et al., 1994, 1995; Müller et al., 1998; Sherwood and Sheath, 2000; Wang et al., 2000; Zuccarello and West, 2002; Gurgel et al., 2004). However, no studies have been published to date using the *rbc*L gene for phylogenetic reconstruction in the Corallinales. In our hands, amplification of the *rbc*L locus has been chancy at best, and it seems that primers in current use are not well suited to amplification of the gene from coralline algae. While in itself intriguing, this has impeded the use of this gene to test phylogenetic hypotheses in this order.

Another marker that has recently been successfully applied to the red algae is the *psb*A gene. This gene codes for the D1 protein of photosystem II, a core part of the photosynthetic machinery. While Yoon et al. (2002) found this gene to be effective in recovering deep phylogenetic branches in the algae, it has also been used to good effect to resolve intrageneric relationships within *Campylaephora* (Ceramiaceae, Rhodophyta) by Seo et al. (2003) and within *Griffithsia* (Ceramiaceae, Rhodophyta) by Yang and Boo (2004). While slightly shorter than the *rbc*L gene, it shares with the *rbc*L a plastid cellular location, and is therefore inherited uniparentally and independently of the nuclear encoded nSSU.

As part of a study of non-geniculate coralline algae in central New Zealand, we have investigated the use of the *psb*A gene for species identification and phylogenetic reconstruction within New Zealand members of the order Corallinales. We have obtained sequence data for the *psb*A and nSSU genes for 33 Corallinales taxa including representatives of three families and 13 genera, and compared the efficiencies of these genes for phylogenetic reconstruction and recognition of genera and species within this flora separately and together. We show that the *psb*A and nSSU datasets are congruent, and that the combined dataset is able to resolve groups within the Corallinales with significant support at a range of phylogenetic depths. Our data show that members of *Spongites yendoi* and *Mesophyllum erubescens* identified using current taxonomic criteria based on reproductive anatomy are genetically diverse and warrant further study with a view to discriminating segregate taxa.

2. Materials and methods

2.1. Collections and taxon selection

Non-geniculate coralline algae samples were collected from the central New Zealand region as part of a survey of species from this region that has been in progress since 2001. To extend taxon sampling, several geniculate and non-geniculate specimens collected from outside the central New Zealand region were also included, extending the geographic range of collections to Cape Reinga at the northernmost tip of the North Island, Stewart Island in the south and the Chatham Islands to the east of the New Zealand mainland. Table 1 gives collection information for New Zealand specimens used in this study. Specimens were inspected, catalogued and dried rapidly in desiccant silica gel, within a few hours of collection where possible. Author names for taxa are included in Tables 1 and 2. Species identifications are based on Harvey et al. (2005) for all New Zealand non-geniculate taxa and on Adams (1994) for geniculate taxa. All New Zealand specimens used in the

[1] *Abbreviations used:* nSSU, nuclear small-subunit ribosomal gene; NGC, non-geniculate coralline.

Table 1
GenBank accession numbers, collection information and voucher numbers for New Zealand samples sequenced in the course of this study

Specimen	Location	Date	Collectors	WELT No.	Genbank Accession No.	
					*psb*A	nSSU
Sporolithaceae						
Sporolithon durum (Foslie) Townsend and Woelkerling	Cable Bay, Nelson, South I	20 March 2003	S. Brown and N. Alcock	A027045	DQ167909	EF628211
Rhodolith D'Urville I	Catherine Cove, D'Urville I	6 January 2003	S. Brown	A027275	DQ167875	EF628212
Heydrichia homalopasta Townsend and Borowitzka	Port Hutt, Chatham I	23 February 2004	W.A. Nelson	A027268	DQ167931	EF628210
Hapalidiaceae						
Unidentified NGC Kaikoura	Rakautara Stream, Kaikoura, South I	5 November 2002	W.A. Nelson and C. Boedeker	A027274	DQ167883	EF628213
Unidentified NGC Stewart I	Lee Bay, Stewart I	18 October 2001	W.A. Nelson, T.J. Farr, J.E. Broom and L.E. Phillips	A027281	EF628243	EF628214
Phymatolithon repandum (Foslie) Wilks and Woelkerling Kaikoura	Kaikoura, South I	5 November 2002	J.X. Mei	A026988	DQ167879	EF628216
P. repandum Chatham I	Heaphy Shoal, Chatham I	20 February 2004	W.A. Nelson, K.F. Neill and T.J. Farr	A027261	DQ167946	EF628215
Synarthrophyton schielianum W.J. Woelkerling and M.S. Foster	Port William, Stewart I	31 October 2004	W. Freshwater, W.A. Nelson, J.E. Broom and S. Cooper	A027277	DQ168017	EF628217
Mesophyllum sp. Chatham I	Okawa Point, Hansen Bay, Chatham I	22 February 2004	W.A. Nelson	A027265	DQ167918	EF628218
M. erubescens (Foslie) M. Lemoine Whahariki Beach	Whahariki Beach, Golden Bay, South I	19 March 2003	W.A. Nelson, T.J. Farr, K.F. Neill and J. Dalen	A027246	DQ167874	EF628219
M. erubescens Golden Bay 1	Paton's Rock, Golden Bay, South I	19 March 2003	A. Harvey and R. Harvey	A027266	DQ167876	EF628220
M. erubescens Golden Bay 2	Taupo Point, Golden Bay, South I	18 March 2003	N. Alcock and S. Brown	A027263	DQ167893	EF628221
M. erubescens Chatham I	Point Durham, Chatham I	24 February 2004	K.F. Neill	A027264	DQ167929	EF628222
M. erubescens Wellington	Island Bay, Wellington, North I	10 September 2002	W.A. Nelson, J.X. Mei and C. Boedeker	A026956	DQ167884	EF628223
M. printzianum Woelkerling and A.S. Harvey Chatham I	Whangatete Inlet, Chatham I	23 February 2004	W.A. Nelson, K.F. Neill and T.J. Farr	A027270	DQ167935	EF628224
Corallinaceae						
Cheilosporum sagittatum (J.V. Lamouroux) Areschoug	Tatapouri, Gisborne, North I	2 August 2003	W.A. Nelson, K.F. Neill and T.J. Farr	A027286	DQ167881	EF628226
Haliptilon roseum (Lamarck) Garbary and H.W. Johansen Port William Stewart I	Port William, Stewart I	31 October 2004	W. Freshwater, W.A. Nelson and J.E. Broom	A027279	EF628244	EF628228
H. roseum Lee Bay Stewart I	Lee Bay, Stewart I	30 October 2004	W.A. Nelson	A027280	EF628245	EF628229
Jania sp. Gisborne	Kaiti Beach, Gisborne, North I	2 August 2003	W.A. Nelson	A027287	DQ167886	EF628227
Jania sp. Cape Reinga	Te Werahi Beach, Cape Reinga, North I	25 October 2003	W.A. Nelson and J.E. Broom	A027288	DQ167885	EF628225
Corallina officinalis Linnaeus Wellington	Island Bay, Wellington, North I	8 June 2004	W.A. Nelson and K.F. Neill	A027278	DQ168010	EF628232
Arthrocardia sp. Otago	Campbell Point, Otago, South I	19 February 2003	W.A. Nelson	A027282	DQ168011	EF628231

Arthrocardia sp. Cape Reinga	Te Werahi Beach, Cape Reinga, North I	25 October 2003	W.A. Nelson and J.E. Broom	A027289	EF628246	EF628230
Lithophyllum stictaeforme (J.E. Areschoug) Hauck	Ranger Point, Wellington, North I	18 May 2004	K.F. Neill and A.-N. Loerz	A027273	DQ167970	EF628241
Lithophyllum sp.	Kapowairua, Spirits Bay, North I	26 October 2003	W.A. Nelson and T.J. Farr	A027276	DQ167941	EF628242
Lithophyllum sp.	Whahariki Beach, South I	19 March 2003	W.A. Nelson, T.J. Farr, K.F. Neill and J. Dalen	A027018	DQ167872	EF628240
Hydrolithon improcerum (Foslie and Howe) Foslie	Pourerere, Tuingara Pt, North I	26 January 2004	W.A. Nelson, T.J. Farr and K.F. Neill	A027029	DQ168006	EF628239
Spongites yendoi (Foslie) Chamberlain Wainui Golden Bay 1	Wainui, Golden Bay, South I	18 March 2003	W.A. Nelson, T.J. Farr, K.F. Neill and J. Dalen	A027260	DQ167903	EF628233
S. yendoi Banks Peninsula	Taylor's Mistake, Banks Peninsula, South I	28 November 2003	S. Miller	A027269	DQ167907	EF628234
S. yendoi Chatham I 1	Heaphy Shoal, Chatham I	20 February 2004	W.A. Nelson	A027262	DQ167982	EF628235
S. yendoi Wainui Golden Bay 2	Wainui, Golden Bay, South I	18 March 2003	K.F. Neill	A027271	DQ167905	EF628236
S. yendoi Kaikoura	Kaikoura, South I	5 November 2002	W.A. Nelson, T.J. Farr and K.F. Neill	A027272	DQ167869	EF628237
S. yendoi Chatham I 2	Heaphy Shoal, Chatham I	20 February 2004	W.A. Nelson and K.F. Neill	A027267	DQ167979	EF628238

analyses are deposited at WELT (Herbarium, Museum of New Zealand Te Papa Tongarewa, Wellington).

The *psb*A and nSSU sequences of 33 representative New Zealand specimens were selected for analysis in order to compare efficacy of identification and phylogenetic reconstruction based on these two genes. Specimens included common taxa identified in central New Zealand (Harvey et al., 2005). We also included a subtidal rhodolith specimen collected off D'Urville Island that was sterile and therefore not amenable to identification based on traditional taxonomic characters, and two specimens that were identified as members of the Hapalidiaceae, but for which generic placement was unclear. All taxa selected for nSSU sequencing had non-identical *psb*A sequences except for the two unidentified Hapalidiaceae specimens.

2.2. DNA extractions, PCR amplification and sequencing

DNA was extracted from dried material using a Qiagen Tissue DNA Extraction Kit (Qiagen GmbH, Hilden) with a modified protocol. Coralline tissue was removed from the substrate, ground to a powder, and approximately 0.4 ml of extraction buffer and 25 mAU of proteinase K was added. After incubation at 65 °C for 4 h or overnight, 0.4 ml of lysis buffer was added and the sample was incubated at 70 °C for 10 min. Following centrifugation at full speed in a bench-top centrifuge for 10 min, the supernatant was removed, 0.4 ml of ethanol was added and the whole sample spun through a Qiagen column, washed and eluted in 0.2 ml elution buffer as per the manufacturers instructions. Extracts were diluted 1:100 for PCR amplification.

The nSSU gene was amplified in a single reaction using primers G01 and G04, and sequenced with primers G10, G02, G04 and G06 (Saunders and Kraft, 1994). Reactions were performed in a Stratagene Robocycler in a volume of 25 μl, under cycling conditions 94 °C 2 min, 30 cycles of 94 °C for 15 s, 50 °C for 30 s, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The *psb*A locus was amplified using primers *psb*AF1 and *psb*AR2 (Yoon et al., 2002) and sequenced using the same primers. Products were purified using exonuclease I/shrimp alkaline phosphatase digestion, and sequenced using standard methods on an ABI 13730 sequencer. All sequences were lodged in GenBank; accession numbers for sequences from New Zealand specimens are given in Table 1.

2.3. Sequence and phylogenetic analyses

Three datasets were constructed for the New Zealand taxa, consisting of each gene separately and a combined dataset of the two genes concatenated. Sequences from two Ceramiaceae taxa were included as outgroups for these analyses. The choice of outgroup sequences was limited by the availability of *psb*A sequences for appropriate taxa. Both *psb*A and nSSU sequences were available for *Centroceras clavulatum*. For the nSSU analysis a sequence attributed to *Antithamnion densum* was used as an outgroup,

Table 2
Additional nSSU sequences from GenBank included in the taxon-replete analysis

Order, family, subfamily and species	GenBank Accession No.
Ceramiales	
Ceramiaceae	
Ceramioideae	
Antithamnion densum (Suhr) M.A. Howe	AY643485
Centroceras clavulatum (C. Agardh) Montagne	AY155521
Colaconematales	
Colaconemataceae	
Colaconema daviesii (Dillwyn) Stegenga	AF079788
Nemaliales	
Liagoraceae	
Nemalion helminthoides (Velley) Batters	L26196
Palmariales	
Palmariaceae	
Palmaria palmata (Linnaeus) Kuntze	Z14142
Corallinales	
Sporolithaceae	
Heydrichia woelkerlingii Townsend, Chamberlain and Keats	U61253
Heydrichia homalopasta Townsend and Borowitzka AUS	AF411629
Sporolithon durum (Foslie) Townsend and Woelkerling NSW AUS	AF411626
Sporolithon durum SA AUS	U61254
Hapalidiaceae	
Choreonematoideae	
Choreonema thuretii (Bornet) Schmitz	AY221254
Melobesioideae	
Clathromorphum compactum (Kjellman) Foslie	U60742
Clathromorphum parcum (Setchell and Foslie) Adey	U61252
'Leptophytum' acervatum	U62119
'Leptophytum' ferox	U62120
Lithothamnion glaciale Kjellman	U60738
Lithothamnion tophiforme Unger	U60739
Mastophoropsis canaliculata (W.H. Harvey) Woelkerling	U62118
Mesophyllum engelhartii (Foslie) Adey SA	U61256
Mesophyllum erubescens (Foslie) Lemoine Brazil	U61257
Phymatolithon laevigatum (Foslie) Foslie	U60740
Phymatolithon lenormandii (Areschoug) Adey	U60741
Synarthrophyton patena (J.D. Hooker and W.H. Harvey) Townsend	U61255
Corallinaceae	
Corallinoideae	
Arthrocardia filicula (Lamarck) H.W. Johansen	U61258
Bossiella californica ssp. *schmittii* (Manza) H.W. Johansen	U60945
Bossiella orbigniana ssp. *dichotoma* (Manza) H.W. Johansen	U60746
Calliarthron cheilosporioides Manza	U60943
Calliarthron tuberculosum (Postels and Ruprecht) E.Y. Dawson	U60944
Cheilosporum sagittatum (J.V. Lamouroux) Areschoug AUS	U60745
Corallina elongata Ellis and Solander	U60946
Corallina officinalis Linnaeus	L26184
Haliptilon roseum (Lamarck) Garbary and H.W. Johansen AUS	U60947
Jania rubens (Linnaeus) J.V. Lamouroux	U61259
Jania verrucosa J.V. Lamouroux	U62113
Serraticardia macmillanii (Yendo) Silva	U62114
Lithophylloideae	
Amphiroa sp. Aus	U62115
Amphiroa sp. SA	U62116
Amphiroa fragilissima (Linnaeus) J.V. Lamouroux	U60744
Amphiroa hancockii Taylor	AY234233
Amphiroa tribulus Foslie and Howe	AY234234
Lithophyllum incrustans Philippi	AF093410
Lithophyllum kotschyanum (Unger) Foslie	U62117

Table 2 (*continued*)

Order, family, subfamily and species	GenBank Accession No.
Lithothrix aspergillum J.E. Gray	U61249
Titanoderma pustulatum (J.V. Lamouroux) Nägeli	AF093409
Mastophoroideae	
Hydrolithon onkodes (Heydrich) Penrose and Woelkerling	AY234237
Hydrolithon pachydermum (Foslie) Bailey, Gabel and Freshwater	AY234235
Hydrolithon samoense (Foslie) Keats and Chamberlain	AY234236
Metamastophora flabellata (Sonder) Setchell clone 1	AY234239
Neogoniolithon brassica-florida (Harvey) Setchell and L.R. Mason	AY233346
Neogoniolithon spectabile (Foslie) Setchell and L.R. Mason	AY234238
Spongites yendoi (Foslie) Chamberlain SA	U60948
Metagoniolithoideae	
Metagoniolithon chara (Lamarck) Ducker	U60743
Metagoniolithon radiatum (Lamarck) Ducker	U61250
Metagoniolithon stelliferum (Lamarck) Ducker	U61251

Abbreviations showing location are retained after the authority where appropriate to allow the reader to locate the sequence in Fig. 5.

however no *psb*A sequences are available for this taxon and so for the *psb*A analysis we used a sequence attributed to *Antithamnion* sp. In the combined analysis, therefore, the '*Antithamnion*' outgroup sequence is a concatenation of sequences from two congeneric but not necessarily conspecific taxa.

In order to evaluate the phylogenetic relationships of New Zealand taxa with other members of the Corallinales, a fourth dataset was constructed by aligning nSSU sequences from our study with Corallinales nSSU sequences of substantial length from GenBank. To provide a broader phylogenetic context for the analysis we included nSSU sequences from three additional orders of Rhodophyte lineage 2 (Harper and Saunders, 2001): *Nemalion helminthoides* (L26196, Ragan et al., 1994), *Palmaria palmata* (Z14142) and *Colaconema daviesii* (AF079788, formerly *Audouinella daviesii*, Harper and Saunders, 2002). The analysis of Bailey et al. (2004) included a sequence designated Corallinales sp. CB-2003 (AY247408) from a single specimen which preliminary morphological analysis suggested was a member of *Sporolithon* Heydrich, but which was not identified to species level. This sequence was not resolved as a member of any of the three Corallinales subfamilies in their analysis, but was instead placed as a single long branch between the Sporolithaceae and Hapalidiaceae. In order to avoid possible long branch attraction artefacts, and since the sequence was based on only a single collection, we have chosen not to include it in our analysis.

Sequences were aligned by eye using Se-AlV2.0a7b (A. Rambaut, http://evolve.zoo.ox.ac.uk/). Unalignable regions of the nSSU matrices were removed for phylogenetic analysis and calculations of pairwise distances. An appropriate model of sequence evolution for each dataset was estimated using the Akaike Information Criterion (AIC) implemented in ModeltestV3.0.6 (Posada and Crandall, 1998; GTR+I+Γ in all cases), and this model was used for all distance and likelihood calculations. All four datasets were analysed using maximum parsimony (MP) and maximum-likelihood (ML) optimality criteria, and Bayesian analysis. MP trees were estimated with PAUP*4.0b10 (Swofford, 2002). MP analyses were conducted using a heuristic search strategy with 100 replicates of random-order sequence addition followed by tree bisection reconnection (TBR) branch swapping. Bootstrap support was estimated with 1000 replicates, each of 10 replicates of random-order sequence addition followed by TBR branch swapping, for the three smaller datasets; MP bootstrap analysis for the taxon-replete nSSU dataset was prohibitively time-consuming and was not performed. Maximum-likelihood analyses were conducted using PHYML v2.2.4 (Guindon and Gascuel, 2003) under the model of sequence evolution chosen by Modeltest for that dataset, with concurrent estimation of parameters for invariant sites and gamma-modelled rate heterogeneity. Support was estimated using 1000 bootstrap replicates for the first three datasets, and 500 replicates for the taxon-replete dataset. Maximum-likelihood analysis of the ingroup taxa only was performed under the same model of sequence evolution as for the larger dataset, and bootstrap support was assessed under 500 replicates.

PAUP*4.0b10 was used to calculate pairwise distances between sequences, and to conduct the Partition Homogeneity Test (PTH test, also known as Incongruence Length-Difference or ILD test; Farris et al., 1995) on the combined dataset, with data divided into two partitions according to the nSSU or *psb*A origin of the sequence, using 1000 replicates and with invariant characters excluded. Saturation in the *psb*A dataset was explored by plotting ML distances using parameters from Modeltest against raw sequence difference for each codon position.

Bayesian analyses were carried out using MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003) to run four Metropolis-coupled MCMC chains (one cold and three incrementally heated, temperature parameter = 0.2). Two independent MrBayes analyses were run under the GTR+I+Γ model of sequence evolution for 2,000,000 generations for the single-gene analyses of New Zealand specimens, 4,000,000 generations for the combined dataset, and

1,000,000 generations for the taxon-replete nSSU dataset. For the combined dataset the data were partitioned by gene and parameters were optimised independently for each partition. Model parameters were treated as unknown and were estimated in each analysis. Chains were initiated with random starting trees and trees were sampled every 100 generations. Appropriate burn-in values were determined by inspection of plots of log-likelihood against generation time for each run. Trees obtained before this value were discarded, and the remaining trees were used to calculate 50% majority rule consensus trees, in which each clade posterior probability (PP) value is represented by the proportion of trees containing that clade.

3. Results

3.1. Phylogenetic datasets

The New Zealand raw nSSU dataset consisted of 1560 characters, reduced to 1462 characters in the phylogenetic matrix on removal of unalignable regions. Of the characters retained in the phylogenetic matrix, 297 were variable and 230 were parsimony-informative. The *psb*A dataset was slightly smaller, at 853 characters, however it was more variable, containing 293 variable and 243 parsimony-informative characters. In percentage terms, 20.3% of the characters in the nSSU phylogenetic matrix were variable and 15.7% were parsimony-informative, compared to 34.3% variable and 28.5% parsimony-informative in the *psb*A matrix. More than 80% of the variable sites in the *psb*A dataset occurred in the third codon position; the plot of ML distances (calculated under GTR+I+Γ) against raw sequence difference suggested that the *psb*A dataset was becoming saturated at the third position at the highest levels of sequence divergence within our taxon set (data not shown). The combined dataset contained 2315 characters, of which 1725 were constant, 590 variable and 473 parsimony-informative. These three datasets each contained 33 ingroup taxa and two outgroup taxa. The taxon-replete dataset of nSSU sequences consisted of 85 taxa, five of which were outgroup taxa, and 1449 characters. Of these, 1054 characters were constant and 307 were variable and parsimony-informative.

3.2. NZ taxa single-gene analyses

The results of the nSSU single-gene analysis are shown in Fig. 1. Six nodes in total on the nSSU tree are recovered with bootstrap support of 100% under both ML and MP analyses, and posterior probability of 1.0 under Bayesian analysis (100/100/1.0). The nSSU analysis generated a tree with generally high bootstrap support and posterior probabilities for nodes along the 'backbone' of the tree—that is for deep divergences in the dataset. Both the Hapalidiaceae and the Corallinaceae are recovered as well-supported monophyletic groups, although support for the Corallinaceae is higher than for the Hapalidiaceae under ML and MP (100/100/1.0 vs 79/85/0.99). The Sporolithaceae are not recovered as a monophyletic group in this analysis, although both *Sporolithon durum* and *Heydrichia homalopasta* are excluded from the other two families (bootstrap and PP support for a monophyletic group of Corallinaceae and Hapalidiaceae is 100/100/1.0). Support for a relationship between *Heydrichia* and the non-Sporolithacean families is conflicted among the three methods of analysis: Bayesian analysis recovers this relationship with a PP of 0.98 while bootstrap support under ML is 74%, and bootstrap support under MP is less than 50%.

Some internal nodes in the nSSU analysis are well-supported. Notably members of genus *Lithophyllum* Philippi are recovered as a monophyletic group at 100% bootstrap support for both MP and ML and a PP of 1.00, as are members of *Spongites yendoi* (Foslie) Y.M. Chamberlain. Within *S. yendoi* two clades are recovered with moderate to high support. Monophyly of the Janieae—*Cheilosporum* (J. Decaisne) G. Zanardini, *Haliptilon* (J. Decaisne) J. Lindley and *Jania* J.V.F. Lamouroux—is well-supported (99/100/1.00), but *Jania* itself is not monophyletic: two specimens from two different locations, which also differed morphologically, are resolved apart from one another. Within the Hapalidiaceae an unidentified taxon from eastern New Zealand is basal to the family (84/94/1.00) and two specimens of *Phymatolithon repandum* (Foslie) Wilks and Woelkerling are resolved together (99/100/1.00). *Mesophyllum* is not resolved as monophyletic under ML analysis, and monophyly of this group has only low support under MP although there is some support for monophyly under Bayesian analysis (56/–/0.9). Within *Mesophyllum*, *Mesophyllum erubescens* (Foslie) M. Lemoine is divided into two well-supported clades, and a specimen identified as *M. printzianum* Woelkerling and A.S. Harvey is resolved within one of these clades.

The results of the *psb*A single-gene analysis are shown in Fig. 2. Pairwise distances between taxa were larger for the *psb*A dataset than for the nSSU dataset, and this is reflected both in the longer branch lengths on the *psb*A phylogram and in the differing bootstrap support for nodes between the two analyses. Six nodes in total on the *psb*A tree are recovered with support of 100/100/1.0, but only two of these nodes are also supported at 100/100/1.0 under the nSSU analysis: the monophyletic ingroup of Corallinales, and the association of the two unidentified non-geniculate specimens from Kaikoura and Stewart Island. In general, support in the *psb*A tree is higher for nodes towards the tips of the tree than for nodes along the backbone, and clades that are well-supported under nSSU analysis also find some support under *psb*A. As under nSSU, the Sporolithaceae is not recovered as a monophyletic group, but in the *psb*A analysis a relationship between *Sporolithon* and the non-Sporolithacean taxa is recovered with weak bootstrap support under MP and ML and near-significant support under Bayesian analysis (68/65/0.94). The Corallinaceae and Hapalidiaceae are recovered as monophyletic, but with lower support than on the nSSU

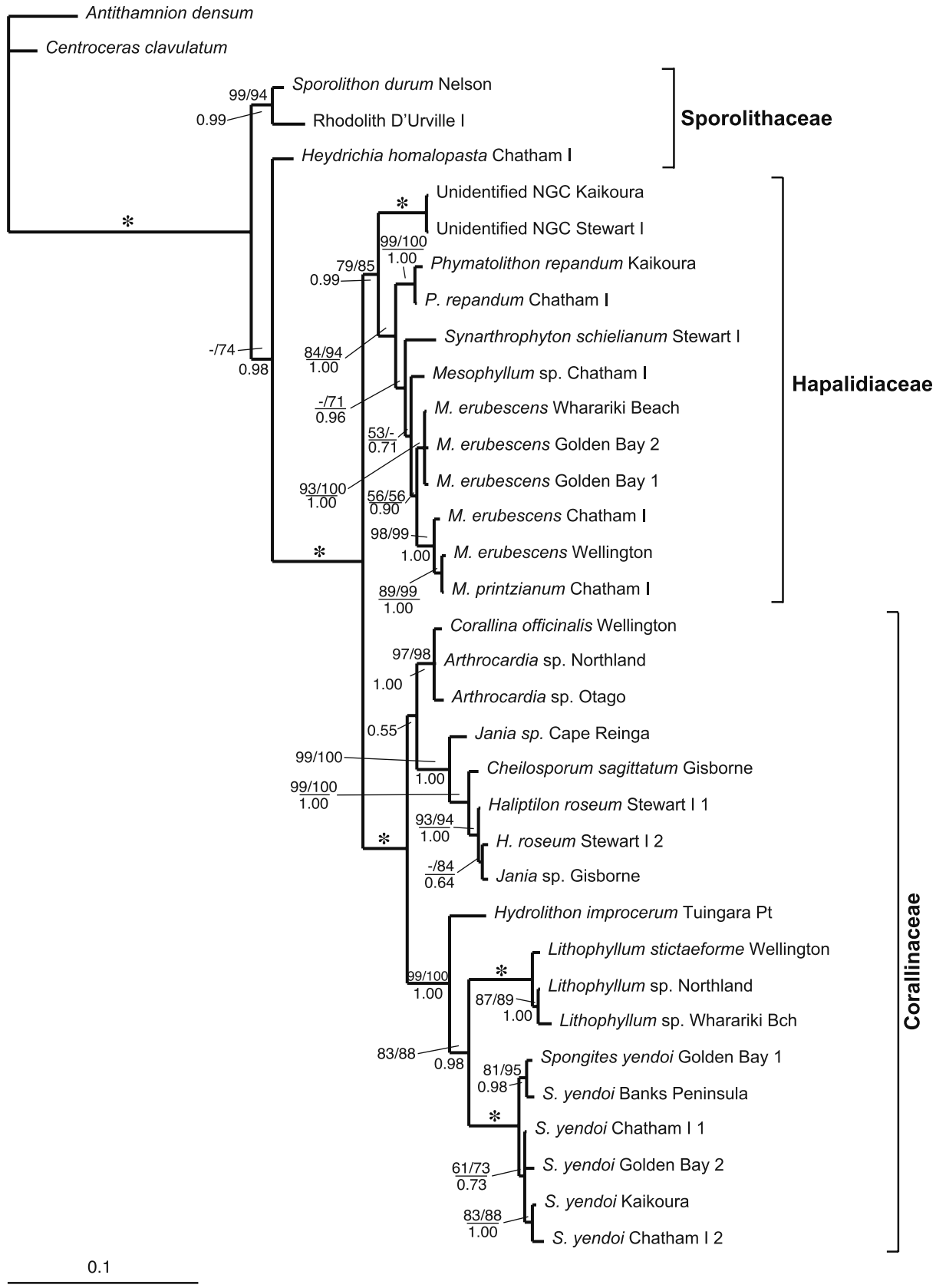

Fig. 1. Bayesian phylogram calculated from the New Zealand taxa nSSU dataset. Numbers above the branch are MP/ML bootstrap support values, respectively, the Bayesian posterior probability values are shown below the branch. The scale bar refers to substitutions per site. Asterisks mark clades that are supported at 100% in all three analyses.

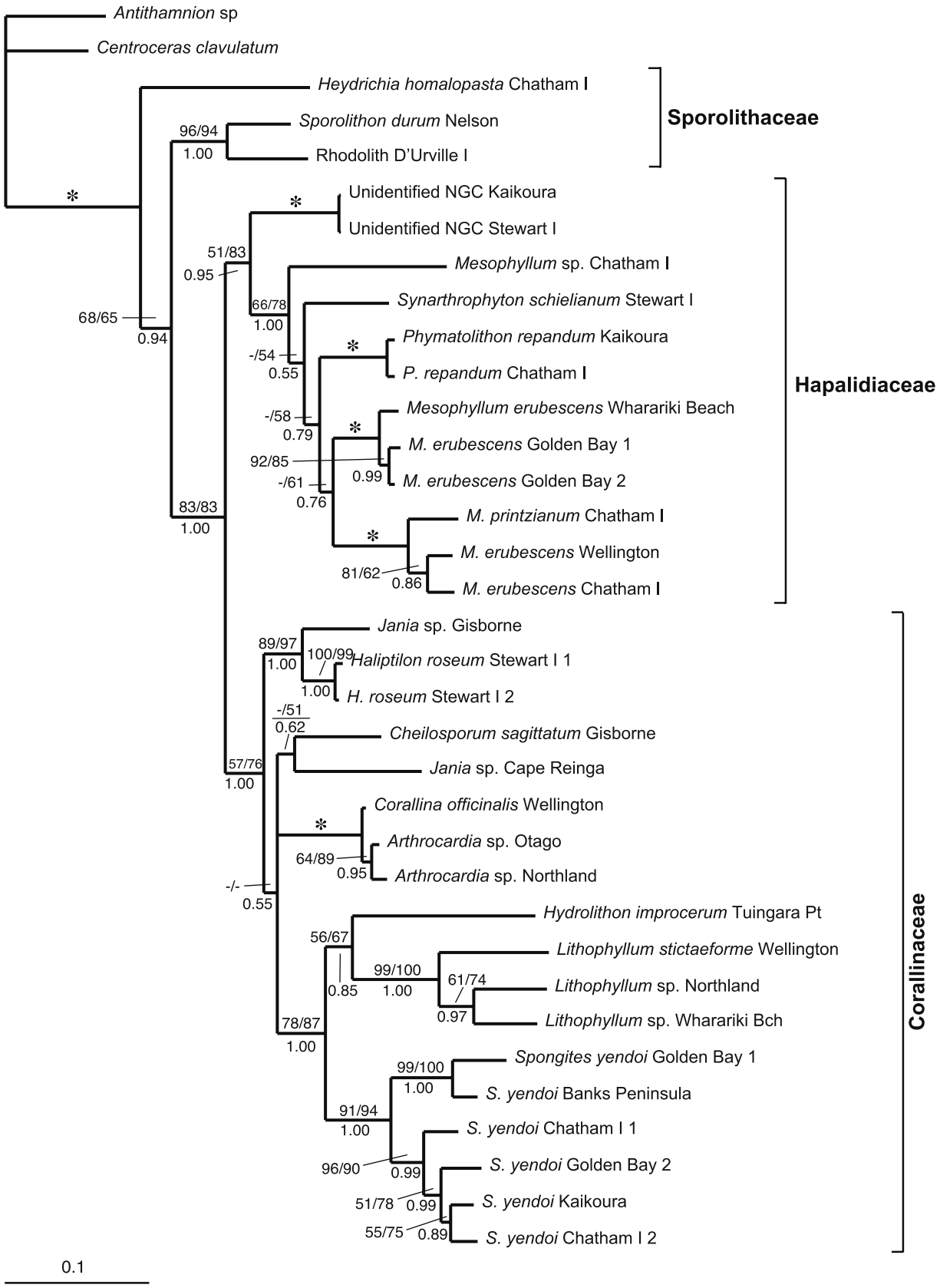

Fig. 2. Bayesian phylogram calculated from the New Zealand taxa *psb*A dataset. Numbers above the branch are MP/ML bootstrap support values, respectively, the Bayesian posterior probability values are shown below the branch. The scale bar refers to substitutions per site. Asterisks mark clades that are supported at 100% in all three analyses.

tree. Support is high for the division of *Mesophyllum erubescens* into two clades; this is also well-supported under nSSU.

There is little significant conflict between the topologies recovered in the *psb*A and nSSU single-gene analyses. Fig. 3 displays the ML topologies recovered for both genes, drawn as a cladogram with nodes having bootstrap support below 60% collapsed. In general the topologies are congruent or not significantly different. Clades which appear in one tree are either present in the other tree, or are resolved as polytomies. The major difference is in the relationships of the Sporolithacean taxa. Neither analysis recovers a monophyletic Sporolithaceae. While both recover a well-supported relationship between *Sporolithon durum* and the D'Urville Island rhodolith, they differ in placing either this clade or *Heydrichia homalopasta* as a sister group to the remaining members of the order. Bootstrap support for these nodes is only moderate, at 74% and 65% for nSSU and *psb*A, respectively. Since the monophyly of the Sporolithaceae is not contentious, and in view of the results of the taxon-replete nSSU analysis (Fig. 5) in which monophyly of the Sporolithaceae is strongly supported, we consider that this result most likely reflects the limited taxon sampling in this dataset. All taxa of the Sporolithaceae are excluded from both the Hapalidiaceae and Corallinaceae by both our single-gene New Zealand datasets.

3.3. NZ taxa combined analysis

Results of the PTH test indicated there is no significant conflict between the two datasets ($P = 0.101$) and analysis of the combined *psb*A + nSSU dataset (Fig. 4) generates a tree with better resolution than either gene alone. A total of 12 nodes receive 100% support under all three methods of analysis (MP, ML and Bayesian)—double that for either gene alone, and including two clades that were not supported at that level in either analysis: the *Hydrolithon*/*Lithophyllum*/*Spongites* clade, and the clade associating *Spongites yendoi* Golden Bay 1 with *S. yendoi* Banks Peninsula. Each of these clades received 99/100/1.00 support in one of the single-gene analyses.

The Sporolithaceae are not recovered as a monophyletic group, but support for an association between *Heydrichia*

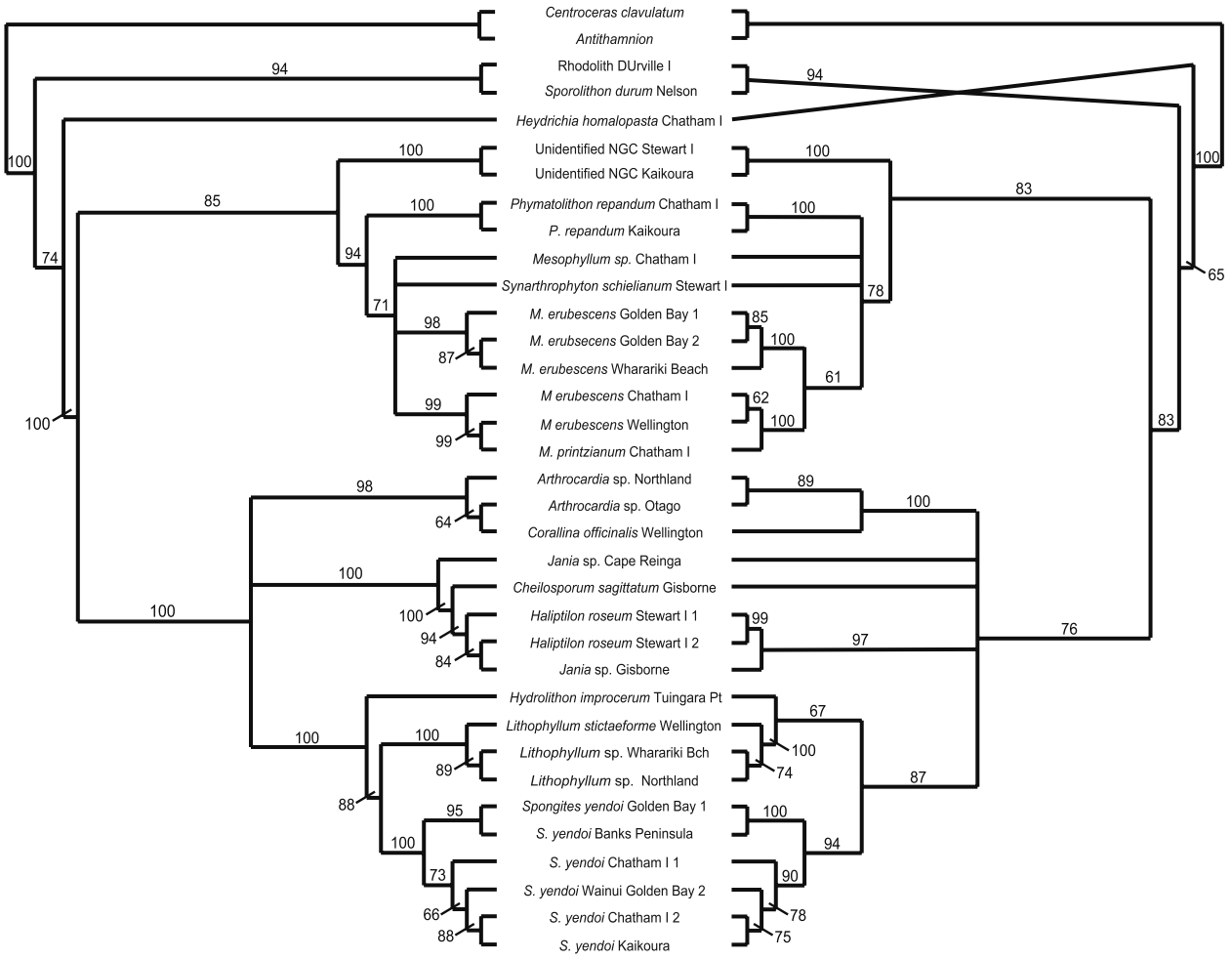

Fig. 3. Comparison of maximum-likelihood cladograms from the single-gene nSSU and *psb*A analyses. ML bootstrap support values >60% are shown above the branch. The nSSU cladogram is to the left and the *psb*A cladogram to the right. Nodes with bootstrap support less than 60% are collapsed.

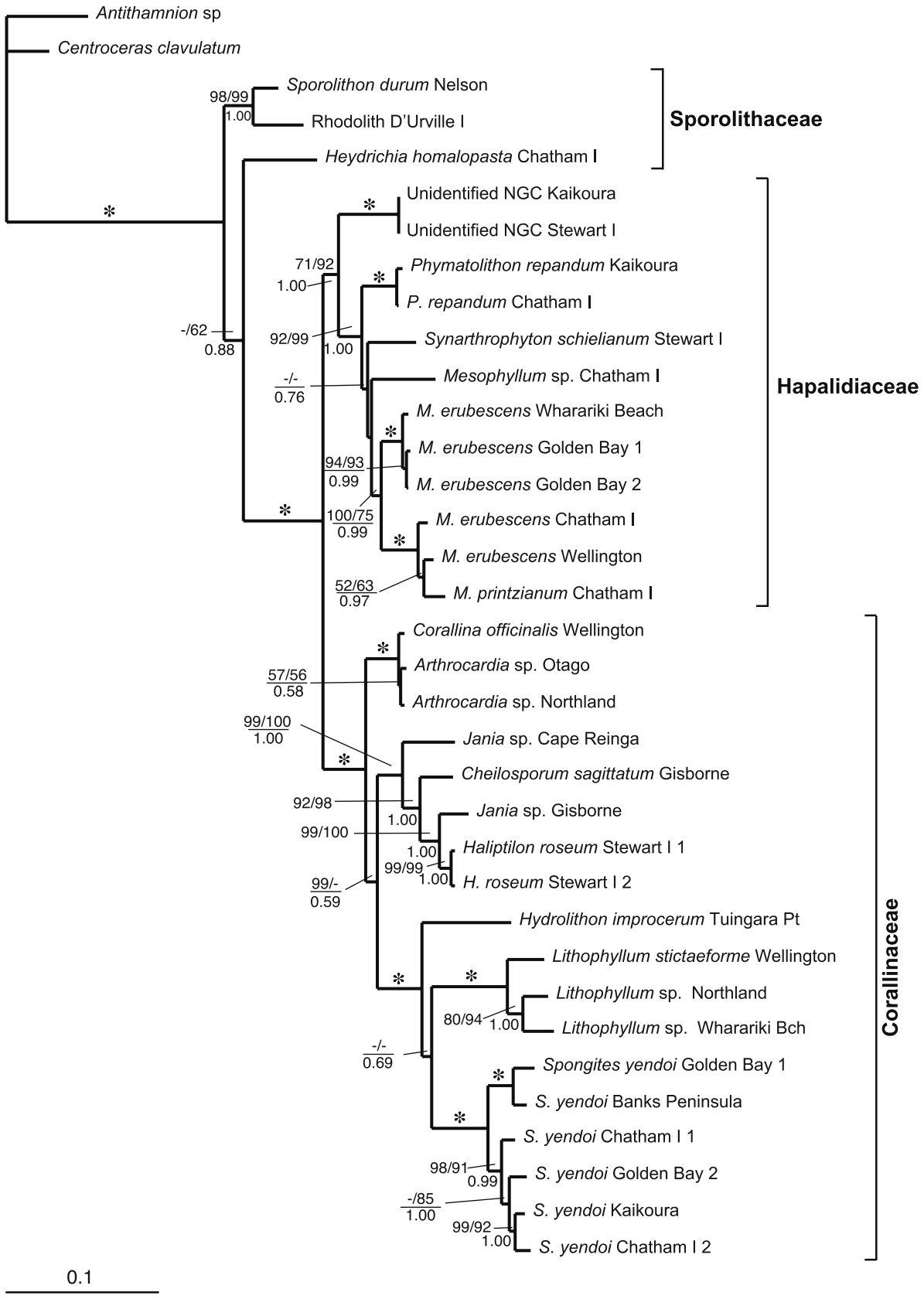

Fig. 4. Bayesian phylogram calculated from the New Zealand taxa combined nSSU and *psb*A dataset. Numbers above the branch are MP/ML bootstrap support values, respectively, the Bayesian posterior probability values are shown below the branch. The scale bar refers to substitutions per site. Asterisks mark clades that are supported at 100% in all three analyses. NGC, non-geniculate coralline.

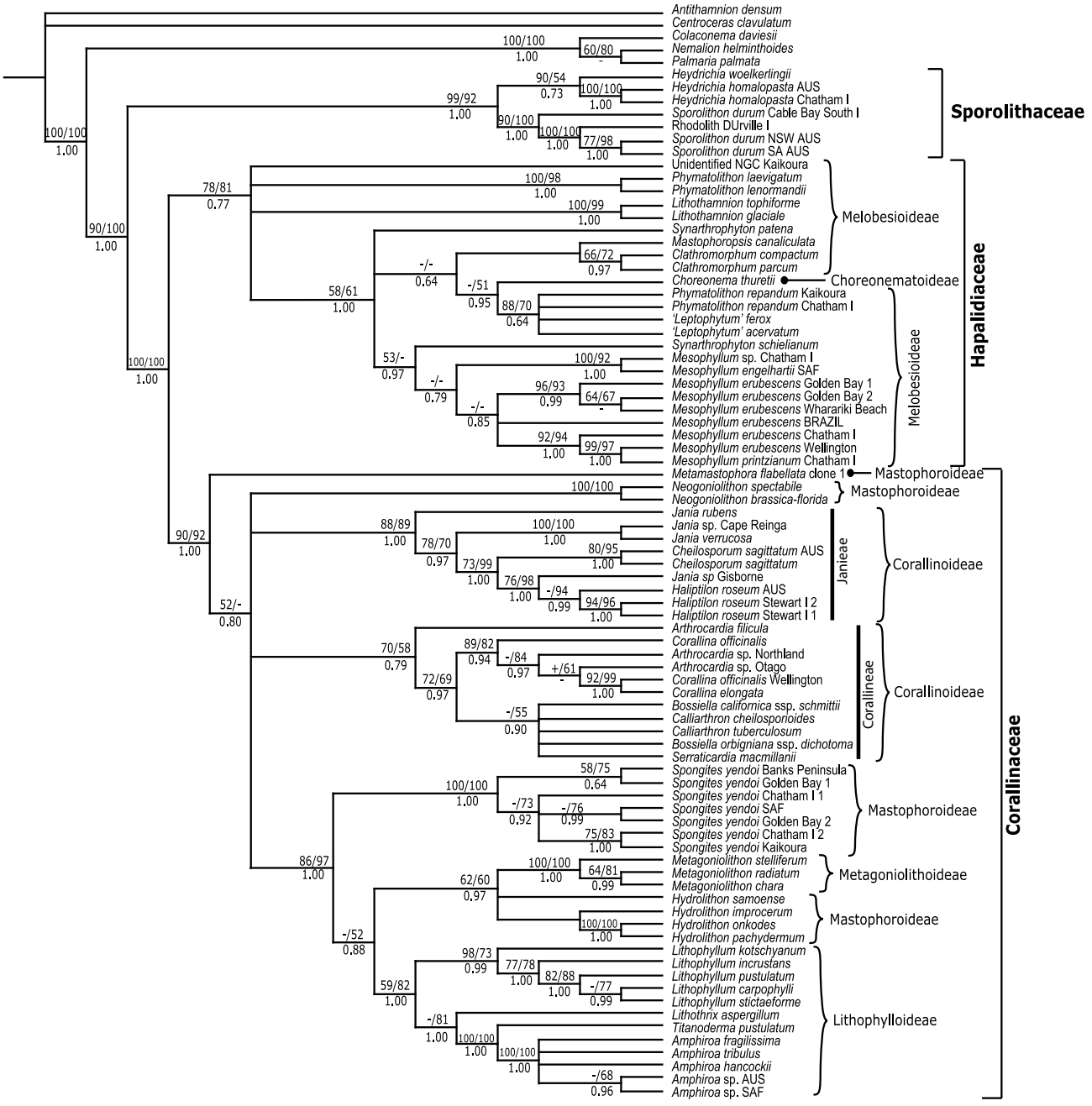

Fig. 5. Maximum-likelihood cladogram inferred from analysis of nSSU data for the taxon-replete dataset. Percentage bootstrap support under NJ (left) and ML (right) are shown above the branches, and Bayesian PP values below. Nodes that are not supported at >50% in at least one analysis are collapsed. Abbreviations: AUS, Australia; SAF, South Africa; SA AUS, South Australia; NSW AUS, New South Wales Australia; NGC, non-geniculate coralline.

homalopasta and the remaining Corallinales excluding *Sporolithon* is not significant. The analysis fails to recover a monophyletic Sporolithaceae, but does not significantly support non-monophyly—rather there is insufficient data to draw reliable conclusions.

Clades that receive strong support under one of the single-gene analyses generally receive increased support under combined analysis. For example support for a monophyletic Hapalidiaceae in the combined analysis exceeds that found in either single-gene analysis under ML and Bayesian methods, although MP bootstrap support is slightly reduced from 79% in the nSSU analysis to 71%. There is very strong support for the monophyly of Hapalidiaceae taxa excluding the Unidentified NGC, however relationships between *Phymatolithon repandum*, *Synarthrophyton* and the *Mesophyllum* taxa are not clearly resolved. The very short branch lengths seen in this group suggest that genetic variability in this group is relatively low, and neither nSSU nor *psb*A contains enough variation to reliably resolve these relationships. Nevertheless *Mesophyllum erubescens* is resolved into two well-supported clades, one of which also contains a specimen identified as *M. printzianum*.

Within the Corallinaceae, the Janiae are resolved as monophyletic with strong support (99/100/1.0) but *Jania* itself is not monophyletic. Two specimens identified as *Arthrocardia* are very closely related to one another and to *Corallina officinalis* from New Zealand. *Hydrolithon improcerum*, *Lithophyllum* and *Spongites yendoi* are resolved as monophyletic, an association also recovered by Bailey et al. (2004). *Lithophyllum* is resolved as monophyletic with strong support.

3.4. Taxon-replete analysis under nSSU

The taxon-replete dataset allows us to place the New Zealand specimens in a global context. We added three further outgroup taxa from three additional orders of Rhodophyte lineage 2 in order to provide a better context for this analysis. GenBank accession numbers for nSSU sequences included in the analysis in addition to the New Zealand sequences are given in Table 2. In broad terms our analysis agrees with that of Bailey et al. (2004). Our taxon set extends theirs by the inclusion of *Heydrichia homalopasta* and *Choreonema thuretii*, which were sequenced by Harvey et al. (2003), *Synarthrophyton schielianum*, *Lithophyllum stictaeforme*, and further representative specimens of *Lithophyllum*, *Spongites*, *Mesophyllum*, *Corallina*, *Phymatolithon*, *Sporolithon*, *Jania* and *Haliptilon*.

Heydrichia and *Sporolithon* are both resolved as monophyletic, and New Zealand specimens are resolved with congeneric specimens from Australia. The monophyly of the Sporolithaceae is strongly supported, as it was in the analysis of Bailey et al. (2004).

The Hapalidiaceae are resolved as a monophyletic group with moderate bootstrap support (78% under NJ, 81% under ML) and Bayesian posterior probability of 0.77. The unidentified non-geniculate coralline taxon from Kaikoura is resolved with the Hapalidiaceae. This is consistent with morphology—this specimen has a multiporate conceptacle roof which is a diagnostic character of this family (Harvey et al., 2003). This specimen is clearly resolved as separate from the remaining 8 Hapalidiaceae taxa included in our analysis. Within the Hapalidiaceae, resolution of genera and taxa is generally poor. Two sequences from specimens of *Phymatolithon repandum* from New Zealand are not resolved with *Phymatolithon laevigatum* and *Phymatolithon lenormandii*, but are instead resolved with moderate support with '*Leptophytum' ferox* and '*Leptophytum' acervatum*. Two *Synarthrophyton* taxa are not resolved as monophyletic, and there is no bootstrap support under ML or NJ for the monophyly of *Mesophyllum*. *Choreonema thuretii* is resolved in a clade with *Phymatolithon*/'*Leptophytum*' taxa, which would render the Melobesioideae paraphyletic, but support for this relationship is very weak and has no known morphological basis. The conceptacle roof in *Choreonema* is very different to other Hapalidiaceae taxa and we do not consider evidence for this placement to be significant.

The Corallinaceae are well-supported as a monophyletic group. The Mastophoroideae, represented in our analysis by *Metamastophora*, *Neogoniolithon*, *Spongites*, and *Hydrolithon*, are confirmed as non-monophyletic, as was reported by Bailey et al. (2004). Both *Metamastophora* and *Neogoniolithon* are resolved on very long branches basal to the Corallinaceae. *Metamastophora* is resolved as a sister taxon to the remaining Corallinaceae taxa, although with very low support, and the two *Neogoniolithon* taxa are resolved within the Corallinaceae but are not resolved with members of any other genus. Our analysis does not exclude the possibility that these two are sister genera, although neither is there significant evidence in favour of such a relationship. The placement of these two genera is not robust. *Hydrolithon*, in contrast, is resolved within a well-supported clade containing members of *Amphiroa, Titanoderma*, and *Lithophyllum* (Lithophylloideae), *Metagoniolithon* (Metagoniolithoideae) and *Spongites* (*Mastophoroideae*), but *Hydrolithon* and *Spongites* are not resolved together within this clade, rather *Hydrolithon* is resolved as more closely related to *Metagoniolithon*.

There is no significant support for monophyly of the subfamily Corallinoideae despite the obvious synapomorphy for this group of a geniculate growth habit. The two tribes Janieae and Corallineae are each resolved as monophyletic. There is significant support (87/97/1.00) for an association of the remaining non-geniculate genera *Spongites*, *Metagoniolithon*, *Hydrolithon* and members of the Lithophylloideae. *Lithophyllum* is resolved as a monophyletic group separate from *Titanoderma pustulatum*.

4. Discussion

Multi-gene analyses are becoming the norm in phylogenetic reconstruction (De Clerck et al., 2006; Lane et al., 2006; Yoon et al., 2006). Our data show the efficacy of *psb*A for phylogenetic reconstruction in the Corallinales, and demonstrate the advantages of a multi-gene analysis combining *psb*A and nSSU sequence data. Despite relatively limited taxon sampling, phylogenetic trees based on the combined dataset are well-resolved at both basal and interior nodes, providing good bootstrap support for at least two of the three families, recovering *Lithophyllum*, *Haliptilon roseum*, *Arthrocardia* and *Spongites* as monophyletic groups and showing genetic diversity within specimens currently placed in *Spongites yendoi* and *Mesophyllum erubescens*.

The *psb*A gene is more variable than nSSU, showing substantial variation between genera, and between taxa. Within a given taxon, however, variation can be small. For instance, identical *psb*A sequences have been obtained for specimens identified as *Corallina officinalis* from Whangarei in the northern North Island, and from Lee Bay in Stewart Island at the south of the main New Zealand archipelago. Maximum sequence diversity observed within *C. officinalis* in New Zealand is 3 nucleotide substitutions (data not shown). In contrast, within specimens

identified according to current taxonomic criteria as *Mesophyllum erubescens* sequence variation is considerable, ranging from 0 to 60 nucleotide substitutions, suggesting the presence of cryptic species. The relationships of these and other New Zealand taxa will be explored in more detail in future papers. Genetic diversity within a species will be reduced if the species is young, or if the species has undergone a significant population bottleneck followed by a range expansion, such as a relatively recent introduction, either natural or anthropogenic. The *psb*A gene offers a window on intraspecific variation that has not previously been available for members of the Corallinales. It will be of great interest to compare sequences of New Zealand specimens with those from overseas taxa, particularly for species that are considered to be cosmopolitan, such as *C. officinalis*; nSSU sequences of New Zealand material identified as *C. officinalis* are more similar to a sequence in GenBank attributed to *C. elongata* (U60946, collected in South Africa) than to a GenBank sequence attributed to *C. officinalis* (Fig. 5).

Extensive genetic variation within a single taxon, such as we observe here in *M. erubescens* and *Spongites yendoi*, which exceeds interspecific variation observed between other recognised species, suggests that cryptic species exist within these taxa as currently defined. Harvey et al. (2005) describe *M. erubescens* as a highly variable species. They also note the presence of intermediate forms between *M. erubescens* and *M. printzianum*, which they place together as a species complex pending further taxonomic study. Our analysis does not resolve *M. erubescens* as distinct from *M. printzianum*, but does indicate two well-supported monophyletic groups within the *erubescens*/*printzianum* complex. Specimens of *S. yendoi* are similarly resolved into two well-supported monophyletic groups, including two specimens from the same location that are resolved in two different clades. The taxonomic status of these monophyletic groups awaits clarification by further anatomical studies. However, it is worth noting that the variation within these clades (up to 65 bp for *S. yendoi* and 60 bp for *M. erubescens*) is of the order of that observed between *Haliptilon roseum* and *Cheilosporum sagittatum* (56–60 bp), or between *H. roseum* and *Jania* sp. Gisborne (44–46 bp).

The taxon-replete nSSU analysis allows us to position the New Zealand taxa against taxa from other parts of the world and to test the monophyly of the Hapalidiaceae. Our study, in contrast to that of Bailey et al. (2004), supports the monophyly of the Hapalidiaceae. These conflicting results may be due to more extensive taxon sampling in the present study, but are also influenced by our choice of different outgroup taxa and the exclusion from analysis of Bailey's sequence Corallinales sp. CB-2003 (AY247408), derived from an unidentified specimen from South Australia which in the analysis of Bailey et al. (2004) was resolved on a long branch between the Sporolithaceae and the Hapalidiaceae. We chose not to use *Rhodogorgon carriebowensis* as an outgroup taxon as this sequence has a number of unusual features, making alignment difficult and suggesting that the taxon has been subject to extensive selection pressure and may not retain ancestral characters relevant to the analysis of the Corallinales.

The Hapalidiaceae was originally established by Gray, 1864 and subsequently resurrected and emended by Harvey et al. (2003) to include Corallinales taxa with tetrasporangia bearing zonately arranged spores, and with tetrasporangia/bisporangia borne in conceptacles, producing apical plugs, developing beneath multiporate plates, and not borne individually within calcified sporangial compartments. The family includes three subfamilies, Melobesioideae, Choreonematoideae and Austrolithoideae, the first two of which are represented in our analysis. The family is well-supported on anatomical grounds including LM, SEM and TEM studies (Harvey et al., 2003), although results of molecular studies have been conflicting (Harvey et al., 2003; Bailey et al., 2004). Our analyses support the Hapalidiaceae as monophyletic, with strongest support in the combined *psb*A/nSSU analysis (Fig. 4). The Hapalidiaceae taxa in our analyses are more closely related to one another than are the members of the Corallinaceae, as evidenced by the shorter branch lengths within the Hapalidiaceae clade (Figs. 1, 2 and 4), and the correspondingly poorer resolution of genera and taxa within the family, particularly under nSSU analysis. The analysis of more variable sequence data such as *psb*A from further representatives of the Hapalidiaceae is urgently needed to clarify relationships within the family.

The status of *Phymatolithon* and '*Leptophytum*' has been contentious as summarised in Harvey et al. (2003). Our nSSU analysis shows that *Phymatolithon* as currently understood is not monophyletic. Specimens identified as *Phymatolithon repandum* from New Zealand are not resolved with sequences from collections of *P. laevigatum* and *P. lenormandii* from Dorset, England, but rather form a well-supported monophyletic clade with two sequences from South African specimens attributed to '*Leptophytum' ferox* and '*Leptophytum' acervatum*. Further exploration of synapomorphic characters for these clades is warranted.

Neogoniolothon and *Metamastophora* are resolved within the Corallinaceae, but relationships to other taxa in the family are unclear. These taxa are placed on very long branches (data not shown). Long branches provide particular challenges to phylogenetic reconstruction under maximum parsimony methods but also under model-based methods when model assumptions are violated (Felsenstein, 1978; Huelsenbeck, 1997; Anderson and Swofford, 2004). In our analysis, the placements of *Neogoniolothon* and *Metamastophora* together at the base of the Corallinaceae may be due to long branch attraction and should be considered provisional.

In this analysis, as in the analysis of Bailey et al. (2004), the Mastophoroideae, even excluding *Neogoniolothon* and *Metamastophora*, are not resolved as monophyletic. We agree with Bailey et al. (2004) that whilst it is premature to make taxonomic changes, these are clearly warranted.

The taxonomic status of *Titanoderma* has been questioned since it was originally distinguished from *Lithophyllum* on the basis of the size and shape of cells comprising basal filaments (Foslie, 1909, 1990 In their study Campbell and Woelkerling (1990) found that this character could not reliably separate plants and subsumed *Titanoderma* in *Lithophyllum*. Bailey (1999) showed that the nSSU sequence of *Titanoderma pustulatum* was significantly different from that of *Lithophyllum* species and that *Titanoderma* was more closely related to *Amphiroa* than to *Lithophyllum*. This result is supported in our analysis. The status of *Titanoderma* and the characters that might distinguish it from *Lithophyllum* are beyond the scope of this study and certainly merit further attention from phycologists.

In summary, our analyses have demonstrated that the *psb*A gene is useful as a phylogenetic marker within the Corallinales, and provides considerably finer resolution than the nSSU gene. As might be predicted, combined analyses provide better resolution, providing substantial support for both deep and shallow nodes of the tree. The three families currently defined in the Corallinales are supported as monophyletic in our analyses, however resolution of genera, particularly within the Hapalidiaceae, is still unclear in some instances. This may be improved by more extensive taxon sampling, but it is likely that another marker more informative than nSSU may be required in combination with *psb*A to resolve relationships within this family. Our results challenge the utility of traditional characters for subfamily definitions, particularly within *Mesophyllum* and *Spongites*; these data are valuable in providing a framework against which to test novel taxonomic hypotheses.

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