# Characterizing the phylogenetic distribution of cryptic species in the Rhodophyta using novel gene sequence analysis and molecular morphometrics

by

Michael D.J. Lynch

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## AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

## Abstract

The Rhodophyta (red algae) are an ancient crown group of the Eukarya (ca. 1400-1500 million years), comprised of 5000 - 6000 species. Gametophytes of taxa excluding the speciose Class Florideophyceae are typically of very simple unicellular, filamentous or foliose morphologies. These simple morphologies are often homoplasious (resulting from convergent or parallel evolution) and can be indistinguishable among distinct taxa, leading to cryptic species. As a result, historical morphology-based taxonomy is often not congruent with evolutionary history.

Intraspecific genetic variation is not yet characterized for non-Florideophyceae taxa. Here the intraspecific genetic variation was characterized for a locally endemic, morphologically distinct bangiophyte red alga, *Bangia maxima* Gardner using inter simple sequence repeat (ISSR) patterns from 91 individual filaments across seven local populations. A high degree of genetic variation was observed over very small distances (< 25 cm) and very little genetic exchange was observed between populations. It is possible that *B. maxima* is a true endemic species and its population dynamics may differ from other *Bangia* species.

Metrics of sequence-based identification rely on genetic divergence among isolates to distinguish taxonomic units independent of morphology. Such metrics are especially useful for morphologically simple or cryptic species. The mitochondrial cytochrome oxidase *c* subunit 1 gene has been proposed for the Florideophyceae. An evaluation of this gene as a metric for non-Florideophyceae taxa was undertaken and limited utility was demonstrated in

most lineages of Rhodophyta due to poor or inconsistent amplification and conflicts with nuclear and plastid phylogenies.

Patterns of genetic divergence among taxa are used to infer evolutionary relationships. The nuclear ribosomal small subunit (nSSU rRNA) is the taxonomically broadest pool of gene sequence data for the Rhodophyta. The use of stochastic models of nucleotide evolution is the most common approach to inferring phylogenies using this gene, ignoring much of its evolutionary information as different characters that contribute to secondary structure (e.g. paired nucleotides) are treated independently. The incorporation of structural information leads to more biologically realistic evolutionary models increasing phylogenetic resolution. Parametric models incorporating structural information were used here to more fully resolve phylogenies for all known Rhodophyta lineages. Novel phylogenetic topologies were observed and well supported for each Class within the Rhodophyta resulting in a number of formally proposed or suggested taxonomic revisions. These include phylogenetic resolution of Rhodophyta Classes, support for the introduction of 11 genera within the Bangiales and support for various taxonomic revisions within the Florideophyceae previously proposed but not yet fully adopted.

As structure evolves more slowly than its constituent sequence, secondary structure elements can further resolve evolutionary relationships, especially in lineages as old as the Rhodophyta. A novel encoding of secondary structure elements and subsequent multivariate analysis was performed for all known Rhodophyta nSSU rRNA gene sequences, reinforcing phylogenetic results. Computer programs developed for these analyses are publicly available.

The analyses presented here significantly advanced understanding of the evolutionary distribution of cryptic species within the Rhodophyta. Furthermore, useful methods for the characterization of such species are presented, as is a demonstration of the utility of biologically realistic sequence models parameterizing nSSU rRNA structure in resolving ambiguous phylogenetic relationships. Most importantly, this work also represents a significant improvement toward taxonomy congruent with evolutionary history for the Rhodophyta.

## Acknowledgements

I would like to extend special thanks to my supervisor Dr. Kirsten M. Müller for her dedication, patience, guidance and support during this long journey. To Dr. John C. Semple, Dr. Jonathan D.S. Witt and Dr. Brian Dixon, thank you for your participation in my advisory committee. Your input at various times has been crucial to the success of this work. Furthermore, thank you to Dr. Semple for Latin translations of novel taxa (Chapter 4).

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## List of Abbreviations

| | |
|---|---|
| BPP: | Bayesian posterior probability |
| COI: | gene encoding cytochrome oxidase *c* subunit 1 |
| GTR: | General Time Reversible models of nucleotide evolution |
| ISSR: | inter-simple sequence repeats |
| ML: | Maximum likelihood |
| MLBS: | Maximum likelihood bootstrap |
| MP: | Maximum parsimony |
| MPBS: | Maximum parsimony bootstrap |
| NJ: | Neighbor-joining |
| NJBS: | Neighbor-joining bootstrap |
| nSSU rRNA: | nuclear small subunit ribosomal RNA |
| PCoA: | principal coordinates analysis |
| *rbc*L: | gene encoding the large subunit of the enzyme ribulose 1, 5-bisphosphate carboxylase/oxygenase |
| *s.l.*: | *sensu lato* (broadest sense) |
| *s.s.*: | *sensu stricto* (strictest sense) |
| UPGMA: | unweighted pair group method using arithmetic means |

## Claims of contributions to scientific knowledge

1. Little is known about the intraspecific dynamics of non-Florideophyceae Rhodophyta. Results in Chapter 2 are the first characterization of intraspecific relationships for a member of the Bangiophyceae *sensu lato* (*Bangia maxima* Gardner). This research provided the first insights into local species diversity and distribution patterns for a non-Florideophyceae Rhodophyta.

2. Due to simple morphologies the genus *Bangia* likely contains a number of cryptic species. The marine species *Bangia maxima* Gardner and *Bangia vermicularis* Harvey are two species of *Bangia* with distinct, unique morphologies relative to the vast majority of morphologically indistinguishable isolates within the genus. Results in Chapter 2 represent the first phylogenetic placement of these two species within the broader monophyletic Bangiales. Notably, these species were strongly related to only one of at least four independent, morphologically indistinguishable lineages of *Bangia*. These results clearly established morphological basis for the recognized paraphyly of *Bangia*, stimulating, in part, a taxonomic revision of the entire Bangiales order.

3. Due to the presence of cryptic species within the Bangiophyceae *sensu lato* a molecular marker suitable for discrimination of unique isolates is required. The traditional barcoding gene (mitochondrial cytochrome c oxidase subunit I), a gene showing promise in species discrimination within the more recently derived Florideophyceae, was evaluated for other lineages within the Rhodophyta. This work (Chapter 3) was the first demonstration of concerns using this gene region as the only

DNA barcoding marker for all Rhodophyta. Most significantly, the inability or differential ability to amplify and sequence the gene in many non-Florideophyceae lineages was observed. Additionally, it is unclear whether the gene can discriminate local endemic species (e.g. *Bangia vermicularis* Harvey). The resolution of disagreements between species groupings using this gene and groupings defined by other gene phylogenies (e.g. nSSU rRNA and *rbc*L) remains problematic.

4. Traditional taxonomy of the Rhodophyta, excluding Bangiophyceae *sensu stricto* and Florideophyceae, does not reflect evolutionary relationships, due primarily to simple and homoplasious morphology. By incorporating information on secondary structure of the nSSU rRNA, including length polymorphisms of variable regions as well as evolutionary models that incorporate nucleotide-pairing information, significant advances in the supra-ordinal taxonomy of this group were achieved (Chapter 4). The three primary advances are outlined below (5-7).

5. Presented in Chapter 4 is the strongest phylogenetic resolution of three monophyletic lineages containing Bangiophyceae *sensu lato* taxa (Eurhodophytina, Rhodellophytina and Cyanidiophytina), in support of recent suggested taxonomic revisions within the Rhodophyta (Saunders and Hommersand, 2004; Yoon et al., 2006a).

6. Currently, the ordinal taxonomy within the Rhodellophytina (Bangiophyceae *sensu lato* excluding the Cyanidiales and Bangiales) is unresolved and maintained as a polytomy. The results presented in Chapter 4 strongly support the resurrection of

three classes, the Porphyridiophyceae Kylin ex Skuja 1939, the Rhodellophyceae Cavalier-Smith 1998 and the Stylonematophyceae H.S. Yoon, K.M. Müller, R.G. Sheath, F.D. Ott & D. Bhattacharya 2004.

7. The Porphyridiophyceae is a taxon novel to this research (constituent orders include the Compsopogonales, Erythropeltidales, Rhodochaetales and Porphyridiales). Phylogenetic evidence and molecular morphometric signatures of nSSU rRNA secondary structure presented in Chapter 4 provide significant support for the monophyly of the class and the organization of the constituent ranks.

8. Phylogenetic results in Chapter 4 are a significant improvement over current taxonomy schemes. Taxonomic revisions consistent with these results are formally presented.

9. The phylogenetic distribution of unicellular Bangiophyceae *sensu lato* (Chapter 4), previously unresolved or poorly resolved, provides insight into the development of multicellularity within the Rhodophyta. Assuming the ancestral Rhodophyta morphology was unicellular the results herein suggests that multicellularity evolved three times independently within the Bangiophyceae *sensu lato*, once each along the branches leading to the Bangiales and the Stylonematales and once in the common ancestor of the Porphyridiales and the Compsopogonales, Rhodochaetales and Erythropeltidales. This is a more parsimonious development of multicellularity than would be suggested by other recent phylogenetic work within the group.

10. Analysis of the lengths of the variable loop regions of the nSSU rRNA structure presented in Chapter 4 uncovered a significant, systematic minimization of the nSSU within lineages of Bangiophyceae *sensu lato* taxa other than the Bangiales. This observed structural simplification was only previously characterized in prokaryote taxa correlated with streamlining of rapidly replicating genomes. Asexuality appears to be a contributing factor as the lineages with minimized structures tended to be from known or putatively asexual taxa (e.g. variable loop regions from *Bangia atropurpurea* are minimized relative to other Bangiales taxa).

11. The cryptically sexual extremophilic Cyanidiales, which should have a higher likelihood of mutational changes due to environmental conditions, had remarkably stable loop lengths within the nSSU rRNA secondary structure (Chapter 4). This stability contrasts with the high sequence variation in nSSU rRNA observed across the Cyanidiales and indicates that evolutionary pressures on the conservation of secondary structure may be acting more strongly on variable loop regions than previously recognized.

12. Observed and characterized non-canonical nucleotide pairing signatures from the nSSU rRNA secondary structure very strongly favour the taxonomy presented in Chapter 4. In future work these nucleotide signatures can be used to aid taxonomic characterization of novel Rhodophyta species. More generally, this technique could prove useful in clarifying taxonomy within any group where the age of the lineage in question obscures phylogenetic signal.

13. Chapter 5 contains phylogenetic analysis of the most taxonomically diverse sequence data set of Bangiophyceae *sensu stricto* and Florideophyceae (the subphylum Eurhodophytina) so far constructed. These analyses demonstrated that by incorporating secondary structure information of the nSSU rRNA gene phylogenetic resolution can be significantly increased relative to current single and multigene phylogenies. Results within the Eurhodophytina suggest significant taxonomic revisions outlined in 14-19 below.

14. The genus *Porphyra* is well recognized as polyphyletic relative to *Bangia* (Bangiales); however, the full extent of the relationship between these taxa has been poorly characterized. The phylogenetic analysis and broad taxonomic sampling presented in Chapter 5 identified 16 independent lineages at the genus level, 15 of which had full or nearly full phylogenetic support. In all, there were three filamentous '*Bangia*' and eight foliose '*Porphyra*' lineages that are candidates for the erection of new genera. These results contributed directly to a complete taxonomic revision of the Bangiales (Broom et al., in press).

15. Due to the taxonomic overhaul within the Bangiales, taxonomy presented or suggested in Chapter 5 is now congruent with the evolutionary conversion of the filament (likely ancestral) to foliose (derived) gametophytes of Bangiales taxa. These broad-scale phylogenetic and taxonomic results provide insight into the evolutionary history of gametophyte development as previously suggested by investigations into *Bangia maxima* (Chapter 2).

16. Significantly increased phylogenetic resolution and the broad taxonomic sampling in the Florideophyceae observed in Chapter 5 identified significant departures from previous taxonomic schemes. One of four subclasses, the Nemaliophycidae, previously characterized as monophyletic was strongly supported as two independent lineages in these analyses. Changes were suggested to correct for this, with the erection of the Corallinophycidae *sensu* L. LeGall and G.W. Saunders (2007), containing the orders Corallinales and Rhodogorgonales, and the Nemaliophycidae *sensu stricto*, containing the remaining Nemaliophycidae *sensu lato* orders. Notably, the Corallinophycidae was strongly resolved here with a single gene region (nSSU rRNA) contrasting the multigene analysis required in its original proposal.

17. Several previously uncharacterized or poorly characterized orders were resolved in analyses presented in Chapter 5. Among them, the observation of several non-monophyletic orders and families within the Florideophyceae.

18. Incongruence between phylogeny and taxonomy can sometimes be the result of sequence provenance as opposed to true non-monophyletic taxa, which is exacerbated by the use of public sequence databases such as GenBank. Strong phylogenetic resolution observed in Chapter 5 was achieved using a single orthologous gene (nSSU rRNA) demonstrated several likely cases of sequence annotation errors within the Florideophyceae. The use of such sequences can be problematic as taxonomy is increasingly reliant on data mining approaches to phylogenetics. For example, the inclusion of *Delisea hypneoides* Harvey (Accession #: EF033585) in multigene work (Verbruggen et al., 2010).

19. The novel application of models of sequence evolution incorporating RNA secondary structure information in phylogenetic analyses provided significant insight into the evolutionary relationships among Rhodophyta taxa, taking advantage of an extremely mature and taxonomically reliable data set (Chapters 4 and 5). This contrasts with recent approaches using data from multiple gene sequence regions to infer relationships. While the approach of multiple sequence sets is very useful in the characterization of difficult evolutionary relationships, generation of such data sets can be problematic and their analysis challenging. Here is proposed an alternative approach that, while much more computationally intensive than single gene analyses using stochastic models (e.g. General Time Reversible, GTR), can be performed relatively rapidly with orthology and taxonomic consistency (sequences from a single individual). Results can then accurately inform downstream multigene studies.

20. Generally the structure of molecules such as proteins and RNA evolves at a slower rate than the underlying sequence. Furthermore, some sequence data from evolutionary divergent taxa are often excluded from phylogenetic analysis due to mutational saturation and ambiguous character alignment. Analysis of structural characteristics of molecules, or molecular morphometrics, can utilize discarded information and provide additional support for derived phylogenies. Chapter 6 discusses the design and implementation of the first software tools for the evaluation of RNA morphometrics. These software tools are open source and publicly available.

21. Molecular morphometrics of the nSSU rRNA gene as presented in Chapter 6 provided some support for phylogenies inferred from phylogenetics of sequence data.

The utility of these tools was strongest for non-Eurhodophytina taxa, which occur on longer branches implying more evolutionary distance. The method is extremely fast (computationally instantaneous) and can be applied to any RNA sequence for which the structure of at least one constituent sequence is known.

22. Molecular morphometrics as implemented in Chapter 6 can also rapidly and effectively screen large sets of sequence data for unique secondary structure characteristics, manifested as large distances to the isolate with the unique character (or long branch lengths in UPGMA or Neighbor Joining trees). This approach successfully distinguished isolates currently known to have synapomorphic characters (e.g. Thoreales) and identified previously unknown potential structural characteristics useful for the definition of taxonomic lineages (e.g. *Porphyra purpurea*).

# Chapter 1
# General Introduction

The Rhodophyta (red algae) is a eukaryotic lineage comprised of 5000-6000 predominantly marine species distributed over ca. 700 genera (Guiry and Dhonncha, 2002), ranging from microscopic unicells to large (up to 1 m) multicellular organisms. These organisms represent an ancient crown group of Eukarya, occupying a distinct lineage of the eukaryotic tree of life that diverged from other eukaryotes ca. 1400-1500 million years ago (Ma) (Lim et al., 1996; Yoon et al., 2004). Furthermore, the majority of the lineages within the Rhodophyta had diverged before the putative split of the Chlorophyta (green algae) and the charophyte-embryophyte lineage ca. 1200 Ma (Yoon et al., 2004).

Species of Rhodophyta have highly divergent biochemistry, ultrastructure and morphology, with no single synapomorphy defining the entire group (Garbary and Gabrielson, 1990). There are, however, a number of characters that do not occur simultaneously within any other eukaryotic linage. These include a complete lack of a flagellated stage, basal bodies, a dual-membraned plastid with unstacked thylakoids lacking chlorophyll b and c, and floridean starch as the photosynthetic reserve (Garbary and Gabrielson, 1990; Bhattacharya and Medlin, 1995).

The Rhodophyta are primarily distributed along the coastal and near coastal regions of tropical, temperate and arctic regions (Graham and Wilcox, 2000). They are predominantly free-living organisms contributing significantly to primary production in marine ecosystems and providing structural habitats to some aquatic organisms. Additionally, some species of Rhodophyta are economically valuable as sources of carrageenan

(*Chondrus*) and food, such as Dulce (*Palmaria*) and Nori (*Porphyra*). Nori, in particular, is one of the world's most significant aquaculture crops with an annual multi-billion dollar retail market (Mumford and Miura, 1988; Merrill, 1993). While the economic development of crops such as Nori has progressed further in Asian markets, most notably Japan, there is a recent interest in expanding aquaculture programs in North America, particularly National Science Foundation initiatives in the United States (S. Brawley, personal communication).

### 1.1 Taxonomic within the Rhodophyta

The Rhodophyta is a monophyletic lineage that shares a most recent common ancestor with the Chlorophyta (Van de Peer et al., 1996; Stiller and Hall, 1997; Lewis and McCourt, 2004). The Rhodophyta also have a long independent evolutionary history and represent the first instance of multicellularity and sexuality in the fossil record (Butterfield, 2000, 2001; Butterfield et al., 1990). Due to the morphological simplicity of many species within the Rhodophyta the taxonomy has undergone considerable revision since the introduction of molecular sequence analysis. Traditionally, species were broadly separated into two groups, the classes Bangiophyceae and Florideophyceae within the phylum Rhodophyta (Chapman, 1974; Garbary et al., 1980; Garbary and Gabrielson, 1990; Saunders and Hommersand, 2004) or alternatively the Bangiophycidae and Florideophycidae subclasses within the class Rhodophyceae (Gabrielson et al., 1985).

The much more species rich and morphologically variable Florideophyceae, containing ca. 5800 species (Guiry and Dhonncha, 2002), are widely considered to be monophyletic and likely derived from an ancestral bangiophyte (Garbary and Gabrielson,

1990; Freshwater et al., 1994; Ragan et al., 1994; Saunders and Kraft, 1997; Müller et al., 2001a). The Bangiophyceae *sensu lato* (*s.l.*) is a morphologically simple, highly genetically divergent class with organisms ranging from unicells to multicellular filaments and sheets, and may retain characters present in the ancestral red algae (Garbary and Gabrielson, 1990; Gabrielson et al., 1990, 1985). In contrast to the Florideophyceae, the Bangiophyceae *s.l.* is a paraphyletic taxon, clearly recognized as such with the proliferation of molecular sequence data in phylogenetic analysis (Müller et al., 2001a; Yoon et al., 2006a; Oliveira and Bhattacharya, 2000). Such data provided novel taxonomic information, as these organisms are generally of simple morphology and lack taxonomically stable morphological characters. As a consequence, taxonomy of the Bangiophyceae *s.l.* has been an active area of research in phycology in recent years, such as the recognition of the Porphyridiales *s.l.* as a polyphyletic order corresponding to three independent lineages of unicellular red algae (Saunders and Hommersand, 2004; Yoon et al., 2006a). The definition of the major lineages of red algae using multiple gene sequence data has resulted in a moderately well supported ordinal-level taxonomy for the Rhodophyta considerably updated from traditional taxonomy (Yoon et al., 2006a). Despite these advances, the supraordinal taxonomy of these groups is poorly understood and is often considered a polytomy awaiting further taxonomic resolution (Saunders and Hommersand, 2004; Yoon et al., 2006a). Common historical and modern taxonomic proposals for the Rhodophyta are presented in Table 1-1.

**Table 1-1:** Synopsis of recent supraordinal taxonomic schemes for the Rhodophyta.

| Chapman (1974) | Garbary et al. (1980) | Garbary and Gabrielson (1990) | van den Hoek et al. (1995) | Saunders and Hommersand (2004) | Yoon et al. (2006a) |
|---|---|---|---|---|---|
| | | | | Rhodoplantae | Rhodophyta |
| Bangiophyceae | Bangiophyceae | Bangiophyceae | Bangiophyceae | Cyanidiophyta | Cyanidiophytina |
| Porphyridiales | Porphyridiales | Porphyridiales | Porphyridiales | Cyanidiophyceae | Cyanidiophyceae |
| incl. Cyanidiaceae | incl. *Cyanidium* and *Goniotrichopsis* in Phragmonemataceae | incl. *Cyanidium* and *Goniotrichopsis* in Phragmonemataceae | Cyanidiaceae not discussed | Cyanidiales | Cyanidiales |
| | | | | Rhodophyta | Rhodophytina |
| | | | | Rhodellophytina | Porphyridiophyceae |
| | | | | Rhodellophyceae | Porphyridiales |
| | | | | Porphyridiales 1 | Stylonematophyceae |
| | | | | Stylonemetales | Stylonematales |
| | | | | Porphyridiales | Rhodellophyceae |
| | | | | Metarhodellophytina | Rhodellales |
| | | | | Compsopogonophyceae | Compsopogonophyceae |
| Compsopogonales | Erythropeltidales | Compsopogonales | Compsopogonales | Compsopogonales | Compsopogonales |
| | | | Erythropeltidales | Erythropeltidales | Erythropeltidales |
| Rhodochaetales | Rhodochaetales | Rhodochaetales | Rhodochaetales | Rhodochaetales | Rhodochaetales |
| | | | | Eurhodophytina | |
| | | | | Bangiophyceae | Bangiophyceae |
| Bangiales | Bangiales | Bangiales | Bangiales | Bangiales | Bangiales |
| Florideophyceae | Florideophyceae | Florideophyceae | Florideophyceae | Florideophyceae | Florideophyceae |

## 1.2 Species definition and DNA barcoding in the Rhodophyta

In order to effectively address ecological concerns such as species diversity, population genetics and biogeography, as well as more fully resolve taxonomy, a cohesive and reproducible species concept is required for organisms in the Rhodophyta. Currently, species within the Rhodophyta are almost exclusively defined based on morphological characteristics. The Bangiophyceae *s.l.* (Table 1.1) are morphologically simple algae existing as unicells, single filaments or sheet-like gametophytes. This lack of morphological variation has resulted in a low number of named species considering the age of the lineage and contrasting with the much more species-rich Florideophyceae. Consequently, there is a high potential for cryptic species. For example, all marine isolates of *Bangia* with indistinguishable morphologies are all currently recognized as *Bangia fuscopurpurea*, despite occupying a minimum of four independent evolutionary lineages observed from gene sequence phylogenies (Müller et al., 2005; Nelson et al., 2006; Lynch et al., 2008). Furthermore, distinct endemic species of marine *Bangia* with discernable morphologies, *Bangia vermicularis* and *Bangia maxima*, both have a closer evolutionary relationship with one lineage of *Bangia fuscopurpurea* than that lineage has with any other *Bangia fuscopurpurea* lineage (Lynch et al., 2008). Further complicating the delineation of species in the Rhodophyta is that many species are either asexual or cryptically sexual.

To resolve difficulties in Rhodophyta species identification, a molecular-based tool for species discrimination is desirable. Unfortunately, research into the utility of specific

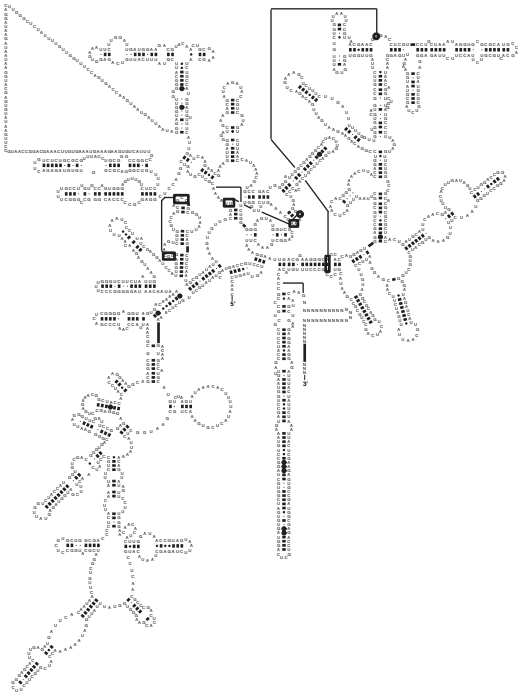
DNA sequence markers is still nascent in the Rhodophyta. Widely acknowledged proposals for the use of DNA sequence tools in species discrimination, e.g., DNA barcoding (Hebert et al., 2003), demonstrate the utility of such tools for animal phyla. The original gene region proposed for DNA barcoding (the 5' region of the cytochrome oxidase *c* subunit 1) was chosen with no *a priori* preference for its use in all taxa and only chosen for its utility within animals. While the use of the cytochrome oxidase *c* subunit 1 gene among other candidates is arbitrary, currently the only investigations into DNA barcoding for the Rhodophyta (primarily Florideophyceae) utilize this gene (Saunders, 2005; Robba et al., 2006). Investigations into the utility of DNA barcoding using existing protocols is required for the Bangiophyceae *s.l.*, considering these organisms have a higher potential for cryptic species due to very simple and often homoplasious morphologies as well as the relative paucity of investigations into species distribution and diversity within the group.

### 1.3 Taxonomic utility of the nSSU rRNA gene

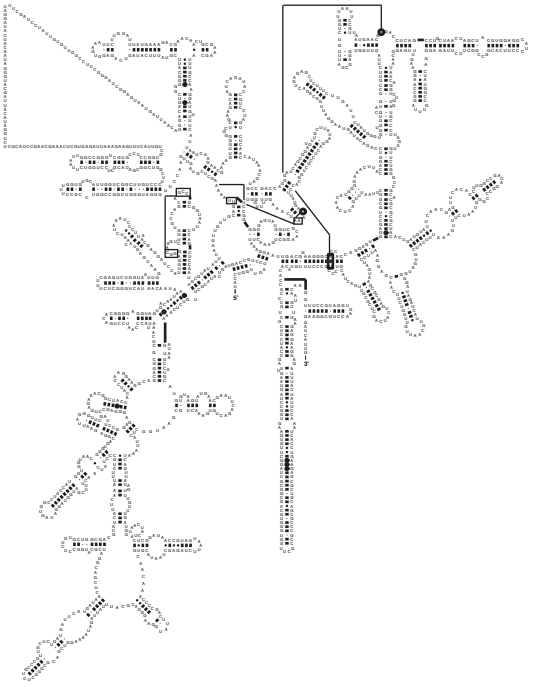
The nuclear small subunit ribosomal RNA (nSSU rRNA) gene region has a long history of use in molecular phylogenetics and systematics (Woese and Fox, 1977), and is the most widely used gene for such studies. The gene possesses several features that make it well suited to such applications. The nSSU rRNA gene is ubiquitous, occurring in all domains (Archaea, Bacteria and Eukarya), orthologous and easy to use in the laboratory environment. Additionally, and perhaps most useful in molecular systematics, the nSSU rRNA contains a secondary structure consisting of nucleotide paired regions (helices) and non-paired loop regions (Figure 1-1) that is generally highly conserved (Van de Peer et al., 1997). Consequently, the evolutionary conservation (or, alternatively, the rate of sequence

divergence) varies across a single sequence, with helical (paired) regions accumulating mutations slowly and looped (unpaired) regions more rapidly. This allows for various degrees of phylogenetic resolution and the evaluation of different ranges of evolutionary distance (e.g. phylum to genus) using a single gene.

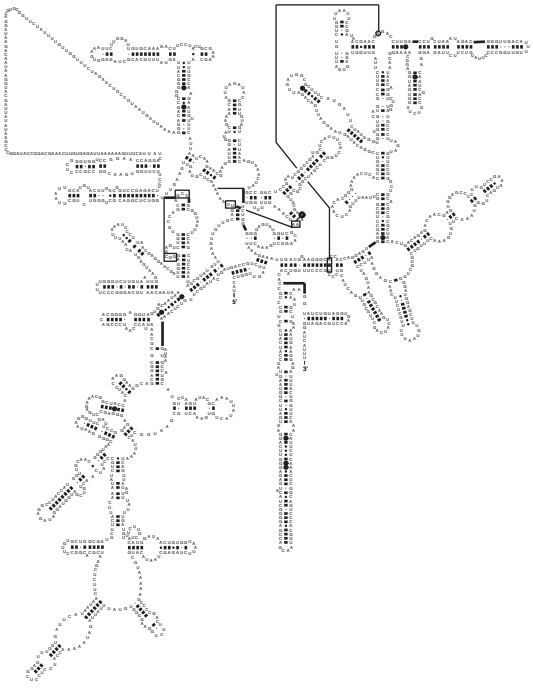
**Figure 1-1:** Structural diagrams of the nuclear small subunit ribosomal RNA representing major crown lineages of Eukarya: (A) *Bangia fuscopurpurea* (Rhodophyta), (B) *Arabidopsis thaliana* (Plantae), (C) *Ustilago maydis* (Fungi), (D) *Homo sapiens* (Metazoa), (E) *Alexandrium fundyense* (Alveolata) and (F) *Thalassiosira eccentrica* (Bacillariophyta), as well as Archaea: (G) *Haloferax volcanii* and (H) Bacteria: *Escherichia coli*. General classifications of Eukarya are consistent with recent proposals (Adl et al., 2005). GenBank accession numbers of corresponding sequence follow species names. Diagrams were inferred from patterns of compensatory mutations and were supplied by the Comparative RNA Website (Cannone et al., 2002).



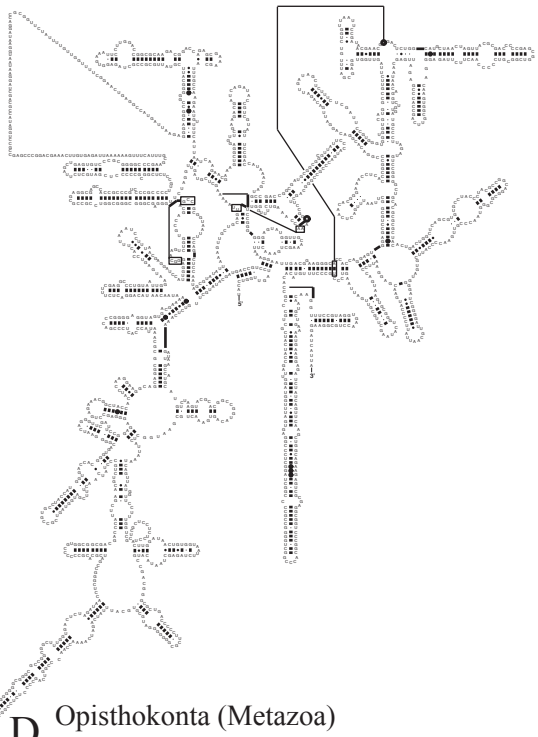
A. Archaeplastida (Rhodophyta)
*Bangia fuscopurpurea* (AF043355)



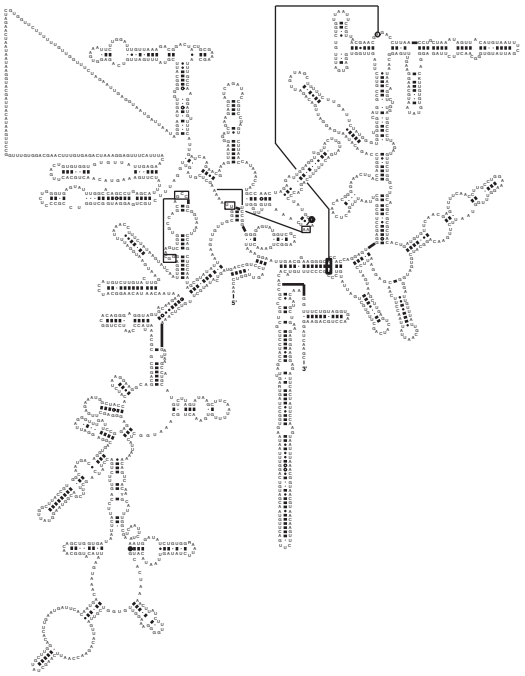
B. Archaeplastida (Plantae)
*Arabidopsis thaliana* (AC006837)



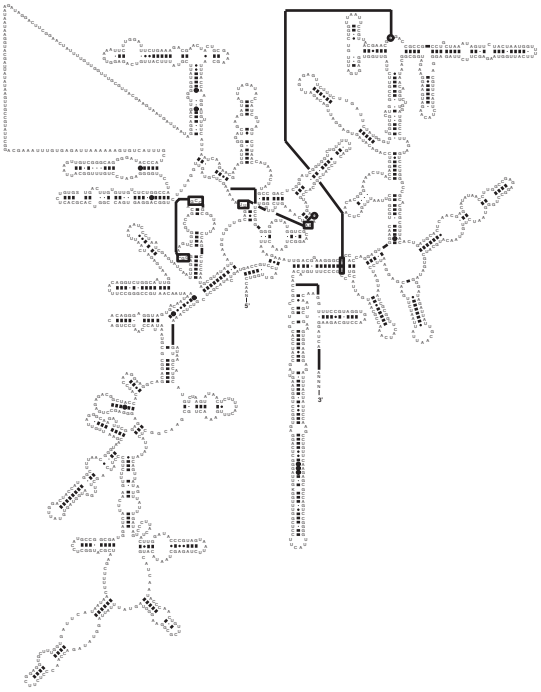
C. Opisthokonta (Fungi)
*Ustilago maydis* (X62396)



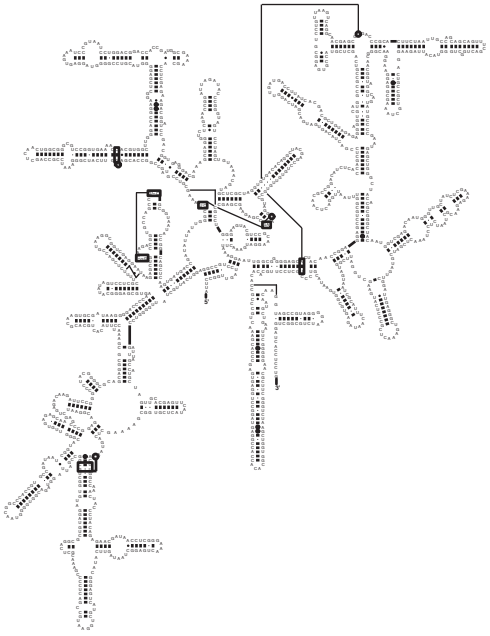
D. Opisthokonta (Metazoa)
*Homo sapiens* (K03432)



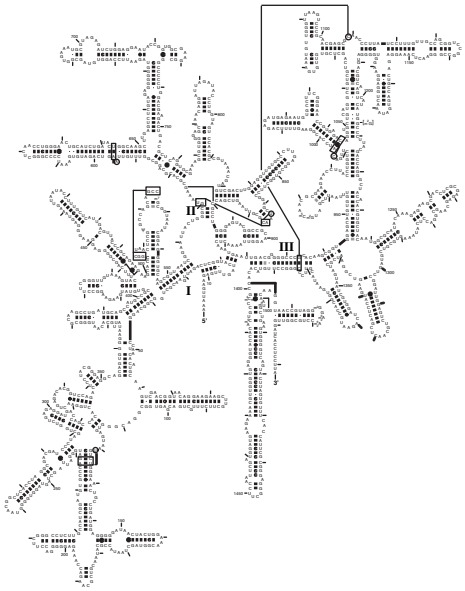
E. Chromalveolata (Alveolata)
*Alexandrium fundyense* (U09048)



F. Chromalveolata (Bacillariophyta)
*Thalassiosira eccentrica* (X85396)



G. Archaea
*Haloferax volcanii* (K00421)




H. Bacteria
*Escherichia coli* (J01695)

The secondary structure of the nSSU rRNA molecule can be accurately predicted using comparative sequence analysis, which identifies compensatory nucleotide mutations across a multiple sequence alignment thereby indicating interacting nucleotides. This allows the identification of conserved structural elements despite variations in underlying nucleotides (Fox and Woese, 1975; Gutell et al., 1985; Woese and Pace, 1993; Cannone et al., 2002). These inference-based models of nSSU rRNA secondary structure correctly identified approximately 97% of base pairs when compared to high-resolution crystal structures of the ribosomal subunits (Ban et al., 2000; Wimberly et al., 2000), indicating a high confidence in predicted homologous sites in sequences aligned using this approach.

The alignment of sequence data is difficult and is the least automated component of phylogenetic sequence analysis. Sequences demonstrating high sequence identity, e.g., closely related sequences, are readily aligned with a high degree of confidence. Unfortunately, as sequence divergence increases so does the complexity of sequence alignment leading to the potential alignment of homoplasious characters, which corrupts downstream phylogenetic sequence analysis. The nSSU rRNA has a predictable and conserved secondary structure across large evolutionary time spans. This conservation of structure enables the delineation in the sequence of interacting nucleotides, which contribute to secondary structure. These sites can then be used as anchor points in sequence alignment, improving overall confidence in the alignment and improving downstream phylogenetic analysis of the gene sequence data. The inclusion of secondary structure information into the alignment of nSSU rRNA sequences has increased the accuracy and efficiency of these alignments and alignment algorithms over sequence identity methods. Additionally,

structural characteristics such as the distribution of interacting nucleotides and the delineation of structural components is widely available due to the continued development of research infrastructure for the analysis of nSSU rRNA gene sequence data (e.g. Ribosomal Database Project (Cole et al., 2009), SILVA (Pruesse et al., 2007) and the Comparative RNA Website (Cannone et al., 2002)).

### 1.3.1 The influence of structural models on phylogenetic analysis of nSSU rRNA sequence data

Nucleotides tend to evolve non-randomly in gene sequences. For example, some positions can accumulate mutations more rapidly than others due to relaxed selective constraints (e.g. a 'wobble' in the third base-pair position of a codon due to the redundant genetic code). Additionally, back mutations, or mutations that return a previously mutated nucleotide back to the ancestral state, can obscure evolutionary distance among sequences. Due to such factors, the evolutionary distance between two sequences cannot be measured accurately by summing the nucleotide differences between them (*p*-distance). Alternatively, models of sequence evolution are used to more accurately infer evolutionary relationships among molecular sequences. While such models exist for both protein and nucleotide sequences, only the latter class of models will be discussed here. Nucleotide models attempt to parameterize the mutational behaviour of nucleotide sequences. For example, an early formulation by Jukes and Cantor (Jukes and Cantor, 1969) assumed equal transition/transversion rates as well as equal equilibrium frequencies for all bases.

Evolutionary models commonly applied to nucleotide sequence data assume that each site evolves independently from every other site. This is a simplification of the process of

sequence evolution, especially of RNA genes as nucleotide sites interact in order to maintain structural elements. While this simplification is generally acknowledged, sequences are still routinely evaluated using these inappropriate models, primarily due to computational limitations. For example, the most computationally intensive widely used stochastic model of sequence evolution is the Generalized Time Reversible (GTR) model (Tavaré, 1986), which has six substitution rate parameters, as well as four equilibrium base frequency parameters (often reduced to two or three). Each of these eight to nine free parameters needs to be evaluated for each nucleotide position in an alignment. Corresponding RNA structural models have many more parameters. For example, the 7A model (Higgs, 2000) contains 26 free parameters, which includes seven frequency parameters (the six nucleotide pairs and a single mismatch pair) and 21 rate parameters that correspond to mutations from one nucleotide pair to another. Consequently, phylogenetic analyses with secondary structure models are considerably more computationally expensive and only feasible for limited sets of sequences using common implementations of the Maximum Likelihood algorithm for phylogenetic analysis (e.g. PAUP* v.4.0 (Swofford, 2003)). Recent advances in Maximum Likelihood-based phylogenetic algorithms, such as RAxML (Stamatakis, 2006) and MrBayes (Huelsenbeck and Ronquist, 2001), allow for more biologically realistic analysis of large data sets of nSSU rRNA gene sequences using reasonable computational resources.

In addition to incorporating secondary structure into sequence analyses, the structural elements themselves can be evaluated in a phylogenetic context. The secondary structure of the eukaryotic nSSU rRNA is highly conserved over broad evolutionary scales (Ali et al., 1999; Van de Peer et al., 1997; Wuyts et al., 2000, 2004; Cannone et al., 2002). Furthermore,

the variations in structure of the nSSU rRNA molecule among sequences have been demonstrated to be phylogenetically informative (Caetano-Anollés, 2001, 2002a, b). As a result, structural deviations from the consensus can be used as taxonomically informative molecular signatures. In past studies the presence of specific secondary structure signatures supported the erection of a red algal class, the Thoreales (Müller et al., 2002); however, a broader application of this approach has not been undertaken within the Rhodophyta. The conservation of such structures, including nucleotide composition and relative size, could provide additional characters or signatures indicative of currently unresolved or poorly resolved taxonomic groups.

### 1.4 Objectives

Taxonomy is the framework for a diverse range of biological investigations, such as population genetics, ecology and the study of infectious disease. Despite a large amount of taxonomic research effort into the Rhodophyta there exists considerable ambiguity in the definition and identification of species, resulting cryptic species and widespread errors in taxonomy. The delineation of species within the Rhodophyta is similarly problematic as there is limited data for biogeographic distributions and inter and intra-specific genetic diversity, especially for the Bangiophyceae *s.l.*. To address taxonomic problems in the Rhodophyta, the objectives of the research outlined in this thesis focus on:

1. The intraspecific genetic variation and population dynamics for the non-Florideophyceae species of Rhodophyta is currently poorly understood. These characteristics will be explored for a highly endemic species, *Bangia maxima*

N.L. Gardner, providing insights in the sexuality, genetic variation and population structure of species within the Bangiales.

2. Current metrics of species discrimination (e.g. DNA barcoding) will be evaluated over a broad taxonomic range within the Bangiophyceae *s.l.*. In addition, the potential for alternative gene regions to supplement current discrimination protocols will be addressed. The prevalence of previously observed cryptic species within the Rhodophyta will be quantified. Biogeographic patterns present in the species distribution of Bangiales isolates will be discussed.

3. In general, Rhodophyta taxonomy is not consistent with phylogeny. The novel large-scale evaluation of existing nSSU rRNA gene sequence data can provide insights into phylogenetic relationships within the Rhodophyta that are currently not resolved. A phylogenetically robust ordinal and supraordinal taxonomy will be proposed supported by a combination of biologically realistic phylogenetic inference and novel molecular morphometrics of nSSU rRNA gene sequence data.

    a. Biologically realistic models of sequence evolution utilizing structural characteristics of RNA molecules will be applied to a taxonomically diverse set of nSSU rRNA gene sequences from the Rhodophyta.

    b. A novel tool for the computational abstraction of the secondary structure of nSSU rRNA molecules will be developed. This tool will be used to identify structural signatures defining taxonomic lineages within the

Rhodophyta. These molecular morphometrics will subsequently be evaluated for congruence with derived phylogenies where species lack known morphological synapomorphies, providing a useful tool for delineating poorly resolved taxonomic classifications.

# Chapter 2

# Phylogenetic position and ISSR-estimated intraspecific variation of *Bangia maxima* (Bangiales, Rhodophyta)

**Relevant publication:** Lynch, M.D.J., R.G. Sheath, and K.M. Müller. 2008. Phylogenetic position and ISSR-estimated intraspecific genetic variation of *Bangia maxima* (Bangiales, Rhodophyta). *Phycologia*. **47**: 599-613.

## 2.1 OVERVIEW

The red alga *Bangia maxima*, in addition to its large size (up to 35 cm long x 6 mm in diameter), was found in this study to be distinguishable from other species of *Bangia* by the character of mature filament apices containing elongate, separated vegetative cells. The phylogenetic position of *B. maxima* was resolved using both the *rbc*L and nuclear SSU rRNA (nSSU rRNA) gene sequences, and the genetic variation within a population was studied using an inter-simple sequence repeat (ISSR) PCR-based DNA fingerprint analysis. In phylogenetic analyses of the plastid *rbc*L gene region and nSSU rRNA gene region, *B. maxima* grouped with local populations of concurrently collected *B. vermicularis* and *B. fuscopurpurea* as well as other collections of *B. fuscopurpurea* from California in a clade of predominantly eastern Pacific isolates. Banding patterns from 13 male filaments from seven littoral boulders for five ISSR primers were used to develop both band presence/absence and distance matrices (using the Dice coefficient). Banding patterns of *B. maxima* isolates were highly polymorphic among different boulders but consistent among individuals from the same boulder, as demonstrated by multivariate analyses (UPGMA, principal coordinates analysis). UPGMA analysis also indicated a limited genetic transfer among boulders. These

results help in clarifying the population genetics of *B. maxima* and further understanding of genetic diversity within the Bangiales.

## 2.2 INTRODUCTION

The order Bangiales (Rhodophyta) is a monophyletic group containing the non-monophyletic genera *Bangia* Lyngbye and *Porphyra* C. Agardh (Müller et al., 1998, 2001a, 2003, 2005; Broom et al., 1999, 2004; Nelson et al., 2005, 2006) as well as several newly described genera, *Pseudobangia* K.M. Müller & Sheath (Müller et al., 2005), *Dione* W.A. Nelson and *Minerva* W.A. Nelson (Nelson et al., 2005). The order is characterized by having a heteromorphic life history with an alternating macroscopic gametophyte and microscopic sporophyte (conchocelis stage). The gametophyte of *Porphyra* is a sheet-like thallus 1-2 cells thick and is distributed in marine intertidal and upper subtidal areas throughout temperate regions (Lindstrom and Cole, 1992). In contrast, *Bangia* is a distally multiseriate filament (Sheath and Cole, 1984) and is ubiquitously distributed in marine intertidal areas as well as in some freshwater habitats, such as the Laurentian Great Lakes in North America and scattered rivers and lakes in Europe and Asia (Müller et al., 2003). Recently, the monotypic genus *Bangiadulcis* was proposed to encompass freshwater *Bangia*, previously known as *B. atropurpurea* (Nelson, 2007). Although *B. atropurpurea* has unique small chromosomes (Müller et al., 2003), the proposal of the genus *Bangiadulcis* was premature, as there are no other distinguishing features of freshwater *Bangia* species and the taxonomic classification of the Bangiales is currently ambiguous. The authors proposing the *Bangiadulcis* generic name arrived at similar conclusions and the taxonomic revision was withdrawn (Silva and Nelson, 2008). Consequently, freshwater *Bangia* species should be maintained as *Bangia*

*atropurpurea* and distinct from the marine *Bangia fuscopurpurea* (Müller et al., 2003). The other three genera are filamentous, *Dione* is marine with wide filaments (up to 150-211 μm) (Nelson et al., 2005), *Pseudobangia* is marine with several chloroplasts per cell (Müller et al., 2005) and *Minerva* is marine but has no distinguishing features except slightly smaller cell diameters (Nelson et al., 2005).

Traditionally, *Bangia* species have been largely delineated on the basis of morphological characters (e.g. pigmentation, filament diameter and length); however, numerous molecular studies (Müller et al., 1998, 2001, b, 2003, 2005; Broom et al., 2004) have demonstrated cryptic diversity within this genus. Additionally, *Bangia* can acclimate to a wide range of salinities as noted by Den Hartog (Den Hartog, 1972) and Geesink (Geesink, 1973), and it has been proposed that freshwater *B. atropurpurea* (Roth) C. Agardh and marine *B. fuscopurpurea* (Dillwyn) Lyngbye be synonymized. This synonomy, however, has been refuted based on molecular data (Müller et al., 1998, 2003) as well as differences in chromosome morphology (Müller et al., 2003).

The extensive diversity of marine filamentous members of the Bangiales certainly requires further study. For example, in a study of the nuclear small subunit ribosomal RNA (nSSU rRNA) gene sequence from New Zealand marine *Bangia* isolates were up to 7.1 % different over approximately 1750 nucleotides (Broom et al., 2004), consistent with genetic variation in other studies of the Bangiales (Müller et al., 1998, 2001b, 2003, 2005). Despite the considerable genetic variation within the marine Bangiales, few studies have been undertaken to assess variability at the population level. Typically, intraspecific variation in a local population is too low to analyze with sequence variation (e.g. nSSU rDNA or ITS

regions). Hence, other more highly variable markers, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) or microsatellites must be used. Both PCR-RFLP and RAPD have been used to study *Porphyra* isolates (Niwa et al., 2005; Weng et al., 2005; Park et al., 2007). Additionally, PCR-RFLP techniques using the RUBISCO spacer and nuclear ribosomal internal transcribed spacer regions have been applied to distinguish cultivars of *P. tenera* Kjellman and *P. yezoensis* Ueda as well as natural populations of *P. tenera* (Niwa et al., 2005). Low intraspecific variation was also demonstrated for *P. yezoensis* forma *narawaensis* A. Miura using AFLP (Niwa et al., 2004). To date there have not been any studies involving populations of *Bangia* using these highly variable molecular markers.

Although most molecular markers are useful for studying population-level genetic structure, each has certain limits. Microsatellites can be expensive and require knowledge of the target genome and microsatellite primers have not been developed for the genus *Bangia*. In addition, both the AFLP and PCR-RFLP techniques are often not sensitive enough and some authors have questioned the reproducibility of RAPD results (Perez et al., 1998). Inter simple sequence repeats (ISSRs) are an alternative low-cost molecular marker that exploit the presence of microsatellites abundant throughout the genome by PCR amplifying the genomic region between like microsatellites. ISSRs have been shown to be abundant, highly reproducible and polymorphic, therefore potentially informative (Gupta et al., 1994; Zietkiewicz et al., 1994; Bornet and Branchard, 2001; Bornet et al., 2004). They are widely used in studies of population genetics and have been successfully used to analyze phytoplankton populations (Bornet et al., 2004). However, only a few studies have applied

this technique to the Rhodophyta (Vis, 1999; Hall and Vis, 2000, 2002). Studies of the freshwater genus *Batrachospermum* demonstrated unique banding patterns for each gametophytic thallus studied from a single stream (Vis, 1999) and streams at different locations showed significant genetic divergence (Hall and Vis, 2002).

The use of dominant molecular markers, such as RAPDs and ISSRs, makes the application of common genetic statistics (e.g. $H_e$, $F_{st}$, $G_{st}$) difficult as both heterozygosity and homozygosity are scored equally. However, the study of the haploid gametophyte of *Bangia* circumvents this problem. In addition, since banding patterns are so variable in ISSR studies, even within a population, $F_{st}$ and $G_{st}$ values can tend towards one, artificially inflating results. Moreover, high mutation rates possible for some microsatellites (see Ellegren, 2004 for review) can increase band polymorphism in a population. This can be exaggerated further by ISSR amplification because two microsatellite regions contribute to band presence. This situation potentially makes interpretation of $F_{st}$ (and analogous) values difficult and conclusions suspect. Consequently, such analyses are avoided here.

*Bangia maxima* N.L. Gardner is an extremely localized species, which is only known from littoral boulders in Bolinas Bay, California, USA (Gardner, 1927). This species is the only readily distinguishable morphotype of *Bangia* due to its large size (up to 35 cm long x 6 mm in diameter) and multiseriate ribbon-like growth form. Type localities for *B. maxima* and another *Bangia* species, *B. vermicularis* Harvey, are geographically close; however, *B. maxima* is much larger and less contorted than *B. vermicularis* (Gardner, 1927). According to Harvey (Harvey, 1853), *B. vermicularis* filaments are approximately 5 cm in length, undulating, with a slender linear-clavate form and a variable number of cuniform cells

radiating from a central cavity. Despite the greater structural complexity, Harvey (1853) noted a morphological similarity to *B. fuscopurpurea*. The relationship of *B. vermicularis* and *B. maxima* with other marine *Bangia* collections is worth exploring in terms of potential phylogenetic associations, to further clarify taxonomic relationships. *Bangia vermicularis* has not been analyzed for its phylogenetic position within the Bangiales and although *B. maxima* appears to group with other marine *Bangia* species (Yoon et al., 2006a), little could be concluded about the biogeographic groupings of these species due to limited taxon sampling.

This study addresses the phylogenetic position of *Bangia maxima* as well as other *Bangia* samples collected from California, including *B. vermicularis* and *B. fuscopurpurea*. In addition, ISSR analyses will examine the genetic variation within a population of the red alga *B. maxima* located on seven separate littoral boulders in Bolinas Bay, California collected in April 2004. Characterization of the magnitude and patterns of genetic diversity within this species will address patterns of population interbreeding and mixing as well as increase understanding of genetic variation for endemic species within the Bangiales.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Sample collection and microscopy

*Bangia maxima* filaments were collected from seven intertidal boulders in Bolinas Bay, Marin County, California in April 2004 (37° 54’ N 122° 41’ W). Boulders at the site are closely positioned into two main groups separated by approximately 1.5 m, with the largest boulder being centrally located (Figures 2-1, 2-6). An additional isolate of immature *B. maxima* was collected from a shoreline location approximately 0.5 km from the intertidal boulders. Filaments were observed microscopically using the Olympus BX41 light

microscope (Olympus America Inc., San Diego, CA) and images were photographed with the Olympus MicroFire camera system. Measurements were made with Rincon image analysis software (Imaging Planet, Goleta, CA). Voucher specimens are available from K. M. Müller upon request. Other collections from California included in the present study are listed in Table 2-1.

**Figures 2-1 to 2-5**. Habitat and vegetative morphology of *Bangia maxima*, *B. fuscopurpurea* and *B. vermicularis isolates* used in this study. **2-1.** Boulders with *Bangia maxima* population at Bolinas Bay, California, USA. Arrow points to large boulder shown in Fig. 2-2. **2-2.** Large filaments of *B. maxima* on a boulder in Bolinas Bay, California as indicated in Fig. 2-1, scale bar = 20 cm. **2-3.** Light micrograph of vegetative portion of a filament of *B. maxima* showing non-abutting, elongate cells in the periphery of a mature thallus, scale bar = 175 μm. **2-4.** Filaments of *B. vermicularis* on one boulder in San Francisco Bay, California, scale bar = 2.5 cm. **2-5.** Light micrograph of *B. fuscopurpurea* from Solana Beach, California, consisting of densely packed, quadrate cells, scale bar = 70 μm.

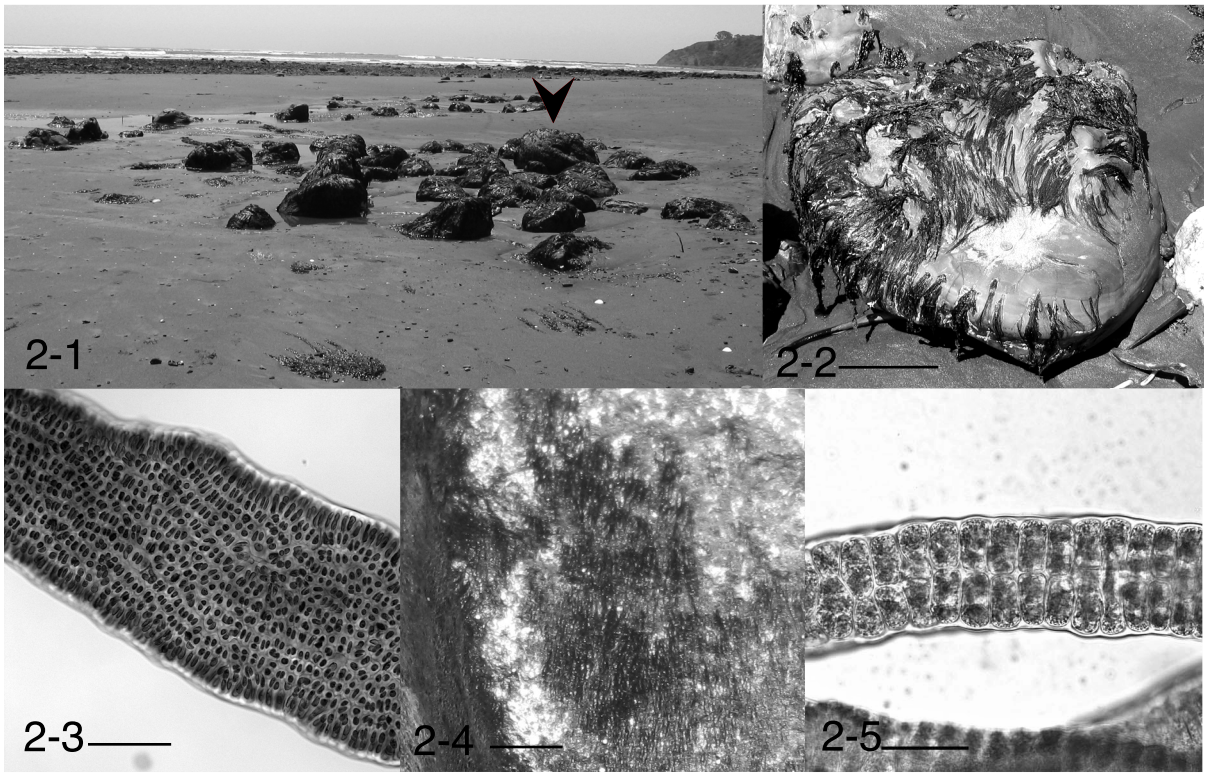

2-1
2-2
2-3
2-4
2-5

**Figure 2-6.** Schematic diagram of the seven boulders at Bolinas Bay, California on which *B. maxima* was observed, each approximately the same height and completely submerged at high tide. The diagram includes the isolated population of *Bangia fuscopurpurea* (Bolinas Bay) observed higher up on the shoreline but still completely submerged at high tide. Distances between each of the boulders are given in metres and the angles to which the boulders were located from each other are approximate.

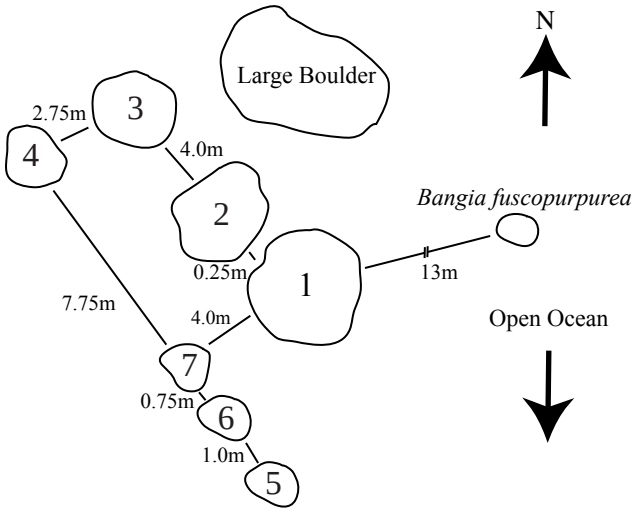

N
Large Boulder
3
2.75m
4
4.0m
2
Bangia fuscopurpurea
0.25m
1
13m
7.75m
4.0m
Open Ocean
7
0.75m
6
1.0m
5

**Table 2-1. *Bangia* isolates from this study collected at marine locations in California by K.M. Müller and R.G. Sheath. N/A: information not available. Type localities are indicated by *.**

| Species | Location | Collection Date | Latitude, Longitude |
|---|---|---|---|
| *Bangia fuscopurpurea* | Bolinas Bay, California<br>- on boulder near *B. maxima* | 27 April 2004 | 37° 54' N, 122° 41' W |
| *Bangia fuscopurpurea* | Solana Beach, California | 28 March 2007 | 32° 59' N, 117° 16' W |
| *Bangia maxima* | * Bolinas Bay, California | 27 April 2004 | 37° 54' N, 122° 41' W |
| *Bangia maxima*<br>(immature) | * Bolinas Bay, California<br>- shore, ~ 0.5 km from *B. maxima* | 27 April 2004 | N/A |
| *Bangia vermicularis* | * San Francisco Bay, California | 28 April 2004 | N/A |

### 2.3.2 DNA extraction and amplification

Genomic DNA of *B. maxima* was extracted from 13 male filaments from each of seven boulders (91 in total), as well as several populations of Californian marine *Bangia* (Table 2-1) using a phenol/chloroform protocol (Saunders, 1993). The genes encoding for the nuclear small subunit ribosomal RNA (nSSU rDNA) and the large subunit of RUBISCO (*rbc*L) were amplified for use in phylogenetic analyses. PCR reactions were performed in a 50 µL volume with 1X PCR buffer, 2.75 mM $MgCl_2$, 0.2 mM of each dNTP, one unit of Fisherbrand™ Taq polymerase (Fisher Scientific Canada Co., Ottawa, ON, Canada) and 0.4 µM of each primer (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Amplifications were performed on an Eppendorf Mastercycler gradient thermocycler (Eppendorf Canada Ltd., Mississauga, ON, Canada) with 35 cycles of 93°C denaturation for 1 min, 51° C annealing for 1 min and 72° C extension for 2 mins, with a 2 min pre-denaturation step at 95° C and a final extension at 72° C for 7 mins. For improved amplification, the SSU rDNA reactions were performed in two segments, 400 nt fragment at the 5' end using the primer pair G01.1 and G10.1 and the remainder using G02.1 and G15.1 (Müller et al., 1998). Due to intron presence, G02.1 – G15.1 amplicons were also sequenced with the universal NS4 reverse primer (White et al., 1990). Amplification of the *rbc*L gene region was performed using Comp1 forward and Comp2 reverse primers (Rintoul et al., 1999). Amplicons were purified using the QiaQuick PCR purification system (Qiagen Inc., Mississauga, ON, Canada) and sequenced with the ABI 3130XL capillary sequencer (Applied Biosystems Canada, Streetsville, ON, Canada).

For the ISSR amplification, male filaments were exclusively used to ensure genetic contribution of only one individual as many of the female plants were fertilized. Thirteen *B. maxima* filaments from seven boulders were subjected to ISSR-PCR amplification using each of five ISSR primers (Table 2-2) performed in a 25 μL volume with 1X PCR buffer, 2 mM $MgCl_2$, 0.3 mM of each dNTP, 1.5 units of Fisherbrand™ Taq polymerase (Fisher Scientific Canada Co., Ottawa, ON, Canada) and 4 μM of primer (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). ISSR reactions were performed using an Eppendorf MasterCycler gradient thermocycler (Eppendorf Canada Ltd., Mississauga, ON, Canada) with 35 cycles of 94° C denaturation for 30 s, 44° C annealing for 45 s and 72° C extension for 1 min 30 s with an initial denaturation step for 2 min at 94° C and a final extension for 10 min at 72° C. PCR products were visualized on an ethidium bromide 0.5 μg $mL^{-1}$ stained 2% agarose gels in 1xTAE and photographed with a digital camera. Band molecular weights were calculated using the GeneTools image analysis software (Syngene, Frederick, MD) and the O'RangeRuler 100bp ladder (Cat. #SM0623, Fermentas Canada Inc., Burlington, ON, Canada) was used as a molecular weight standard. For each primer, duplicate reactions were performed for each unique banding pattern and only reproducible bands were scored. No weight was given for band intensity. Bands migrating to the same position in the gel, inspected visually and cross-referenced with molecular weights, were considered homologous, which was sufficient for classifying the majority of bands. Bands that did not have obvious homologues were binned based on their calculated molecular weights using the protocol of Hong and Chuah (Hong and Chuah, 2003). A presence/absence matrix was constructed from the pooled banding patterns of all five ISSR primers.

**Table 2-2. Primers (Wolfe *et al.*, 1998) and number of amplified bands used to amplify inter simple sequence repeat molecular markers for seven populations of *Bangia maxima* from boulders in Bolinas Bay, CA, USA.**

| Primer Name | Sequence | No. of amplified bands |
|---|---|---|
| ISSR8 | $(GA)_6GG$ | 4 |
| ISSR10 | $(GA)_6CC$ | 38 |
| ISSR12 | $(CAC)_3GC$ | 34 |
| ISSR13 | $(GAG)_3GC$ | 34 |
| ISSR15 | $(GTG)_3GC$ | 33 |

### 2.3.3 Sequence and ISSR analyses

Sequences of the nSSU rRNA and *rbc*L genes were each aligned with sequences acquired from GenBank, including all unique *Bangia* (based on a 99 % sequence identity threshold) and multiple *Porphyra* sequences known to intercalate with *Bangia* clades (Müller et al., 2001a, 2005), using MUSCLE v.3.6 (Edgar, 2004). Non-Eurhodophytina taxa were selected and used as outgroups. Alignments were then manually inspected using JalView v.2.2.1 (Waterhouse et al., 2009). Taxon, genus and species designations were kept consistent with GenBank flatfiles unless revisions to the nomenclature of the species have been published. Further information on sequences used in this study is available in Table 2-3. The nucleotide model of evolution for each alignment was determined using ModelTest v.3.7 (Posada and Crandall, 1998). Maximum Likelihood (ML) analysis was performed with 10 replicates of a heuristic search using random addition of sequences. To provide support for tree topologies 1000 Maximum Parsimony (MP) and Neighbor-Joining (NJ) bootstrap replicates were performed using PAUP* v4b10 (Swofford, 2003). Bayesian posterior probability support for tree nodes was also calculated using MrBayes v.3.1 (Huelsenbeck and Ronquist, 2001) with default prior parameters. Two parallel analyses of four simultaneous chains of which three were heated (Metropolis-coupled Markov chain Monte Carlo) were run until convergence below a standard deviation of 0.05 between the two runs was reached. Trees before that point were discarded as burnin. All trees were visualized using TreeView v.1.6.6 (Page, 1996).

**Table 2-3. GenBank accession numbers and species identifiers/voucher numbers for bangiophyte sequences used in phylogenetic analyses.**

| Collection | Reference | GenBank Accession | |
|---|---|---|---|
| | | SSU | *rbc*L |
| *Bangia atropurpurea* | Hanyuda *et al.,* (2004) | AB114638 | - |
| *B. atropurpurea* (AT17=Austria) | Müller *et al.,* (1998), Müller *et al.,* (2003) | AF169339 | AF169327 |
| *B. atropurpurea* (AT22=Austria) | Müller *et al.,* (2003) | - | AF169333 |
| *B. atropurpurea* (IR=Ireland) | Müller *et al.,* (1998) | AF043365 | AF043371 |
| *B. atropurpurea* (IT=Italy) | Müller *et al.,* (1998) | AF043365 | AF043370 |
| *B. atropurpurea* (BI12=British Isles) | Müller *et al.,* (1998) | AF043365 | AF043373 |
| *B. atropurpurea* (GL=Great Lakes) | Müller *et al.,* (1998) | AF043365 | AF043370 |
| *B. atropurpurea* (NL=Netherlands) | Müller *et al.,* (2003) | - | AF169330 |
| *Bangia atropurpurea** | Shimomura *et al.,* unpublished | D88387 | - |
| *Bangia fuscopurpurea* (SAGB 59.81) | Schlösser (1994), Müller *et al.,* (2001b) | AF342745 | AY119771 |
| *B. fuscopurpurea* (SWE=Sweden) | Müller *et al.,* unpublished | AF175528 | - |
| *B. fuscopurpurea* (NJ=New Jersey) | Müller *et al.,* unpublished | AF169335 | AF169328 |
| *B. fuscopurpurea* (WA=Washington) | Müller *et al.,* (2003), Müller *et al.,* unpub. | AF169336 | AF169329 |
| *B. fuscopurpurea* (BC1=British Columbia) | Müller *et al.,* (1998) | AF043360 | AF043372 |
| *B. fuscopurpurea* (TX=Texas) | Müller *et al.,* (1998) | AF043361 | AF043377 |
| *B. fuscopurpurea* (NF=Newfoundland) | Müller *et al.,* (1998) | AF043357 | - |
| *B. fuscopurpurea* (NS=Nova Scotia) | Müller *et al.,* (2003) | AF169337 | AF169331 |
| *B. fuscopurpurea* (MA=Massachusetts) | Müller *et al.,* (1998) | AF043362 | AF043369 |
| *B. fuscopurpurea* (Greece) | Müller *et al.,* unpublished | AF175533 | - |
| *B. fuscopurpurea* (Mexico) | Müller *et al.,* unpublished | AF169334 | - |
| *B. fuscopurpurea* (Nice, France) | Müller *et al.,* unpublished | AF175535 | - |
| *B. fuscopurpurea* (NC=North Carolina) | Müller *et al.,* (1998) | AF043363 | AF043368 |
| *B. fuscopurpurea* (AUS=Australia) | Müller *et al.,* unpublished | AF175531 | - |
| *B. fuscopurpurea* (Helgoland) | Müller *et al.,* unpublished | AF175532 | - |
| *B. fuscopurpurea* (AK=Alaska) | Müller *et al.,* (1998) | AF043355 | AF043366 |
| *B. fuscopurpurea* (NH=New Hampshire) | Müller *et al.,* (1998) | AF043353 | AF043366 |
| *B. fuscopurpurea* (RI=Rhode Island) | Müller *et al.,* (1998) | AF043354 | AF043378 |
| *B. fuscopurpurea* (GLD=Greenland) | Müller *et al.,* (1998) | AF043355 | AF043366 |
| *B. fuscopurpurea* (NFF=Ferryland, Nfld) | Müller *et al.,* (1998), Müller *et al.,* unpub. | AF169338 | AF169332 |
| *B. fuscopurpurea* (NWT=Northwest Territories) | Müller *et al.,* (1998) | AF043355 | AF043366 |
| *B. fuscopurpurea* (ANT=Antarctica) | Müller *et al.,* unpublished | AF175530 | - |
| *B. fuscopurpurea* (Norway) | Müller *et al.,* unpublished | AF175536 | - |
| *B. fuscopurpurea* (BC2=British Columbia) | Müller *et al.,* (1998) | AF043359 | AF043376 |
| *B. fuscopurpurea* (CA=California) | Müller *et al.,* (1998) | AF043356 | AF043374 |
| *B. fuscopurpurea* (Taiwan) | Müller *et al.,* unpublished | AF175529 | AF168654 |
| *B. fuscopurpurea* (Ireland) | Müller *et al.,* (1998) | AF175534 | - |
| *B. fuscopurpurea* (Baltic) | Müller *et al.,* unpublished | - | AF168655 |

| | | | |
|---|---|---|---|
| *B. fuscopurpurea* (OR=Oregon) | Müller *et al.,* (1998) | AF043358 | AF043367 |
| *Bangia* sp. (Alaska) | Lindstrom & Fredericq (2003) | - | AF452422 |
| *B.* sp. (BRM NZ) | Broom *et al.,* (2004) | AY184346 | - |
| *B.* sp. (BFK NZ) | Broom *et al.,* (2004) | AY184338 | - |
| *B.* sp. (BNS NZ) | Broom *et al.,* (2004) | AY184345 | - |
| *B.* sp. (BCP NZ) | Broom *et al.,* (2004) | AY184336 | - |
| *B.* sp. (BDS NZ) | Broom *et al.,* (2004) | AY184337 | - |
| *B.* sp. (BWP NZ) | Broom *et al.,* (2004) | AY184348 | - |
| *B.* sp. (BCH NZ) | Broom *et al.,* (2004) | AY184335 | - |
| *B.* sp. (BMW NZ) | Broom *et al.,* (2004) | AY184344 | - |
| *B.* sp. (BGA NZ) | Broom *et al.,* (2004) | AY184341 | - |
| *Bangia gloiopeltidicola* | Niwa *et al.,* unpublished | AB053490 | - |
| *Bangiopsis subsimplex* | Müller *et al.,* (2001b) | AF168627 | - |
| *Bangiopsis subsimplex* | Yoon *et al.,* (2002) | - | AY119772 |
| *Boldia erythrosiphon* | Holton *et al.,* (1998), Rintoul *et al.,* (1999) | AF055299 | AF087122 |
| *Chroodactylon ornatum* | Starr & Zeikus (1993) | AF168628 | - |
| *Chroodactylon ornatum* | Yoon *et al.,* (2006) | - | DQ308429 |
| *Compsopogonopsis leptoclados* | Rintoul *et al.,* (1999) | AF087123 | AF087120 |
| *Dione arcuata* | Broom *et al.,* (2004) | AY184343 | - |
| *Erythrocladia* sp. | Ragan *et al.,* (1994); Rintoul *et al.,* (1999) | L26188 | AF087117 |
| *Erythrotrichia carnea* | Ragan *et al.,* (1994); Rintoul *et al.,* (1999) | L26189 | AF087118 |
| *Flintiella sanguinaria* | Müller *et al.,* (2001b) | AF168621 | - |
| *Flintiella sanguinaria* | Yoon *et al.,* (2002) | - | AY119774 |
| *Minerva aenigmata* | Broom *et al.,* (2004) | AY184347 | - |
| *Porphyra acanthophora* | Oliveira *et al.,* (1995) | L26197 | - |
| *P. amplissima* | Yamazaki *et al.,* unpublished | AB015791 | - |
| *Porphyra amplissima* | Klein *et al.,* (2003) | - | AF021034 |
| *P. birdiae* | Klein *et al.,* (2003) | - | AF319460 |
| *P. capensis* | Milstein & Oliveira (2005) | AY766361 | - |
| *P. carolinensis* | Freshwater *et al.,* (1994) | - | U04041 |
| *P.* cf. *leucosticta* | Müller *et al.,* unpublished | AF175538 | - |
| *P. cinnamomea* | Broom *et al.,* (1999) as BRU107 | AH008010 | - |
| *P. coleana* | Broom *et al.,* (1999) as PAP032 | AF136423 | - |
| *P. conwayae* | Lindstrom & Fredericq (2003) | - | AF452427 |
| *P. cuneiformis* | Lindstrom & Fredericq (2003) | - | AF452428 |
| *P. dentate* | Kunimoto *et al.,* (1999) | AB013183 | AB118579 |
| *P. dioica* | Klein *et al.,* (2003) | - | AF081291 |
| *P. fallax* | Müller *et al.,* unpublished | AF175541 | - |
| *P. haitanensis* | Kunimoto *et al.,* (1999) | AB013181 | - |
| *P. hollenbergii* | Lopez-Vivas *et al.,* unpub. | - | AY794401 |
| *P. kanakaensis* | Lindstrom & Fredericq (2003) | - | AF452431 |
| *P. katadae* | Kito *et al.,* unpub. | - | AB118583 |
| *P. leucosticta* | Müller *et al.,* (2001a) | AF175557 | - |
| *P. leucosticta* | Müller *et al.,* (2001b) | AF342746 | - |

| | | | |
|---|---|---|---|
| *P. suborbiculata* | Broom *et al.,* (1999) as *P. lilliputiana* | AF136424 | - |
| *P. lucasii* | Farr *et al.,* (2003) | AY139685 | AY139687 |
| *P. occidentalis* | Lindstrom & Fredericq (2003) | - | AF452436 |
| *P. onoi* | Yamazaki *et al.,* unpublished | AB015794 | - |
| *P. perforata* | Lindstrom & Fredericq (2003) | - | AF452438 |
| *P. pseudolanceolata* | Lindstrom & Fredericq (2003) | - | AF452439 |
| *P. pseudolanceolata* | Müller *et al.,* (2001a) | AF175543 | - |
| *P. pseudolinearis* | Yamazaki *et al.,* unpub. | AB015793 | - |
| *P. pseudolinearis* | Hong unpublished | AF116913 | - |
| *P. purpurea* | Ragan *et al.,* (1994) | L26201 | - |
| *P. purpurea* (*rediviva*) | Lindstrom & Fredericq (2003) | - | AF514280 |
| *P. rakiura* | Farr *et al.,* (2003) | AY139682 | - |
| *P. rosengurtii* | Brodie *et al.,* (2007) | - | AY486349 |
| *P. schizophylla* | Lindstrom & Fredericq (2003) | - | AF452443 |
| *P. spiralis* var. *amplifolia* | Oliveira & Ragan (1994) | L26177 | - |
| *P. tenera* | Park *et al.,* (2007) | AB235852 | - |
| *P. torta* | Müller *et al.,* unpub. | AF175552 | - |
| *P. torta* | Lindstrom & Fredericq (2003) | - | AF452445 |
| *P. umbilicalis* | Müller *et al.,* (2005) as *P. sp.* HG | AF175549 | - |
| *P. variegata* | Lindstrom & Fredericq (2003) | - | AF452447 |
| *P. yezoensis* | H. Kito *et al.,* unpublished data | - | AB118589 |
| *P. yezoensis* | Yiu *et al.,* unpub. | AY131005 | - |
| *P. yezoensis* | Yamazaki *et al.,* (1996) | D79976 | - |
| *Pseudobangia kaycoleia* | Müller *et al.,* (1998, 2005) | AF043364 | - |
| *Rhodochaete parvula* | Zuccarello *et al.,* (2000) | AF139462 | - |
| *Rhodochaete parvula* | Yoon *et al.,* (2002) | - | AY119777 |
| *Smithora naiadum* | Rintoul *et al.,* (1999) | AF087129 | AF087119 |

A similarity matrix was constructed from ISSR band presence/absence data using the Dice coefficient: Dice (x, y) = $2a\bullet(2a + b + c)^{-1}$ where *a* is the number of shared bands between samples *x* and *y*, *b* is band presence in individual *x* but not in *y* and *c* is band presence in *y* but not in *x*. The Dice coefficient does not consider matches of band absence between samples, which tend to overestimate relatedness. Unweighted pair group method with arithmetic averages (UPGMA) clustering was performed on distance data (1 – Dice similarity coefficient) using the Molecular Evolutionary Genetics Analysis (MEGA) v.3.1 software package (Kumar et al., 2004).

To corroborate clustering results, Principal Coordinates Analysis (PCoA) was performed using the *labdsv* package (Roberts, 2006) as implemented in the r-project for statistical computing (R Development Core Team, 2006). To visualize variation in the data the first three principal coordinates were plotted against each other.

In order to test the genetic isolation of boulder populations of *B. maxima* due to between-boulder distance, a Mantel test of geographic vs. Dice coefficient distances was performed using the Arlequin v.3.11 software package (Excoffier et al., 2005).

## 2.4 RESULTS

### 2.4.1 Morphology

*Bangia maxima* was observed at the type locality in Bolinas Bay on seven boulders in close proximity to each other (Figures 2-1, 2-6), with the exception of a small immature population (~0.5 km away) identified through sequence analysis and confirmed by morphological analysis based on lack of fully differentiated reproduction (spores or gametangia). The population located on the seven boulders was composed of separate sexually reproductive

male and female filaments intermingled on the rocks (Figure 2-2). The average length of the male filaments was 81.6 ± 21.6 mm and ranged from 40 to 156 mm (102 filaments measured) and the average length of the female filaments was 106.7 ± 25.2 mm and ranged from 60 to 175 mm (104 filaments measured). The average width of the male filaments was 342.0 ± 114.7 µm and ranged from 109.4 - 527.8 µm (25 filaments measured) and the average width of the female filaments was 569.9 ± 154.0 µm and ranged from 399.9 - 921.5 µm (25 filaments measured). Distal ends of *B. maxima* filaments were multiseriate (Figure 2-3) like typical mature thalli of this genus. However, a unique combination of features was observed in these collections, namely the cells were not abutting and quadrate, as is typical in *Bangia,* but rather they were separated by gelatinous wall material and were elongate along the longitudinal axis (cell diameter 6.3-10.2 µm with a mean of 8.9 µm; cell length 18.2-24.0 µm with a mean of 19.2µm). Other Californian samples used in this study were also examined and did not have this set of features (e.g. Fig. 2-5 for Solana Beach). Hence, these characteristics represent another way to distinguish *B. maxima* from other marine populations of this genus.

### 2.4.2 Phylogenetic analyses

The general time reversible model was selected as the most suitable model of nucleotide evolution for both the nSSU rRNA and *rbc*L genes (GTR+I+G with a gamma distribution shape parameter of 0.4894 (nSSU rRNA)/1.2613 (*rbc*L) and a 0.3348 (nSSU rRNA)/0.4856 (*rbc*L) proportion of invariant sites). The log likelihood scores of the best ML trees were 16629.33 (Figure 2-7) and 10919.76 (Figure 2-8). Phylogenetic reconstruction of both nSSU rRNA (Figure 2-7) and the *rbc*L gene (Figure 2-8) demonstrated topologies similar to Müller

*et al.,* (2005), and consequently nomenclature for *Bangia* clades was maintained. *Porphyra* was polyphyletic relative to the paraphyletic *Bangia* in these analyses, consistent with current literature (Müller *et al.,* 1998, 2001a, 2001b, 2003, 2005; Broom *et al.,* 1999, 2004; Nelson *et al.,* 2005, 2006), and the presence of multiple clades of unidentified marine *Bangia* sp. was well supported by both bootstrap values and Bayesian posterior probability.

There were four groups of marine *Bangia* and one well-supported clade of the freshwater *B. atropurpurea* (nSSU rRNA: 100/100/1.00, *rbc*L: 100/100/1.00, where support values indicate NJ bootstrap (%)/MP bootstrap (%)/Bayesian posterior probability for a clade). *Bangia* clade 1 (Figures 2-7, 2-8) represents Arctic and northwestern Atlantic isolates, with strong support (nSSU rRNA: 64/62/0.97, *rbc*L: 100/100/1.00). This clade also contains an Alaskan isolate and could potentially represent northeastern Pacific *Bangia* isolates as well. Similarly, *Bangia* clade 2 (Figures 2-7, 2-8) was a well-supported clade in nSSU rRNA gene analyses (100/100/1.00) and weakly supported by *rbc*L (-/-/1.00, where - indicates values < 50). However, when excluding two divergent taxa, OR and NC, support increased in the *rbc*L analyses (97/79/1.00). *Bangia* clade 2 contained predominantly eastern Pacific isolates of *Bangia*, with the exception of those from Newfoundland, Nova Scotia and New Jersey. All new samples from this study grouped in this clade. The isolate of *Bangia* located 0.5 km from the intertidal boulders on which *B. maxima* was located exhibited high sequence identity with *B. maxima* over both the nSSU rRNA gene (99.6%) and the *rbc*L gene (100%). This isolate was therefore categorized as *B. maxima* (immature) (Table 2-1), consistent with morphological analyses. Additionally, a southern California *Bangia fuscopurpurea* isolate from Solana Beach resolved as a sister taxon to these samples in both phylogenetic analyses

(nSSU rRNA: 79/73/1.00, *rbc*L: 100/88/1.00). The phylogenetic position of *B. vermicularis* was more ambiguous, although showing affinity to marine *Bangia* from California and well supported as a member of *Bangia* Clade 2 (Figures 2-7, 2-8). In nSSU rRNA gene sequence analysis (Figure 2-7) *B. vermicularis* was weakly supported (65/-/0.72) as sister to *B. fuscopurpurea* from California, British Columbia and Bolinas Bay. Conversely, the position of *B. vermicularis* was not resolved in the *rbc*L analyses beyond its inclusion in *Bangia* Clade 2 (Figure 2-8). Taxa comprising *Bangia* clades 3 and 4 were monophyletic and well supported in NJ and MP analyses (100 % and 70 % respectively, data not shown); however, one taxon, *Bangia* sp. BWP NZ was isolated in ML analyses. Consequently, there is little support for the ML topology of the clade containing *Bangia* clades 3 and 4 in Fig. 2-7 (-/-/0.63). *Bangia* clade 3 (Figure 2-7) exclusively contained isolates from New Zealand (Broom *et al.,* 2004) consistent with previous analyses (Müller *et al.,* 2005). Taxa in this group appear, for the most part, to be monophyletic and sister to the strongly supported *Bangia* clade 4. The phylogenetic resolution of clade 3, however, is questionable and these isolates may correspond to multiple clades of *Bangia* sp. or potentially be combined with *Bangia* clade 4 with more complete taxa sampling. Consequently, *Bangia* clade 3 is maintained as distinct from the well-supported *Bangia* clade 4 in this study. Corresponding *rbc*L sequences for these isolates were not available for analysis. *Bangia* clade 4 (Figures 2-7, 2-8) was strongly supported (100/96/1.00) and contained predominantly temperate Atlantic isolates. Some isolates from clade 4 (Müller *et al.,* 2005) maintained similar positions in nSSU rRNA gene phylogenies, but placed within clade 2 (e.g. NJ) in *rbc*L analyses (Figure 2-8). One isolate from New Zealand, *Bangia* sp. BWP (GenBank Accession number

AY184348), was isolated outside of *Bangia* clades 3 and 4 in ML and Bayesian analyses (Figure 2-7), although it was in *Bangia* clade 3 in NJ bootstrap analysis (82 % support, not shown).

**Figure 2-7.** Maximum likelihood phylogeny using the nuclear small subunit ribosomal RNA gene region of *Bangia maxima*, *B. vermicularis* and nearby *B. fuscopurpurea* collections from Bolinas Bay and Solana Beach described in this study. Selected Bangiales sequences downloaded from GenBank are included. Support values correspond to neighbor-joining bootstrap – 1000 replicates/ maximum parsimony bootstrap – 1000 replicates/ Bayesian posterior probability. Values represented by - are < 50 % bootstrap values or 0.5 for posterior probabilities. Alphanumeric code preceding each taxa label corresponds to the GenBank accession number for that sequence. Code following taxa labels refers to known specimen identifiers (see Table 2-3). [1], *B. fuscopurpurea* from AK, NH, RI, GLD, NFF, NWT, ANT, Norway; [2], a marine *Bangia* isolate identified as *B. atropurpurea* in GenBank.

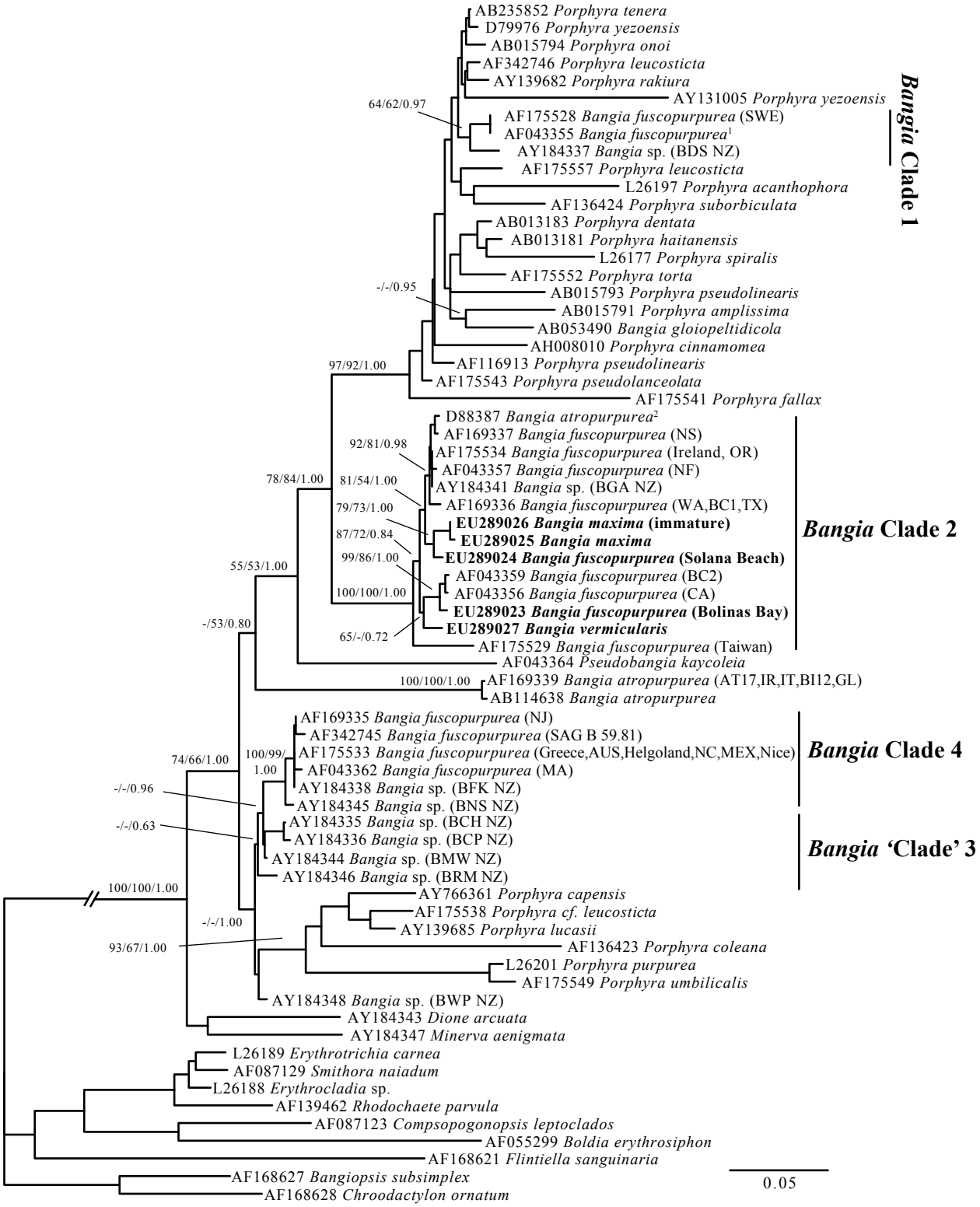
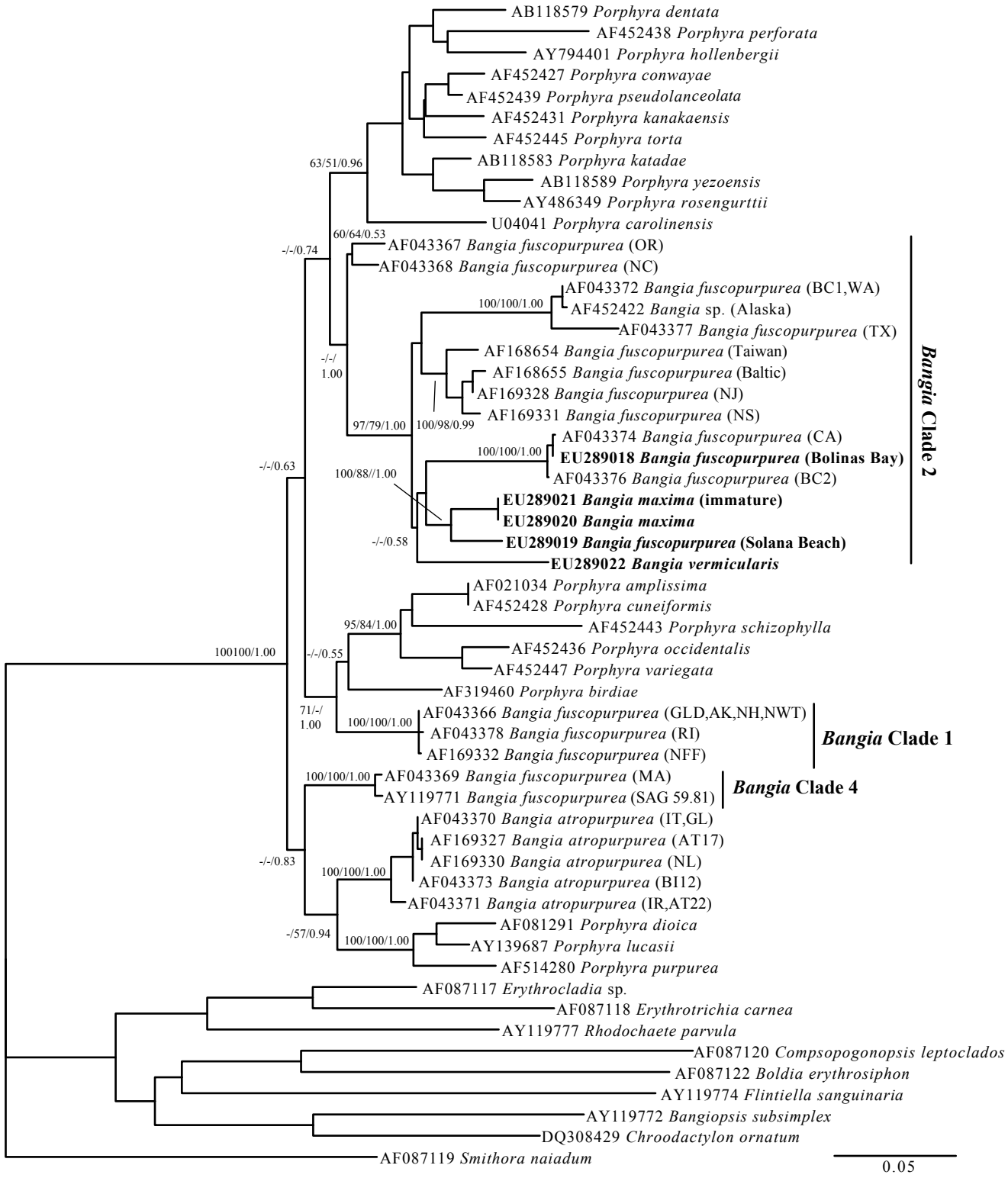

AB235852 Porphyra tenera
D79976 Porphyra yezoensis
AB015794 Porphyra onoi
AF342746 Porphyra leucosticta
AY139682 Porphyra rakiura
AY131005 Porphyra yezoensis
64/62/0.97
AF175528 Bangia fuscopurpurea (SWE)
AF043355 Bangia fuscopurpurea[1]
AY184337 Bangia sp. (BDS NZ)
AF175557 Porphyra leucosticta
L26197 Porphyra acanthophora
AF136424 Porphyra suborbiculata
AB013183 Porphyra dentata
AB013181 Porphyra haitanensis
L26177 Porphyra spiralis
AF175552 Porphyra torta
-/-/0.95
AB015793 Porphyra pseudolinearis
AB015791 Porphyra amplissima
AB053490 Bangia gloiopeltidicola
AH008010 Porphyra cinnamomea
97/92/1.00
AF116913 Porphyra pseudolinearis
AF175543 Porphyra pseudolanceolata
AF175541 Porphyra fallax
Bangia Clade 1
D88387 Bangia atropurpurea[2]
AF169337 Bangia fuscopurpurea (NS)
92/81/0.98
AF175534 Bangia fuscopurpurea (Ireland, OR)
78/84/1.00
81/54/1.00
AF043357 Bangia fuscopurpurea (NF)
AY184341 Bangia sp. (BGA NZ)
AF169336 Bangia fuscopurpurea (WA,BC1,TX)
79/73/1.00
EU289026 Bangia maxima (immature)
87/72/0.84
EU289025 Bangia maxima
EU289024 Bangia fuscopurpurea (Solana Beach)
55/53/1.00
99/86/1.00
AF043359 Bangia fuscopurpurea (BC2)
100/100/1.00
AF043356 Bangia fuscopurpurea (CA)
EU289023 Bangia fuscopurpurea (Bolinas Bay)
-/53/0.80
65/-/0.72
EU289027 Bangia vermicularis
AF175529 Bangia fuscopurpurea (Taiwan)
Bangia Clade 2
AF043364 Pseudobangia kaycoleia
100/100/1.00
AF169339 Bangia atropurpurea (AT17,IR,IT,BI12,GL)
AB114638 Bangia atropurpurea
AF169335 Bangia fuscopurpurea (NJ)
AF342745 Bangia fuscopurpurea (SAG B 59.81)
74/66/1.00
100/99/1.00
AF175533 Bangia fuscopurpurea (Greece,AUS,Helgoland,NC,MEX,Nice)
AF043362 Bangia fuscopurpurea (MA)
-/-/0.96
AY184338 Bangia sp. (BFK NZ)
AY184345 Bangia sp. (BNS NZ)
Bangia Clade 4
-/-/0.63
AY184335 Bangia sp. (BCH NZ)
AY184336 Bangia sp. (BCP NZ)
AY184344 Bangia sp. (BMW NZ)
AY184346 Bangia sp. (BRM NZ)
Bangia 'Clade' 3
100/100/1.00
AY766361 Porphyra capensis
AF175538 Porphyra cf. leucosticta
-/-/1.00
AY139685 Porphyra lucasii
93/67/1.00
AF136423 Porphyra coleana
L26201 Porphyra purpurea
AF175549 Porphyra umbilicalis
AY184348 Bangia sp. (BWP NZ)
AY184343 Dione arcuata
AY184347 Minerva aenigmata
L26189 Erythrotrichia carnea
AF087129 Smithora naiadum
L26188 Erythrocladia sp.
AF139462 Rhodochaete parvula
AF087123 Compsopogonopsis leptoclados
AF055299 Boldia erythrosiphon
AF168621 Flintiella sanguinaria
AF168627 Bangiopsis subsimplex
AF168628 Chroodactylon ornatum
0.05

**Figure 2-8.** Maximum likelihood phylogeny using the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene region of *Bangia maxima*, *B. vermicularis* and nearby *B. fuscopurpurea* collections from Bolinas Bay and Solana Beach described in this study. Selected Bangiales sequences downloaded from GenBank are included. Support values correspond to neighbor-joining bootstrap – 1000 replicates/ maximum parsimony bootstrap – 1000 replicates/ Bayesian posterior probability. Values represented by - are < 50 % bootstrap values or 0.5 for posterior probabilities. Alphanumeric code preceding each taxa label corresponds to the GenBank accession number for that sequence. Code following taxa refers to known specimen identifiers (see Table 2-3).


AB118579 Porphyra dentata
AF452438 Porphyra perforata
AY794401 Porphyra hollenbergii
AF452427 Porphyra conwayae
AF452439 Porphyra pseudolanceolata
AF452431 Porphyra kanakaensis
AF452445 Porphyra torta
AB118583 Porphyra katadae
AB118589 Porphyra yezoensis
AY486349 Porphyra rosengurttii
U04041 Porphyra carolinensis
AF043367 Bangia fuscopurpurea (OR)
AF043368 Bangia fuscopurpurea (NC)
AF043372 Bangia fuscopurpurea (BC1,WA)
AF452422 Bangia sp. (Alaska)
AF043377 Bangia fuscopurpurea (TX)
AF168654 Bangia fuscopurpurea (Taiwan)
AF168655 Bangia fuscopurpurea (Baltic)
AF169328 Bangia fuscopurpurea (NJ)
AF169331 Bangia fuscopurpurea (NS)
AF043374 Bangia fuscopurpurea (CA)
EU289018 Bangia fuscopurpurea (Bolinas Bay)
AF043376 Bangia fuscopurpurea (BC2)
EU289021 Bangia maxima (immature)
EU289020 Bangia maxima
EU289019 Bangia fuscopurpurea (Solana Beach)
EU289022 Bangia vermicularis
Bangia Clade 2
AF021034 Porphyra amplissima
AF452428 Porphyra cuneiformis
AF452443 Porphyra schizophylla
AF452436 Porphyra occidentalis
AF452447 Porphyra variegata
AF319460 Porphyra birdiae
AF043366 Bangia fuscopurpurea (GLD,AK,NH,NWT)
AF043378 Bangia fuscopurpurea (RI)
AF169332 Bangia fuscopurpurea (NFF)
Bangia Clade 1
AF043369 Bangia fuscopurpurea (MA)
AY119771 Bangia fuscopurpurea (SAG 59.81)
Bangia Clade 4
AF043370 Bangia atropurpurea (IT,GL)
AF169327 Bangia atropurpurea (AT17)
AF169330 Bangia atropurpurea (NL)
AF043373 Bangia atropurpurea (BI12)
AF043371 Bangia atropurpurea (IR,AT22)
AF081291 Porphyra dioica
AY139687 Porphyra lucasii
AF514280 Porphyra purpurea
AF087117 Erythrocladia sp.
AF087118 Erythrotrichia carnea
AY119777 Rhodochaete parvula
AF087120 Compsopogonopsis leptoclados
AF087122 Boldia erythrosiphon
AY119774 Flintiella sanguinaria
AY119772 Bangiopsis subsimplex
DQ308429 Chroodactylon ornatum
AF087119 Smithora naiadum
63/51/0.96
60/64/0.53
-/-/0.74
-/-/
1.00
100/100/1.00
97/79/1.00
100/98/0.99
100/100/1.00
100/88//1.00
-/-/0.58
-/-/0.63
95/84/1.00
100100/1.00
-/-/0.55
71/-/
1.00
100/100/1.00
100/100/1.00
-/-/0.83
100/100/1.00
-/57/0.94
100/100/1.00
0.05

### 2.4.3 ISSR analyses of *Bangia maxima*

Highly polymorphic ISSR primers were chosen to magnify the differences between conspecific samples, and some primers with low polymorphisms were not included in this study. Each of the five primers gave consistent and reproducible banding patterns, four of which demonstrated a high number of polymorphic bands (> 30). Bands below 150 bp were discarded, leaving 144 well-separated, polymorphic banding sites. There was an average of 28.6 polymorphic bands per primer (Table 2-2). One primer, ISSR8, amplified few bands with low variability in *B. maxima*; however, a primer similar in sequence, ISSR10, consistently resulted in the most polymorphic markers. Overall, band presence/absence was more consistent within boulder samples than among different ones. Physical distances among boulders were small (Figure 2-6) and no clear correlation between physical and ISSR-estimated genetic distance using the Dice coefficient was observed based on the Mantel test ($p >= 0.05$).

The similarity values calculated from the Dice coefficient ranged from 0.887 to 0.0308 and the cophenetic coefficient of the UPGMA analyses was 0.808. Cluster patterns further indicated that, with some exceptions, isolates from individual boulders grouped consistently together (Figure 2-9). Two predominant clusters were evident in UPGMA analysis, diverging at the 0.7 distance level, one contained boulders 2 and 3 and the other contained boulders 4, 5, 6 and 7. Isolates from boulder 1 were split between the first group (boulders 2 and 3) and sister to all members of the second group, indicating some genetic affinity to both main populations. Although isolates from the same boulder tended to be

positioned together, there were exceptions, most commonly for boulders 1, 2 and 4 (Figure 2-9). Terminal branch lengths of the UPGMA analysis and highly polymorphic banding patterns indicated that a large proportion of the variation was among individuals. Consequently, bootstrapping the data matrix was not useful in determining clade support and is therefore not included here.

Principal coordinates analysis (PCoA) performed on the Dice coefficient distance matrix demonstrated structure in the data generally similar to UPGMA analysis (Figure 2-10). For better visualization the distribution of points representing *B. maxima* isolates the ordination was rotated around the third principal coordinate, of which three different rotations demonstrating distinct separations of isolates are presented (Figure 2-10). The two main clades in the UPGMA analysis (Figure 2-9) were not distinctly observable in the PCoA ordination (Figure 2-10), likely due to presenting a limited scope of the total variation in the data. General clade composition of the boulders was, however, observable. Isolates from boulders 3, 5 and 6, which formed internally consistent clades in UPGMA clustering formed distinct groupings of points in PCoA. Isolates from boulders 5 and 6 demonstrated a similar pattern of distribution, consistent with the isolates clustering relatively closely together in UPGMA. Isolates from boulder 7, which clustered together in UPGMA, showed no discernable structure in PCoA. While isolates from each the remaining boulders did not group distinctly together in the PCoA ordination, the isolates did demonstrate similar distributions to UPGMA. For example, eight isolates from boulder 4 form a distinct cluster that grouped near isolates from boulders 5 and 6. Overall, although only 40.6 % of the

variation in the data set is accounted for in the three dimensions plotted (Figure 2-10), much of the distribution of *B. maxima* isolates in the ordination was consistent with UPGMA.

**Figure 2-9.** UPGMA cluster diagram generated from distance data derived from the Dice similarity coefficient (1 – Dice similarity) for *Bangia maxima* isolates from seven littoral boulders in Bolinas Bay, CA, USA. Patterned boxes are used to emphasize clustering of isolates and numbers 1-7 correspond to the boulders indicated in Fig. 2-6.

Boulders 1-7
(Fig. 2-6)

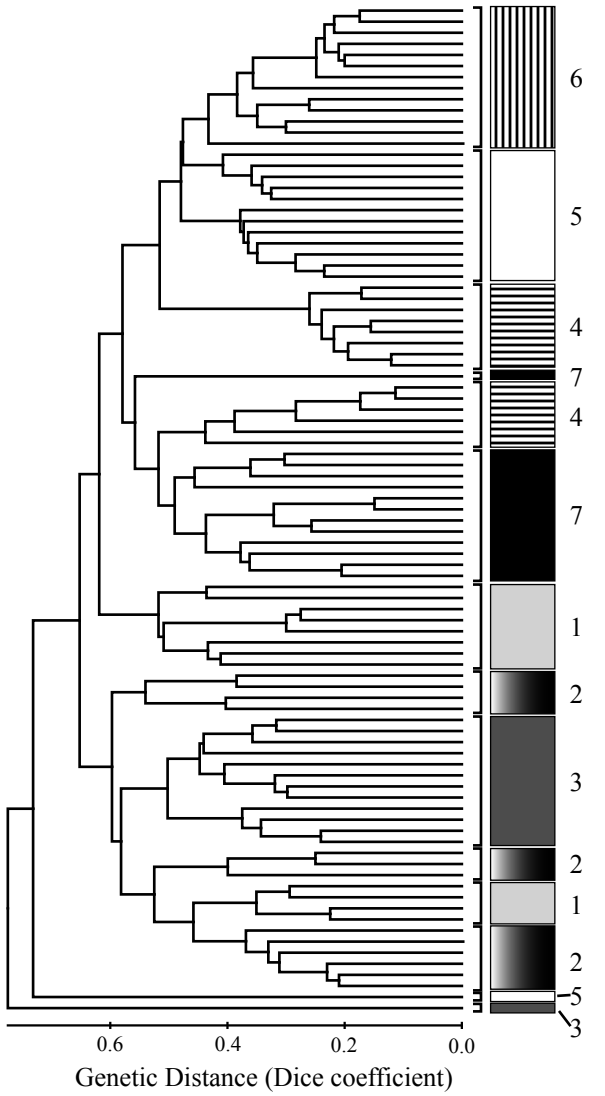
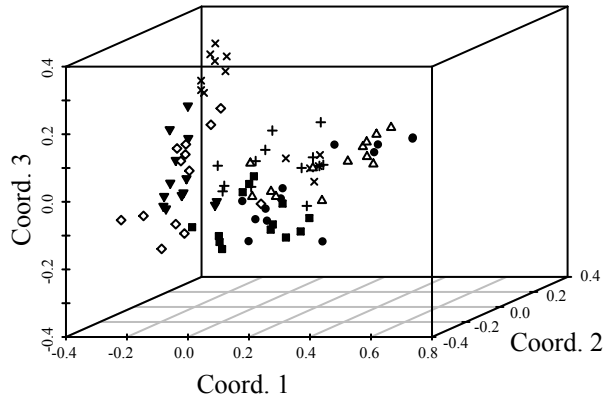
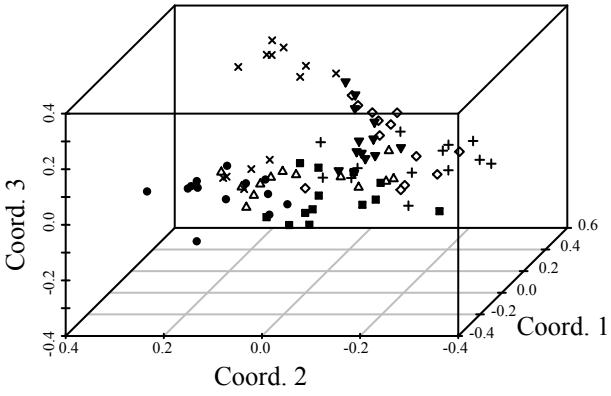
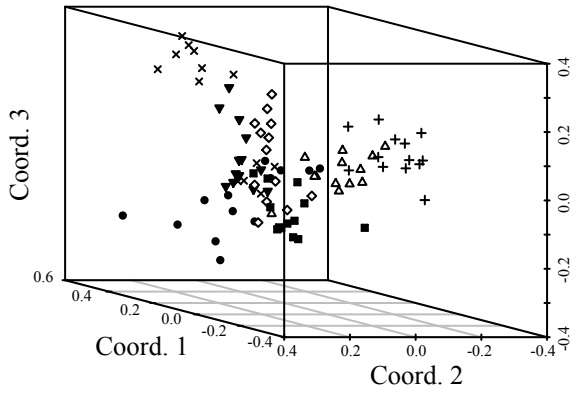


**Figure 2-10.** Principal coordinates analysis based on distance data derived from the Dice similarity coefficient (1-Dice similarity) for *Bangia maxima* isolates from seven littoral boulders in Bolinas Bay, CA, USA: Boulders 1 (●); 2 (△); 3 (+); 4 (×); 5 (◇); 6 (▼); 7 (■). To better visualize the distribution of points representing *B. maxima* isolates, three different perspectives of the ordination are presented, each a different rotation around the third principal coordinate. The first three principal coordinates account for 40.6% of the variation in the data.


Coord. 3
0.4
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
Coord. 1
0.4
0.2
0.0
-0.2
-0.4
Coord. 2


Coord. 3
0.4
0.2
0.0
-0.2
-0.4
0.4
0.2
0.0
-0.2
-0.4
Coord. 2
0.6
0.4
0.2
0.0
-0.2
-0.4
Coord. 1


Coord. 3
0.6
0.4
0.2
0.0
-0.2
-0.4
Coord. 1
0.4
0.2
0.0
-0.2
-0.4
Coord. 2

## 2.5 DISCUSSION

### 2.5.1 Morphology

This study reveals a new set of features to distinguish *B. maxima* from other species of the genus, other than its large size and hollowness at maturity, namely the non-abutting and elongate cells in the mature portions of the thallus. The greater spacing of cells, in part, accounts for the large diameter of filaments of this species. Furthermore, the polyphyly of *Porphyra* within the Bangiales (Müller et al., 1998, 2005; Nelson et al., 2006; Broom et al., 2004) suggests that there has been repeated convergence of the bladed form in several clades, suggesting a relatively simple transition from the filamentous form. Additionally, in recent phylogenetic analyses (Nelson et al., 2006) *B. gloiopeltidicola* Tanaka, a Japanese obligate epiphyte of *Gloiopeltis* J. Agardh from Japan, phylogenetically resolved more closely with bladed taxa than other filamentous ones based on nSSU rRNA gene sequence data. This finding is in agreement with phylogenetic analyses performed here (Figure 2-7). Consequently, the thallus organization of *B. maxima*, with its large, multiseriate filaments, which are distally hollow, may represent a growth form intermediate between the smaller filamentous (e.g. typical *Bangia*) and bladed thallus morphologies (e.g. *Porphyra*). It must be clarified, however, that this condition in *B. maxima* is a derived feature in this clade and therefore not directly observed as a transitional stage in the development between filamentous and bladed thallus morphologies.

An EST-based study of gene expression between gametophyte (bladed thallus) and sporophyte (filamentous) forms of *Porphyra yezoensis* has indicated many differences

between the life cycle stages, although relatively few genes appear to be implicated in cellular organization (Asamizu et al., 2003). Similarly, Stiller and Waaland (1993) hypothesized that few genes may control the plane of cellular division in the Bangiales, which determines filamentous or bladed growth. It therefore seems likely that there are relatively few genetic controls between bladed and filamentous morphologies in the Bangiales. This situation is further mirrored in the number of layers that make up the thallus of *Porphyra*. Most distromatic species of *Porphyra* are monophyletic (Lindstrom and Fredericq, 2003); however, there are some exceptions. The distromatic species, *P. papenfussii*, is paraphyletic in phylogenetic analyses of the *rbc*L gene region (Lindstrom, 2008a) and in another study (Klein et al., 2003) the distromatic *P. amplissima* and *P. miniata* were not monophyletic in nSSU rRNA gene sequence analyses. Additionally, *P. amplissima* contains both monstromatic and distromatic isolates (Brodie et al., 1998), further indicating that the thickness of the *Porphyra* thallus is not a taxonomically informative character. These results are analogous to paraphyly of the green alga *Ulva* (bladed thallus) relative to *Enteromorpha* (tube-like thallus) (Tan et al., 1999; Hayden et al., 2003). *Ulva* and *Enteromorpha* were traditionally considered closely related but separate genera, but paraphyly demonstrated by molecular phylogenetic analyses (Tan et al., 1999; Hayden et al., 2003) argued for their unification (Hayden et al., 2003). This unification is also supported by culture-based evidence of alternation between the two morphologies for single isolates (Provasoli and Pintner, 1980). Similar to *Bangia* and *Porphyra* paraphyly/polyphyly, there are no clear synapomorphies for clades based on molecular phylogenies (Tan et al., 1999; Hayden et al., 2003), which is probably due to morphological simplicity. While a

synonomizing of *Porphyra* and *Bangia* is unlikely, the hollow distal end of *B. maxima* and *B. vermicularis* and phylogenetic distribution of the genera appears to indicate thallus morphology is homoplasious and the genera should not be delineated solely on this character. Indeed, based on the long evolutionary history of the Bangiales and the evolutionary distance within the order demonstrated here and in previous studies (Müller et al., 2001, 2005), unifying *Bangia* and *Porphyra* would likely be rejected in favour of erecting multiple genera within the order as has been previously suggested (Müller et al., 2005; Nelson et al., 2006).

### 2.5.2 Phylogenetic position of *Bangia maxima*

There were four distinct groups of *Bangia* in nSSU rRNA gene sequence analysis (Figure 2-7), three of which were present in the *rbc*L gene sequence analyses (Figure 2-8), consistent with paraphyly demonstrated in other studies (Müller et al., 1998, 2001, b, 2003, 2005; Broom et al., 1999, 2004; Nelson et al., 2005, 2006). Each clade probably represents multiple species/genera with similar morphologies and potentially cryptic species diversity. This concept is further supported by the position of both *B. maxima* and *B. vermicularis* clustering with some *B. fuscopurpurea* isolates within *Bangia* clade 2 (Figures 2-7, 2-8). While these *Bangia* species are morphologically distinguishable from typical *Bangia* collections, the genetic and phylogenetic difference between either of the isolates and *B. fuscopurpurea* within clade 2 is much smaller than among *B. fuscopurpurea* isolates from different clades (Figures 2-7, 2-8).

*Bangia maxima* and *B. vermicularis* were initially reported from their type localities near San Francisco, California and the latter has also been reported from British Columbia (Oates and Cole, 1992). Both appear to cluster with *Bangia* isolates primarily from the

northern Pacific coast of North America. It is highly probable, considering the very restricted distribution of *B. maxima* and the geographic proximity of its closest phylogenetic relatives (e.g. *B. fuscopurpurea* Solana Beach), that *B. maxima* evolved from an eastern Pacific population of *B. fuscopurpurea*. A similar situation is likely for *B. vermicularis* as it is consistently sister to California/British Columbia isolates of *B. fuscopurpurea*. Notably, despite the gross morphological similarity between *B. maxima* and *B. vermicularis,* as noted by Gardner (Gardner, 1927), the species are not particularly close phylogenetic relatives (Figures 2-7, 2-8). Additionally, as *B. maxima* has not been observed outside of Bolinas Bay, California, it may be an endemic species and further field observations should be used to confirm this.

Some *Bangia* taxa phylogenetically positioned within clade 2 are from regions other than the eastern Pacific, including isolates from the Baltic, western Pacific (Taiwan), northwestern Atlantic (Nova Scotia, Newfoundland) and northeastern Atlantic (Ireland). This finding is likely due to vector-assisted transport, proposed previously by Müller *et al.* (1998, 2003). Alternatively, the temperate distribution of isolates from this clade may indicate taxa adapted to more temperate conditions, contributing to their distribution on both coasts of North America. Despite some inconsistencies, there is a discernable geographic distribution of taxa within each of the *Bangia* clades – transarctic (clade 1), eastern Pacific (clade 2), south Pacific (clade 3) and temperate Atlantic (clade 4). It is therefore unlikely that *B. fuscopurpurea* is a single species with a cosmopolitan distribution. There are probably multiple cryptic species in the marine *Bangia* species complex, similar to a proposal by Müller *et al.* (2003) and Nelson *et al.* (2005). One isolate of *Bangia fuscopurpurea,* NJ*,* had a

conflicting placement between the nSSU and *rbc*L sequence analyses (Figures 2-7, 2-8). This observation is in agreement with previous phylogenetic analyses (Müller et al., 1998, 2003) and is an unexplained result consistent after multiple sequencing efforts from the same individual, perhaps caused by differential rates of evolution for each of the genes studies or a past hybridization event.

### 2.5.3 ISSR analyses of *Bangia maxima*

The ISSR primers used in this study and effectively separating isolates predominantly contained $(CG/C)_n$ motifs in contrast with embryophyte genomes, which have been shown to have an abundance of $(AT)_n$ motifs (Lagercrantz *et al.,* 1993). Although few $(AT)_n$ motif primers were screened, this could potentially demonstrate a subtle difference between microsatellite regions of Bangiophyceae and embryophytes. The high variation among *B. maxima* isolates may potentially have been affected by inconsistent amplifications of SSRs in the plastid genome. This possibility, however, is unlikely as plastid SSRs are typically mononucleic repeats (Powell et al., 1995) and would therefore not be amplified by these primers.

There was a high diversity of polymorphic banding for four of the ISSR primers in *B. maxima*, as well as consistent and clear differences observed among all boulders (Figures 2-9, 2-10). These results are similar to those from a study of the freshwater red alga *Batrachospermum* in an Ohio creek (Hall and Vis, 2000, 2002) in which 165 individuals from 11 streams each had unique banding patterns over 7 ISSR primers, including the 5 used here. Moreover, when a stream was divided into three segments separated by 50 m, 79% of

the observed banding variation occurred within a segment (< 10 m), similar to boulder-level variation demonstrated in this study. High genetic variability over low spatial scales has precedent in the red algae. The use of RAPD markers demonstrated high variability over < 0.25 $m^2$ in the red alga *Delisea pulchra* (Greville) Montagne (Wright *et al.,* 2000), although overall the genotypes were less variable than observed here, probably because the population of *D. pulchra* studied was considered to be asexual. Additionally, dioecious species, such as *B. maxima*, tend to have higher population differentiation (Sosa and Lindstrom, 1999).

Sexual reproduction is likely to be correlated with increased heterogeneity of genetic markers (Houliston and Chapman, 2003). Consequently, the low level of within-boulder heterogeneity is likely to be indicative of either asexual reproduction (Wright *et al.,* 2000) or inbreeding. *Bangia maxima* isolates were observed to be sexual and thus inbreeding within boulders is likely to have contributed to banding homogeneity. Additionally, ISSR distances are sufficiently large to suggest sexual reproduction with limited crossing among boulders. Dispersal in the bangialean red algae, by fragmentation or spore/gamete release, is dependent on water currents as all life stages of red algae lack flagella. Consequently red algal gametes can have short dispersal distances, as demonstrated for two species of the florideophyte genus *Gelidium* (Sosa et al., 1998). Similarly, Engel *et al.* (Engel et al., 1999) demonstrated most mating events for *Gracilaria gracilis* occurred within approximately 5 m, suggesting a strong negative effect of distance on mating success. The boulders on which *B. maxima* are located are also partially sand-submerged for part of the year, reducing the overall influence of water currents in gamete dispersal, further decreasing dispersal distance and raising the likelihood of inbreeding. Additionally, *Bangia* is typically located on smooth rocky

substrates and not as common on rougher ones. The sandy nature of Bolinas Bay results in few suitable substrates beyond the boulders on which *B. maxima* are located (Figure 2-1), further contributing to the isolation of the populations to these suitable boulders. Conversely, dispersal may not be limited to short distances in some red algae, as has been proposed for the freshwater *Batrachospermum helminthosum* in North American streams across its distribution (Hall and Vis, 2002). Factors such as water currents and animal transport are likely to have contributed to this larger dispersal range observed in *B. helminthosum*. It is possible, therefore, that gamete dispersal is more efficient in stream ecosystems than littoral ones and that long-distance dispersal is unlikely for *B. maxima*. Despite factors contributing to within-boulder homogeneity, limited out-crossing among boulders is likely, particularly for boulders 1, 2 and 4 (Figures 2-9, 2-10).

Long terminal branch lengths in UPGMA analysis (Figure 2-9) indicates that much of the genetic variation in *B. maxima*, as defined by ISSRs, is among individuals. This observation is consistent with other ISSR studies (Hall and Vis, 2002; Bornet et al., 2004). The strong localized (within boulder) pattern of ISSR banding, as demonstrated in UPGMA (Figure 2-9) and PCoA (Figure 2-10) analyses, indicates that there is likely little sexual cross-fertilization among boulders. Instances of isolates not clustering within boulders were probably either due to cross-fertilization among boulders or asexual migration of individuals. Additionally, as ISSRs can be extremely variable (Hall and Vis, 2002; Bornet et al., 2004; Roux et al., 2007), some differences among isolates may be the result of linear inheritance as opposed to recombination. Predominantly only isolates from boulders 1, 2 and 4 grouped in multiple areas of the cluster diagram (Figure 2-9), indicating cross-fertilization among

isolates from different boulder populations. Boulder 1 is centrally located, which may explain the clustering of isolates from this boulder in both main clades. This clustering is consistent with PCoA in which isolates from boulder 1 occurred in two distinct groups corresponding to the two main clades. In addition, boulder 2 is located very close to boulder 1, which could facilitate exchange between the two. Boulder 4 was at the perimeter of the grouping and greater exposure to tidal currents would account for distribution of spores to other boulders.

Since physical distances among boulders did not correlate with genetic distance, it seems likely that other factors besides distance, including water current activity and boulder submersion, contribute more to gamete dispersal in this population. Bolinas Bay is a sheltered bay and the boulders are often submerged in sand. This submergence is likely to be the largest contributing factor to the within-boulder homogeneity of the samples, as noted previously.

Genetic variation can be high within morphologically indistinguishable species of *Bangia*. This variation, including known paraphyly/polyphyly of *Bangia* and *Porphyra*, is of relevance, not only to taxonomists and ecologists, but researchers in cellular development. The phylogenetic position of morphologically distinguishable *B. maxima* within a clade of the paraphyletic *Bangia* further supports the proposal of multiple cryptic species within the currently recognized genus *Bangia*. Moreover, the morphology of *B. maxima* may further indicate the possibility of homoplasious transition to the bladed thallus within the Bangiales. While genetic variation in species of *Bangia* as a whole, specifically over gene regions such as nSSU rRNA, is well established, more work is necessary to understand the dynamics of genetic variation over small spatial scales. Since *B. maxima* is only known from one

restricted location, it is possible that it is a true endemic species and its population dynamics may be sufficiently different from other *Bangia* species. Consequently, the finding of relatively consistent within-boulder ISSR banding patterns may not be typical of the genus as a whole.

# Chapter 3
# Utility of DNA barcoding in bangiophyte species discrimination

## 3.1 OVERVIEW

The bangiophyte red algae (Rhodophyta) are an ancient and diverse set of organisms with simple morphologies ranging from unicells to filaments and thalli. Such morphological simplicity can make species identification difficult resulting in a high potential for cryptic species. Consequently, comparatively little is known about species distribution and biogeography.

Adhering to proposed barcoding protocols for the Rhodophyta, the use of the mitochondrial cytochrome oxidase *c* subunit I (COI) gene for species discrimination was evaluated for non-Florideophyceae Rhodophyta, with a primary focus on Bangiales isolates. The COI gene from a total of 87 isolates were successfully sequenced. Cryptic diversity was observed in *Porphyra schizophylla* (up to 55 bp) and among marine *Bangia* isolates. This marker demonstrated higher sensitivity for potential cryptic species than more common sequence markers (e.g. nuclear small subunit ribosomal RNA). In contrast, discrimination of a marine *Bangia* from Texas contradicted previous phylogenetic placements of that isolate. Despite demonstrated potential of the COI gene as a sequence marker for DNA barcoding, amplification and sequencing of samples was problematic, especially for non-Bangiales samples. Further development of molecular protocols as well as taxonomically informed sampling is required to establish the COI gene as a viable primary sequence marker for DNA barcoding of the bangiophyte red algae.

## 3.2 INTRODUCTION

The red algae (Rhodophyta) are an ancient lineage of eukaryotes (Butterfield et al., 1990; Butterfield, 2000, 2001) and are one of the three major lineages of photosynthetic Eukaryota (Yoon et al., 2006b). These organisms are significant contributors to global carbon budgets, constitute a significant portion of the eukaryotic flora of marine ecosystems and are integral to understanding the development of sexuality and multicellularity. Despite their significance, the Rhodophyta are poorly studied relative to more conspicuous organisms such as mammals and embryophytes. Taxa in the Rhodophyta have overwhelmingly been classified using the morphological species concept. This has likely resulted in a large number of different (cryptic) species being lumped under a single species name (Lindstrom, 2008a, b), especially in the non-Florideophyceae taxa (herein referred to as the bangiophytes) due to their simple morphologies. For example, the two primary genera within the bangiophyte order Bangiales are *Bangia* and *Porphyra*, and species were distributed among these genera solely based on the gametophyte growth form being either filamentous (*Bangia*) or foliose (*Porphyra*). This practice has resulted in taxa recognized as paraphyletic, containing at least 3 separate clades of marine *Bangia* with indistinguishable morphologies (Müller et al., 2005; Lynch et al., 2008). Recently, aided by molecular sequence analysis, three isolates with morphologies very similar to or indistinguishable from marine *Bangia* have been elevated to new genera within the Bangiales (Müller et al., 2005; Nelson et al., 2005). A further proposal of taxonomy congruent with phylogeny is still required, although a taxonomic revision for the Bangiales is the current focus of an international consortium of phycologists (Bangiales Taxonomy Working Group).

In order to fully characterize bangiophytes in nature as well as support future taxonomic revisions, a rapid and robust method for species identification and discrimination is required. The use of short DNA sequences for species identification was most successfully proposed as DNA barcoding by Hebert et al. (Hebert et al., 2003). This approach to cataloguing and characterizing species has been variously accepted in the biological community. Critics argue that the use of a short fragment of a single gene (e.g. cytochrome oxidase c subunit 1, COI) may not provide enough resolution to accurately discriminate among species, especially over broad taxonomic ranges. Similarly, it is not likely that any single gene will accomplish barcoding objectives for all eukaryotes. In some groups of organisms, such as the flowering plants (Kress et al., 2005), the COI gene is not suitable for species discrimination due to low or high rates of mutation, requiring the addition of secondary genes or the discarding of COI gene altogether. Furthermore, the protocol for amplifying the COI gene, e.g., PCR primers, is not consistent across lineages, which further compromises the utility of the technology.

Since the proposal of COI as a DNA barcoding gene for animals (Hebert et al., 2003), the development of DNA barcoding technologies for other phyla has been an active area of research. Different gene regions have been proposed for use as a DNA barcode with varying levels of success; COI (Seifert et al., 2007) and the nuclear internal transcribed spacer (ITS) (International Subcommission on Fungal Barcoding, 2009) in the fungi and the nuclear ITS region and plastid *trn*H-*psb*A intergenic spacer for flowering plants (Kress et al., 2005). A recent proposal for the use of the COI gene for red macroalgae (Saunders, 2005) has been successful primarily for the more derived Florideophyceae taxa (Saunders, 2005; Robba et

al., 2006). While the Florideophyceae are the most species-rich class of Rhodophyta, more ancestral-like taxa, which are fundamental to understanding the development of sexuality and multicelluarity, have not been adequately evaluated for DNA barcoding.

DNA barcoding also has practical implications for the field of taxonomy as a whole. The application of a sequence-based identification marker to species-level taxonomy has the common side effect of identifying potential cryptic species. While this is an exceptionally useful result, the rate and ease with which these cryptic species are recognized can potentially cause the degeneration of the taxonomy they were meant to aid since many of these new 'species' will not be formally or accurately described (Ebach and Holdrege, 2005). Despite these concerns, DNA barcoding can adhere to the stated goals (discrimination of species) and with improvements made to the approach (e.g. application of appropriate genes for different phyla), it can be a useful tool furthering taxonomic research.

The objectives of this study were two-fold; (a) to establish the utility of the COI gene for DNA identification of non-Florideophyceae taxa and (b) to apply these technologies to a geographically diverse set of non-Florideophyceae taxa (predominantly Bangiales from North America and Europe) providing insights into the biogeography and cryptic species diversity within this group.

## 3.3 METHODS

### 3.3.1 Taxon sampling

Collections of bangiophyte algae primarily from North America, supplemented by culture collection samples, were screened for the amplification of the COI gene. These taxa included

a total of 166 individual isolates representing all known lineages of bangiophyte algae (Yoon et al., 2006a). The majority of these samples (142) belonged to the Bangiales, including the genera *Bangia, Pseudobangia* and *Porphyra* and represented each known independent lineage of marine and freshwater *Bangia* (Müller et al., 2005; Lynch et al., 2008). In addition, as there are no published studies of DNA barcoding within the non-Bangiales bangiophytes, we also attempted to sequence isolates of these taxa in our collections.

### 3.3.2 DNA extraction, PCR amplification and DNA sequencing

Total DNA was extracted from fresh or frozen samples using a modified phenol-chloroform protocol (Saunders, 1993), and a rapid CTAB extraction protocol (Allen et al., 2006) was used for silica gel preserved samples. Mitochondrial COI gene amplification was attempted for all samples using GazF1 (5' TCAACAAATCATAAAGATATTGG 3') and GazR1 (5' ACTTCTGGATGTCCAAAAAAYCA 3') PCR primers following the amplification protocol of Saunders (Saunders, 2005). To facilitate the amplification of problematic samples, additional PCR amplification was attempted with annealing temperatures as low as 47º C. PCR amplicons were then purified using the QIAquick PCR purification kit (cat. # 28106, Qiagen Inc., Mississauga, Canada). Sequencing in both the 5' and 3' direction of all amplicons was performed by the Canadian Centre for DNA Barcoding (http://www.dnabarcoding.ca/) using the GazF1 and GazR1 primers.

### 3.3.3 Data analysis

Sequences from successful amplicons were visualized, edited and assembled using FinchTV v.1.4.0 (Geospiza, Inc., USA). Edited sequences were evaluated for sample contamination by

blastn (Altschul et al., 1997) and sequences belonging to non-target taxa were removed from the data set. Existing Bangiales COI gene sequence data available from GenBank were added to the data set, primarily from Robba *et al.,* (Robba et al., 2006). A calculation of total pairwise nucleotide differences between sequences was performed using a PERL script, which is available upon request. Sequences were aligned using MUSCLE v.3.6 (Edgar, 2004) and manually examined using Seaview v.4 (Galtier et al., 1996). The completed data set contained 107 sequences of the COI gene, 87 unique to this study. Sequences were trimmed to the shortest sequence in the data set resulting in a total alignment of 545 nucleotide sites.

To present phenetic distance between sequences the alignment was subjected to unweighted pair group method with arithmetic mean (UPGMA) using MEGA v.4 (Tamura et al., 2007) using number of character differences between sequences. As pairwise distances were calculated independently, the complete deletion parameter was used to calculate the distance matrix for UPGMA analysis. Consequently, a character site was removed from UPGMA analysis if any sequence contained a gap or unknown character at that site. To provide phylogenetic support for the UPGMA topology, 1000 neighbor joining bootstrap iterations were calculated. The appropriate model of sequence evolution for neighbor joining phylogenetic reconstruction of the COI gene was determined through the AIC test using jModelTest v.0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) and analysis was performed using PAUP* v.4.10 (Swofford, 2003). All trees were visualized using FigTree v.1.2.1 (Rambaut).

## 3.4 RESULTS

In general, amplification of the COI gene was successful for Bangiales samples for both fresh and gel-desiccated material (approximately 75% and 60% respectively) and sequencing yielded a success rate of approximately 80% (Table 3-1). In contrast, only one of 22 non-Bangiales samples was successfully amplified and sequenced (*Boldia* CRON). Sequencing reads were generally of high quality in both directions; however, a marginally higher degree of sequence quality and read length was noted for sequences using the reverse (GazR1) primer. Five of the successful sequencing reads were of contaminant sequences as determined by blastn results, including *Heterozoa* and the aquatic mould *Saprolegnia*. The complete deletion of unknown or gapped sites in UPGMA analysis resulted in a final sequence alignment with a total length of 462 nucleotides. The model of sequence evolution used for Neighbor Joining analysis, as selected by the AIC implemented in jModelTest (Guindon and Gascuel, 2003; Posada et al., 2008), was GTR+I+G with a gamma distribution shape parameter of 0.5720 and a 0.4220 proportion of invariant sites.

**Table 3-1. Isolate and collection information for samples of bangiophyte red algae used in this study.**

| Taxon | Locality | Collector/Collection ID | Citation |
|---|---|---|---|
| *B. atropurpurea* | British Isles (BI12) | K.M Müller | Müller et al. unpublished |
| | Lake Michigan (LM11b) | K.M Müller | Müller et al. unpublished |
| | Lake Michigan (LM15) | K.M Müller | Müller et al. unpublished |
| | Lake Michigan (LM4c) | K.M Müller | Müller et al. unpublished |
| | Lake Michigan (LM5e) | K.M Müller | Müller et al. unpublished |
| | Lake Ontario (BL01-1) | K.M Müller | Müller et al. unpublished |
| | Lake Ontario (Kingston) | K.M Müller | Müller et al. unpublished |
| | Lake Ontario (Port Stanley) | K.M Müller | Müller et al. unpublished |
| | Ysselmeer, The Netherlands (NL) | K.M Müller | Müller et al. (2003) |
| *Bangia* (ambiguous placement) | Port Aransas, Texas (TX) | R.G. Sheath and M. Vis | Müller et al. (1998) |
| *Bangia* (Marine Clade 1) | Argentia, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Bear Cove, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Brigus, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Brigus, Newfoundland (1) | M.D.J. Lynch and K.M Müller | This study |
| | Ferryland, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Foxtrap, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Hant's Harbour, Newfoundland (H) | M.D.J. Lynch and K.M Müller | This study |
| | Hant's Harbour, Newfoundland (L) | M.D.J. Lynch and K.M Müller | This study |
| | Holyrood, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | New Hampshire (NH) | K.M Müller | Müller et al. (1998) |
| | Perry's Cove, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Point No Point, BC | M.D.J. Lynch and R. Young | This study |
| | Torbay, Newfoundland (NF10) | K.M Müller | Müller et al. unpublished |
| | Tors Cove, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Whiteway, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Whiteway, Newfoundland (2006 H) | M.D.J. Lynch and K.M Müller | This study |
| | Whiteway, Newfoundland (2006 L) | M.D.J. Lynch and K.M Müller | This study |
| *Bangia* (Marine Clade 2) | B. vermicularis San Francisco Bay, CA | K.M Müller and R.G. Sheath | Lynch et al. 2008 |
| | Bolinas Bay, CA | K.M Müller and R.G. Sheath | Lynch et al. 2008 |
| | Campbell River, BC | M.D.J. Lynch and R. Young | This study |
| | Illamna Bay, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Ketchikan, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Sedanka Island, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Seppings Island, BC | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Skan Bay, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| *Bangia* (Marine Clade 3/4) | Australia (AUS) | K.M Müller | Müller et al. unpublished |
| | Devon, England (JB212) | J. Brodie JB212 culture no. 245 | Robba et al. (2006) |
| | Devon, England (JB238) | J. Brodie JB238 JB culture no. 263 | Robba et al. (2006) |
| | Dorset, England (JB culture 225) | W. Farnham JB culture no. 225 | Robba et al. (2006) |
| | Ensenada, Mexico (MEX) | K.M Müller | Müller et al. unpublished |
| | Helgoland, Germany (GER) | K.M Müller | Müller et al. unpublished |
| | Italy (IT) | K.M Müller | Müller et al. (1998) |
| | New Jersey (NJ) | K.M Müller | Müller et al. unpublished |
| | Nice, France | K.M Müller | Müller et al. unpublished |
| | North Carolina (NC) | K.M Müller | Müller et al. (1998) |
| | Valencia, Spain | K.M Müller | Müller et al. unpublished |
| *Boldia erythrosiphon* | Champlain Rapids, Ottawa River, Ontario | J. Beaulieu | Rintoul et al. (1999) |
| *Porphyra sp.* | Parker Bay, BC | M.D.J. Lynch and R. Young | This study |
| *P. conwayae* | Kalaloch Beach, WA | M.D.J. Lynch and R. Young | This study |
| | Lincoln City, OR | M.D.J. Lynch and R. Young | This study |
| | Teawit, WA | M.D.J. Lynch and R. Young | This study |
| *P. cuneiformis* | Sunshine Cove, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. dioica* | Devon, England (JB347) | J. Brodie JB347 [752] | Robba et al. (2006) |
| | Glamorgan, Wales (JB137) | J. Brodie JB137 [819] | Robba et al. (2006) |
| | Orkney, Scotland (JB246) | F. Bunker JB246 | Robba et al. (2006) |
| | P. cf. umbilicalis (SCO 2Wb) | K.M Müller | Müller et al. unpublished |
| | P. linearis (SCO 2W) | K.M Müller | Müller et al. unpublished |
| | Sutherland, Scotland (JB314) | J. Brodie JB314 [725] | Robba et al. (2006) |
| *P. kurogii* | Katmai Bay, AK (P145) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. leucosticta* | Cornwall, England (JBLR21) | J. Brodie JBLR21 [769] | Robba et al. (2006) |

| | | | |
|---|---|---|---|
| | Durness, Scotland (JB308) | J. Brodie JB308 [820] | Robba et al. (2006) |
| | Northumberland, England (JB322) | J. Brodie JB322 [733] | Robba et al. (2006) |
| *P. mumfordii* | Devil's Punchbowl, OR (P234) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. nereocystis* | Cape Udak, AK (P262) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. perforata* | Birch Bay, WA (P142) | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Campbell River, BC | M.D.J. Lynch and R. Young | This study |
| | Cordoba Bay, BC | M.D.J. Lynch and R. Young | This study |
| | Crescent City, CA (P232) | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Hudson Bay, MB | J. Witt | This study |
| | Kitty Coleman, BC | M.D.J. Lynch and R. Young | This study |
| | Oregon (OR) | K.M Müller | Müller et al. (1998) |
| | Parker Bay, BC | M.D.J. Lynch and R. Young | This study |
| | Parksville, BC | M.D.J. Lynch and R. Young | This study |
| | Port Angeles, WA | M.D.J. Lynch and R. Young | This study |
| | Three Saints Bay, AK (P264) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. pseudolanceolata* | Rosario Beach, WA | M.D.J. Lynch and R. Young | This study |
| *P. purpurea* | Avonport, NS | N/A | Ragan et al. (1994) |
| *P. rosengurtii* | Sussex, England (JB286)# | I. Tittley JB286 | Robba et al. (2006) |
| *P. schizophylla* | Akun Island, AK (P202) | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Akutan Pt., AK (P150) | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Creyke Pt. BC (P92) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. tasa* | Kiska Island, AK | S.C. Lindstrom | Lindstrom et al. unpublished |
| | Spray Cape, AK (P185) | S.C. Lindstrom | Lindstrom et al. unpublished |
| *P. umbilicalis* | Bauline, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | County Down, N. Ireland (JB329) | C.A. Maggs JB329 | Robba et al. (2006) |
| | Devon, England (JB342) | J. Brodie JB342 [750] | Robba et al. (2006) |
| | Devon, England (JB352) | J. Brodie & L. Robba JB352 [757] | Robba et al. (2006) |
| | Devon, England (JB357) | J. Brodie & L. Robba JB357 [753] | Robba et al. (2006) |
| | Hampton Beach, NH (1) | M.D.J. Lynch and R. Young | This study |
| | Hampton Beach, NH (2) | M.D.J. Lynch and R. Young | This study |
| | Kent, England (JB324) | J. Brodie JB324 [824] | Robba et al. (2006) |
| | Kent, England (JB334) | I. Tittley JB334 [727-749-730] | Robba et al. (2006) |
| | Kintyre, Scotland (JB178) | J. Brodie JB178 [821] | Robba et al. (2006) |
| | Newfoundland (NF16) | K.M Müller | Müller et al. unpublished |
| | Pemaquid Point, MN | M.D.J. Lynch and R. Young | This study |
| | Portugal Cove, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Tors Cove, Newfoundland (1) | M.D.J. Lynch and K.M Müller | This study |
| | Tors Cove, Newfoundland (2) | M.D.J. Lynch and K.M Müller | This study |
| | Tors Cove, Newfoundland (3) | M.D.J. Lynch and K.M Müller | This study |
| | Whiteway, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Witless Bay, Newfoundland | M.D.J. Lynch and K.M Müller | This study |
| | Witless Bay, Newfoundland (VF3) | K.M Müller | Müller et al. unpublished |
| *Pseudobangia kaycoleia* | Virgin Islands (VIS7) | K.M Müller | Müller et al. (2005) |

The discrimination of isolates in UPGMA analysis of the COI gene (Figure 3-1) was consistent with other gene phylogenies (Müller et al., 2001a, 2005; Lindstrom, 2008a; Lynch et al., 2008) and illustrated the potential presence of cryptic species. There were a total of five clades of *Bangia* species similar to previously published *Bangia* phylogenies, and therefore naming conventions for the clades were maintained (Müller et al., 2005; Lynch et al., 2008). Of these groups, the freshwater *Bangia atropurpurea*, with samples from both the Great Lakes and Europe, formed the group with the lowest genetic variation (with 0-8 nucleotide changes among isolates). Of the remaining *Bangia* groups, *Bangia* Clade 1 was made up of isolates from northwest Atlantic (0-2 nucleotide differences among isolates) and an Antarctic isolate (20-22 nucleotide differences relative to northwest Atlantic isolates). *Bangia* Clade 2 contained two separate groups of isolates, one from the northeast Pacific showing high sequence identity (0-5 nucleotide differences), and a second group of sequences from California (1 nucleotide difference). Although these two groups of marine *Bangia* clustered together, they were genetically distant (72 nucleotide differences). Notably, the California sequences included *Bangia vermicularis*, which was the only marine *Bangia* sample in this study that had a morphology distinguishable from other marine *Bangia* isolates. The sequence for *Bangia vermicularis* was nearly identical to the sequence from a nearby (Bolinas Bay, CA) population that had morphology indistinguishable from other marine *Baniga*. One isolate (TX), which had previously resolved in *Bangia* Clade 2 using nSSU rRNA and *rbc*L gene sequence analysis (Lynch et al., 2008), did not group closely with any *Bangia* isolates in this study; it differed by 92-108 nucleotides from *Bangia* Clade 2. *Bangia* Clade 3-4 showed a large degree of sequence variation (a maximum of 53

nucleotide differences between isolates). These isolates had a more temperate distribution in the Atlantic, further south than *Bangia* Clade 1.

Similar to *Bangia*, *Porphyra* isolates formed cohesive, distinct genetic groups. Figure 3-1 suggested at least 6 independent groupings of *Porphyra* species in this data set, i.e., separated by filamentous clades. One grouping contained only isolates of *P. schizophylla*; however, there was considerable sequence diversity among these isolates (up to 46 nucleotide differences). The grouping of *P. leucosticta/P. rosengurtii* contained a loose association (55 nucleotide changes) between an isolate of each species, while the three remaining *P. leucosticta* isolates were identical (in the case of JB308 the sequence was 11 bp shorter). The grouping of northeast Pacific species in the *P. pseudolanceolata* complex (including *P. nereocystis*) contained a group of three unnamed *Porphyra* isolates (likely *P. conwayae* as labeled in Figure 3-1) with identical COI sequences as well as another unknown *Porphyra* isolate (from Parker Bay, BC) with a morphology identical to *P. pseudolanceolata.* *Porphyra kurogii* occurred at the base of this grouping. These sequences differed from each other by a maximum of 62 nucleotides. In another northeast Pacific species, *P. perforata*, samples occurred in two groups, one with from 0-3 nucleotide differences and a relatively close (12 nucleotide differences) sister clade of two identical sequences.

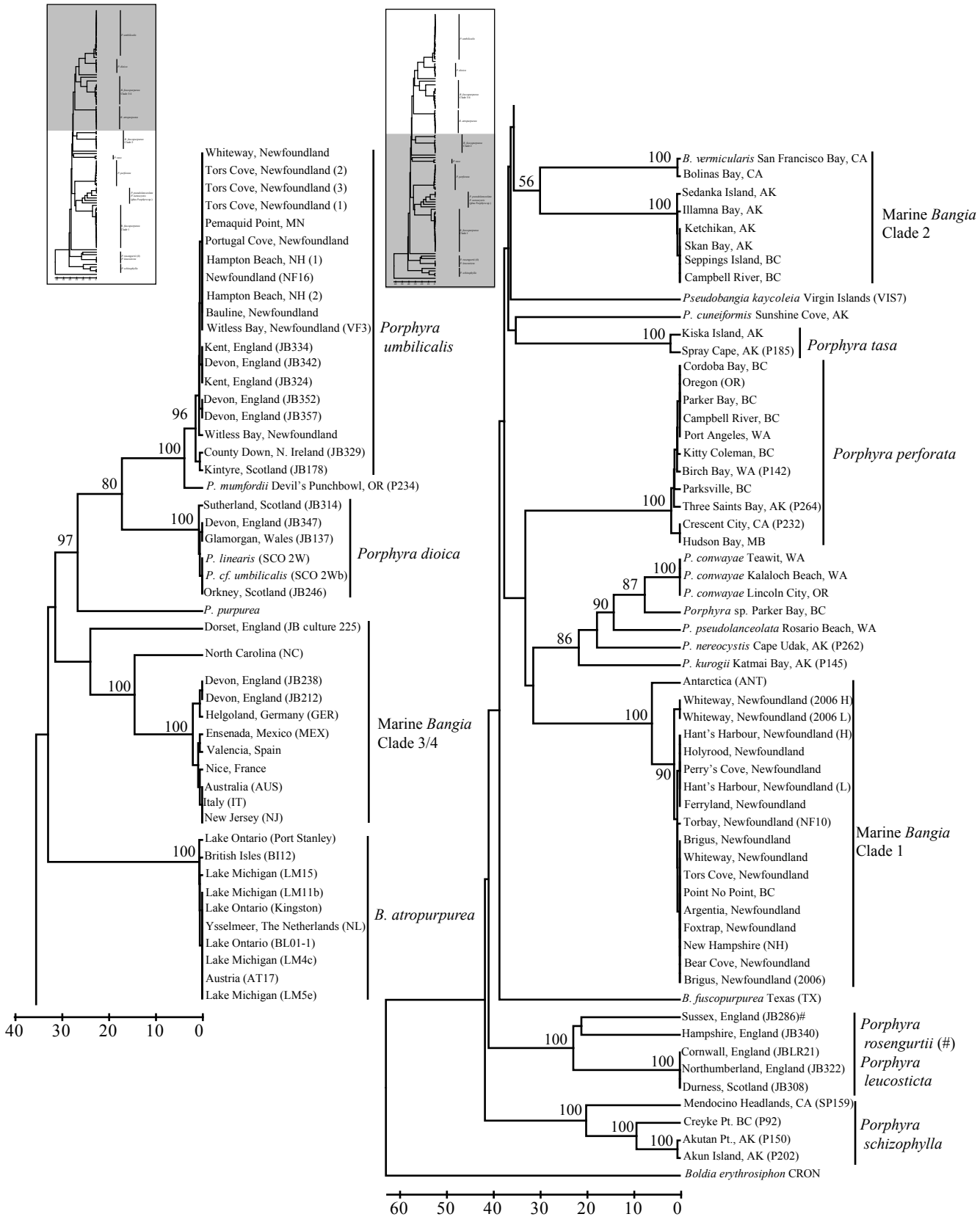
Identification of two *Porphyra* isolates appeared to be incorrect. Within the *P. dioica* group, where all sequences were nearly identical (0-2 nucleotide differences), there were two Scottish isolates identified as *P. linearis* and *P. cf. umbilicalis*. As all samples in this group are from the British Isles it is likely that the isolates are misidentified *P. dioica*. All samples collected in the northwest Atlantic and identified as *P. umbilicalis* grouped with *Porphyra*

*umbilicalis* isolates from the northeast Atlantic (British Isles). Isolates from this group were nearly identical (0-4 nucleotide differences), despite the broad geographical distribution. A sequence at the base of this group, *P. mumfordii* (Oregon) had a consistent 12-15 nucleotide differences from isolates in the *P. umbilicalis* group.

There was a single monotypic genus from the Bangiales included in this study, *Pseudobangia kaycoleia*. This isolate had a minimum of 75 nucleotides differences from all other Bangiales sequences.

The clustering (UPGMA) results were strongly supported by neighbor joining bootstrap analysis (Figure 3-1). Each distinct *Porphyra* group at the species level had greater than 95% bootstrap support, with most such groupings fully supported. There was weaker support for *Porphyra* groups that included multiple species. For example the *Porphyra conwayae/pseudolanceolata/nereocystis/kurogii* grouping had 86% bootstrap support. Similarly, terminal groupings of *Bangia* sequences were often fully supported in bootstrap analysis. In contrast, support decreased in two groups of marine *Bangia* that contained divergent sequences. Marine *Bangia* Clade 2 had two distinct, strongly supported groups corresponding to California isolates and the more northern Alaska and British Columbia isolates, but the association between these two groups was not strongly supported by bootstrap analysis. Similarly, the position of the marine *Bangia* from Dorset, England in Marine *Bangia* Clade 3/4 was not supported by phylogenetic analysis even though the grouping of the remaining sequences in the group had full phylogenetic support.

**Figure 3-1.** Phenogram for the unweighted pair group method with arithmetic mean (UPGMA) clustering analysis of the COI gene sequence data for all taxa. Node support values were derived from 1000 parametric bootstrap replicates using the GTR+I+G model of sequence evolution. Clade number designations for *Bangia* follow Müller *et al.,* (Müller et al., 2005).


Whiteway, Newfoundland
Tors Cove, Newfoundland (2)
Tors Cove, Newfoundland (3)
Tors Cove, Newfoundland (1)
Pemaquid Point, MN
Portugal Cove, Newfoundland
Hampton Beach, NH (1)
Newfoundland (NF16)
Hampton Beach, NH (2)
Bauline, Newfoundland
Witless Bay, Newfoundland (VF3)
Kent, England (JB334)
Devon, England (JB342)
Kent, England (JB324)
Devon, England (JB352)
Devon, England (JB357)
Witless Bay, Newfoundland
County Down, N. Ireland (JB329)
Kintyre, Scotland (JB178)
Porphyra umbilicalis
P. mumfordii Devil's Punchbowl, OR (P234)
Sutherland, Scotland (JB314)
Devon, England (JB347)
Glamorgan, Wales (JB137)
P. linearis (SCO 2W)
P. cf. umbilicalis (SCO 2Wb)
Orkney, Scotland (JB246)
Porphyra dioica
P. purpurea
Dorset, England (JB culture 225)
North Carolina (NC)
Devon, England (JB238)
Devon, England (JB212)
Helgoland, Germany (GER)
Ensenada, Mexico (MEX)
Valencia, Spain
Nice, France
Australia (AUS)
Italy (IT)
New Jersey (NJ)
Marine Bangia Clade 3/4
Lake Ontario (Port Stanley)
British Isles (BI12)
Lake Michigan (LM15)
Lake Michigan (LM11b)
Lake Ontario (Kingston)
Ysselmeer, The Netherlands (NL)
Lake Ontario (BL01-1)
Lake Michigan (LM4c)
Austria (AT17)
Lake Michigan (LM5e)
B. atropurpurea
40 30 20 10 0
B. vermicularis San Francisco Bay, CA
Bolinas Bay, CA
Sedanka Island, AK
Illamna Bay, AK
Ketchikan, AK
Skan Bay, AK
Seppings Island, BC
Campbell River, BC
Marine Bangia Clade 2
Pseudobangia kaycoleia Virgin Islands (VIS7)
P. cuneiformis Sunshine Cove, AK
Kiska Island, AK
Spray Cape, AK (P185)
Porphyra tasa
Cordoba Bay, BC
Oregon (OR)
Parker Bay, BC
Campbell River, BC
Port Angeles, WA
Kitty Coleman, BC
Birch Bay, WA (P142)
Parksville, BC
Three Saints Bay, AK (P264)
Crescent City, CA (P232)
Hudson Bay, MB
Porphyra perforata
P. conwayae Teawit, WA
P. conwayae Kalaloch Beach, WA
P. conwayae Lincoln City, OR
Porphyra sp. Parker Bay, BC
P. pseudolanceolata Rosario Beach, WA
P. nereocystis Cape Udak, AK (P262)
P. kurogii Katmai Bay, AK (P145)
Antarctica (ANT)
Whiteway, Newfoundland (2006 H)
Whiteway, Newfoundland (2006 L)
Hant's Harbour, Newfoundland (H)
Holyrood, Newfoundland
Perry's Cove, Newfoundland
Hant's Harbour, Newfoundland (L)
Ferryland, Newfoundland
Torbay, Newfoundland (NF10)
Brigus, Newfoundland
Whiteway, Newfoundland
Tors Cove, Newfoundland
Point No Point, BC
Argentia, Newfoundland
Foxtrap, Newfoundland
New Hampshire (NH)
Bear Cove, Newfoundland
Brigus, Newfoundland (2006)
Marine Bangia Clade 1
B. fuscopurpurea Texas (TX)
Sussex, England (JB286)#
Hampshire, England (JB340)
Cornwall, England (JBLR21)
Northumberland, England (JB322)
Durness, Scotland (JB308)
Porphyra rosengurtii (#)
Porphyra leucosticta
Mendocino Headlands, CA (SP159)
Creyke Pt. BC (P92)
Akutan Pt., AK (P150)
Akun Island, AK (P202)
Porphyra schizophylla
Boldia erythrosiphon CRON
60 50 40 30 20 10 0

## 3.5 DISCUSSION

The findings here suggest that the COI gene is useful as a primary DNA barcoding candidate in the Bangiales, consistent with previous studies of red algae (Saunders, 2005; Robba et al., 2006); however, in some instances a secondary genetic marker may be required. In order to fully determine the utility of the COI marker across all bangiophyte taxa a modification of current protocols such as the utilization of degenerate or alternative primers is required. Current protocols, most commonly applied to the more species rich Florideophyceae work moderately well for the Bangiales, as there was moderate to highly successful amplification of all lineages attempted. This is not a surprising result as these are sister groups within the Rhodophyta. Despite this success in the Bangiales, there was a non-trivial failure rate of both PCR amplification and DNA sequencing using published barcoding primers and protocols, which was higher than that for the nSSU rRNA gene for the same isolates. The non-Bangiales bangiophytes appear to be even more problematic and more work will be required to consistently obtain COI sequences for these taxa. Interestingly, this is despite the fact that the sequenced mitochondrial genomes for *Cyanidium* and *Cyanidioschyzon* show full identity with the barcoding primers used (GazF1 and GazR1, Saunders, 2005) within the COI gene. As *Cyanidium* and *Cyanidioschyzon* represent the most divergent lineage of bangiophyte red algae, this identity is surprising. The difficulties in obtaining COI sequences observed here need to be overcome before this gene region is confirmed at the most suitable candidate for DNA barcoding of all of the Rhodophyta.

Efforts to improve amplification of COI in samples had mixed results. For example, decreasing the annealing temperature did marginally increase the number of amplicons; however, many of these were contaminants. For example, an amplification of a *Porphyridium apyrenoidium* sample yielded a sequence sharing highest identity with COI sequences from predominantly marine predators of the class Hydrozoa. Many of the non-Bangiales samples were from existing whole genome extractions and could have degraded mitochondrial genomes. This, however, is not likely, as samples from Bangiales preserved and maintained under the same conditions did not demonstrate the same degree of difficulty in PCR amplification and sequencing.

The COI gene sequences reported here suggest even more cryptic species in the Bangiales than are currently recognized among the multiple lineages of *Bangia* (Müller et al., 2005; Lynch et al., 2008). In general the potential for cryptic species is less prominent in the more morphologically diverse *Porphyra* species. Despite this, there are some instances of high sequence diversity within identified *Porphyra* species. *Porphyra schizophylla* demonstrated a large sequence divergence between two isolates from Alaska, which were nearly identical morphologically, and a California isolate, which was the most divergent of the group (Figure 3-1). This was also noted by Lindstrom (Lindstrom, 2008a) using chloroplast-*rbc*L sequence data. The most commonly occurring *Porphyra* species in our collections from the northeast Pacific was *P. perforata.* Based on the sequence of the COI gene, this species appears to exhibit cryptic diversity, as there was two distinct groups of this species, with the California isolate, grouped with a Manitoba isolate, again the most divergent. The sequence identity between the Crescent City, California and Hudson Bay,

Manitoba isolates in this case is notable due to geographic separation. It is possible that one of these two isolates is the result of human transport.

The situation of *P. perforata* contrasts with that of *P. umbilicalis*, which similarly has two distinct genetic groups, isolates JB178 and JB329 in one group and the remaining northern Atlantic isolates in the other. In a previous study focusing on some of the sequences used here, these groups were postulated as an incipient speciation event (Robba et al., 2006), consistent with the results of this study. Furthermore, this appears to be a well-sampled taxon in this study, as samples of *P. umbilicalis* from England (northeast Atlantic) and from New Hampshire to Newfoundland (northwest Atlantic) showed high sequence identity.

My results indicate that each independent grouping of marine *Bangia* in this study requires a different genus designation, if *Porphyra* is not to be collapsed into *Bangia*, and possibly an even more extensive taxonomic realignment including changes to familial taxonomy. It is also apparent that the previously recognized clades are not monotypic and include additional cryptic diversity. The Antarctic isolate of marine *Bangia* is sufficiently divergent from the remaining, closely grouped isolates of Clade 1 to suggest it is a different species. This would be consistent with the wide geographic disjunction between these samples although additional southern Atlantic samples would help confirm these relationships. Similarly, marine *Bangia* Clade 2 also demonstrates strong evidence of two related northeast Pacific species, one more northern and one more southern, as suggested previously (Lynch et al., 2008). The similarity of the Bolinas Bay *Bangia* and the *Bangia vermicularis* sequences despite their morphological differences provides an additional complication for differentiating these species. This may be an instance where the COI gene is

not sufficient for discrimination of distinct species or it may represent an example of interbreeding. The sequence divergence associated with marine *Bangia* clade 3-4 suggests at least three species within this group. The recognition of two *Bangia* isolates (Dorset and NC) lacking any closely related sequences indicates that further sampling of marine *Bangia* for northern European, British, and temperate western Atlantic sites is required.

One sample of marine *Bangia* from Texas occupied a long branch. This is remarkable as previous *rbc*L and nSSU rRNA gene phylogenies clearly placed this isolate within marine *Bangia* clade 2 (Müller et al., 2005; Lynch et al., 2008). There may be a biological basis for such placement; however, the potential for it to be an artifact needs to be addressed. This suggests that an additional DNA barcoding gene might be required for such cases. Unfortunately, these cases can only be identified through phylogenetic analysis of alternative sequence data.

There does appear to be considerable utility in the use of the 5' region of COI gene for species discrimination in the bangiophyte red algae. The gene demonstrated a high degree of sequence identity (typically >99%) for isolates within the same sequence group relative to phylogenies using other genes such as *rbc*L and nSSU rRNA (Müller et al., 2001a, 2005; Broom et al., 2004; Lynch et al., 2008). Similarly, the discrimination between sequence groups was clear (i.e. there was a clear set of sequence 'islands' in the majority of cases). There were, however, some inconsistencies that need to be addressed before the COI gene can be adopted as the sole DNA barcoding region for all of Rhodophyta. Most significantly, the inability to amplify and sequence the gene in many lineages of non-Bangiales bangiophytes needs to be addressed. Additionally, it is unclear whether the gene can

discriminate local endemic species (e.g. *Bangia vermicularis*), and it is unclear what it means when there are disagreements between COI species groupings and those defined by other gene phylogenies.

# Chapter 4
# Supraordinal Taxonomy Within the Rhodophyta: Insights Using nSSU rRNA Secondary Structure Information and *rbc*L Sequence Data

## 4.1 OVERVIEW

The red algae are an early-diverged lineage of Eukaryotes occurring globally in a wide range of habitats. These organisms have been traditionally placed into one division (Rhodophyta) and divided into two subclasses (Bangiophyceae *sensu lato* and Florideophyceae), a taxonomy subject to recent and ongoing revision. Due primarily to the age of the lineage, recent phylogenetic analyses of the Bangiophyceae *sensu lato* have either low or inconsistent support for many ancestral branching events, making taxonomic conclusions problematic. The objectives of this study were to (1) evaluate ordinal and supraordinal taxonomy of the Bangiophyceae *sensu lato* by novel, structurally informed phylogenetic analyses of publicly available sequence data of nuclear and plastid genes and to (2) propose a revised taxonomy for these organisms.

Analysis of structural characteristics of the nuclear SSU rRNA gene was used to improve phylogenetic signal. Additionally, deep phylogenetic relationships were evaluated by focusing analyses on slowly evolving conserved nucleotide sites. Clades of taxa were also screened for orthologous elements of secondary structure including the lengths of loop regions and non-canonical nucleotide pairs, providing support for phylogenetic results.

These analyses increased resolution and metrics of phylogenetic support for higher-order branching in the Bangiophyceae *sensu lato* relative to the taxonomic literature. This increased resolution provided insights into ancient evolutionary events in the Rhodophyta such as branching order, cladogenesis and the taxonomic relationships among the unicellular Rhodophyta. A revised taxonomy is proposed for the Bangiophyceae *sensu lato* specifically reflecting higher-order phylogenetic relationships. Subphylum, class and ordinal proposals are discussed.

## 4.2 INTRODUCTION

The red algae (Rhodophyta) are a globally distributed phylum within the eukaryotic domain containing ecologically and economically significant species. These organisms are an early diverging lineage of eukaryotes present in the fossil record dating 1,250 million years ago (Mya) and include the oldest taxonomically resolved eukaryote *Bangiomorpha pubescens,* which closely resembles the extant genus *Bangia* (Butterfield, 2000; Butterfield et al., 1990). Since they are an early diverging Eukaryotic lineage, the study of red algal systematics provides an insight into early eukaryotic evolution and the evolution of the red lineage of chloroplasts (Oliveira and Bhattacharya, 2000; Müller et al., 2001a; Chu et al., 2004; Keeling, 2004; McFadden and Dooren, 2004; Yoon et al., 2004, 2005, 2006a).

Organisms within the Rhodophyta can be broadly divided among the florideophytes (class Florideophyceae) and the bangiophytes (class Bangiophyceae *sensu lato [s.l.]*). Traditionally, the taxonomy within the Rhodophyta has been based primarily on morphological characters, which are generally subject to homoplasious evolution. Many

characters also exhibit phenotypic plasticity and therefore do not effectively delineate species (Sheath and Cole, 1984; Stiller and Waaland, 1993; Ragan et al., 1994). There are few synapomorphic morphological characters delineating species within the red algae (Garbary and Gabrielson, 1990), and there is no synapomorphy defining the entire group. Furthermore, organisms within the Bangiophyceae *s.l.* exhibit a wide range of morphological variation, from unicells to simple filaments to sheets of cells. Due to this paucity of distinguishing morphological characters and convergence (e.g. the unicellular order Porphyridiales *s.l.* is polyphyletic), current taxonomy of the Bangiophyceae *s.l.* does not reflect phylogeny.

Molecular gene phylogenies have resolved some taxonomic inconsistencies within the Bangiophyceae *s.l.* (Freshwater et al., 1994; Ragan et al., 1994; Oliveira et al., 1995; Müller et al., 1998, 2001a, 2005; Saunders and Hommersand, 2004; Yoon et al., 2006a). Chief among these resolutions is the recognition of the Bangiales as a sister clade to the Florideophyceae and that the Porphyridiales *s.l.* consist of three separate lineages of unicellular red algae (Saunders and Hommersand, 2004; Yoon et al., 2006a). The Bangiophyceae *s.l.* is therefore paraphyletic and taxonomy does not reflect phylogeny. While recent taxonomic treatments have resolved some relationships within the Rhodophyta (Table 1-1), a comprehensive, well-supported and large-scale treatment of supra-ordinal taxonomy is needed for the Bangiophyceae *s.l.*

Phylogenetic resolution from molecular sequence data can be problematic, especially when dealing with large evolutionary time scales as in the Rhodophyta. Consequently, some ancestral nodes in the Bangiophyceae *s.l.* are poorly resolved. While the inclusion of more data (e.g. novel gene sequences) in analyses has successfully resolved difficult phylogenetic

relationships (Soltis et al., 1999; Baldauf et al., 2000; Dunn et al., 2008), these data are often difficult to reliably generate, especially for a morphologically simple group such as the Rhodophyta. Furthermore, much of the phylogenetic signal within the data can be muted if incorrect evolutionary models are applied or the genes are not evaluated independently (Dornburg et al., 2008). Commonly applied models of sequence evolution treat individual characters as independent and fixed mutation events as stochastic. These models have been useful in previous phylogenetic studies of the Rhodophyta; however, they are a simplification of evolutionary processes in RNA. Additionally, while a large and taxonomically diverse data set of nSSU rRNA sequence data for the Bangiophyceae *s.l.* has been developed and analyzed in fragments, the full utility of the data set has yet to be realized. Novel analyses of currently available high-quality sequence data could yield important taxonomic results.

In addition to the utilization of appropriate models of RNA evolution the secondary structure of these organisms can be further evaluated as ‘molecular signatures'. The core structure of the nSSU rRNA is remarkably conserved across much of the Eukarya; however, variable regions including some secondary structures can be highly variable among taxa, already observed within the Rhodophyta (Müller et al., 2002). Furthermore, these variations occur in regions of the sequence alignment that are typically excluded from phylogenetic analysis because of difficulties in alignment resulting from high rates of nucleotide substitution. Since structural patterns evolve more slowly than constituent nucleotides, an analysis of these characters among structurally aligned sequences may provide additional resolution for branching relationships among the Bangiophyceae *s.l.*

The objective of this study was to re-evaluate previously unresolved or poorly resolved ordinal and supra-ordinal taxonomy within the Bangiophyceae *s.l.* by applying models of nucleotide evolution more closely mirroring evolutionary constraints on RNA secondary structure. These analyses were performed on the largest and most taxonomically diverse set of Bangiophyceae *s.l.* nSSU rRNA gene sequences currently available. Further analysis of nSSU rRNA secondary structural signatures will be used to provide additional support for inferred phylogenies. The influence and utility of the incorporation of additional gene (the large subunit of RuBisCO, *rbc*L) sequence information on phylogenetic resolution are also addressed. Taxonomy for the Bangiophyceae *s.l.* congruent with these analyses is proposed.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Sequence alignment construction

Initial nSSU rRNA gene sequence alignments were provided by the Comparative RNA Website (Cannone et al., 2002), containing 392 Bangiophyceae *s.l.* and three Chlorophyta outgroup sequences. Alignments were constructed by adding sequences to existing Eukarya alignments corresponding to structural models of the nSSU rRNA derived from covariation analysis, which identifies conserved helices and base pairs among homologous molecules. Short sequences (< 800 nt) were removed and the remaining sequences were verified against GenBank to ensure sequence annotations were current. In order to limit redundancy in the data set identical sequences were removed leaving one representative sequence for each

group using JalView v.2.4 (Waterhouse et al., 2009), resulting in a total of 269 Bangiophyceae *s.l.* and three Chlorophyta outgroup sequences. The final alignment contained a total of 3303 sites, 991 of which were gap only sites maintained to preserve agreement with known secondary structure models. See Appendix A for sequence information, including GenBank accession numbers and collection information.

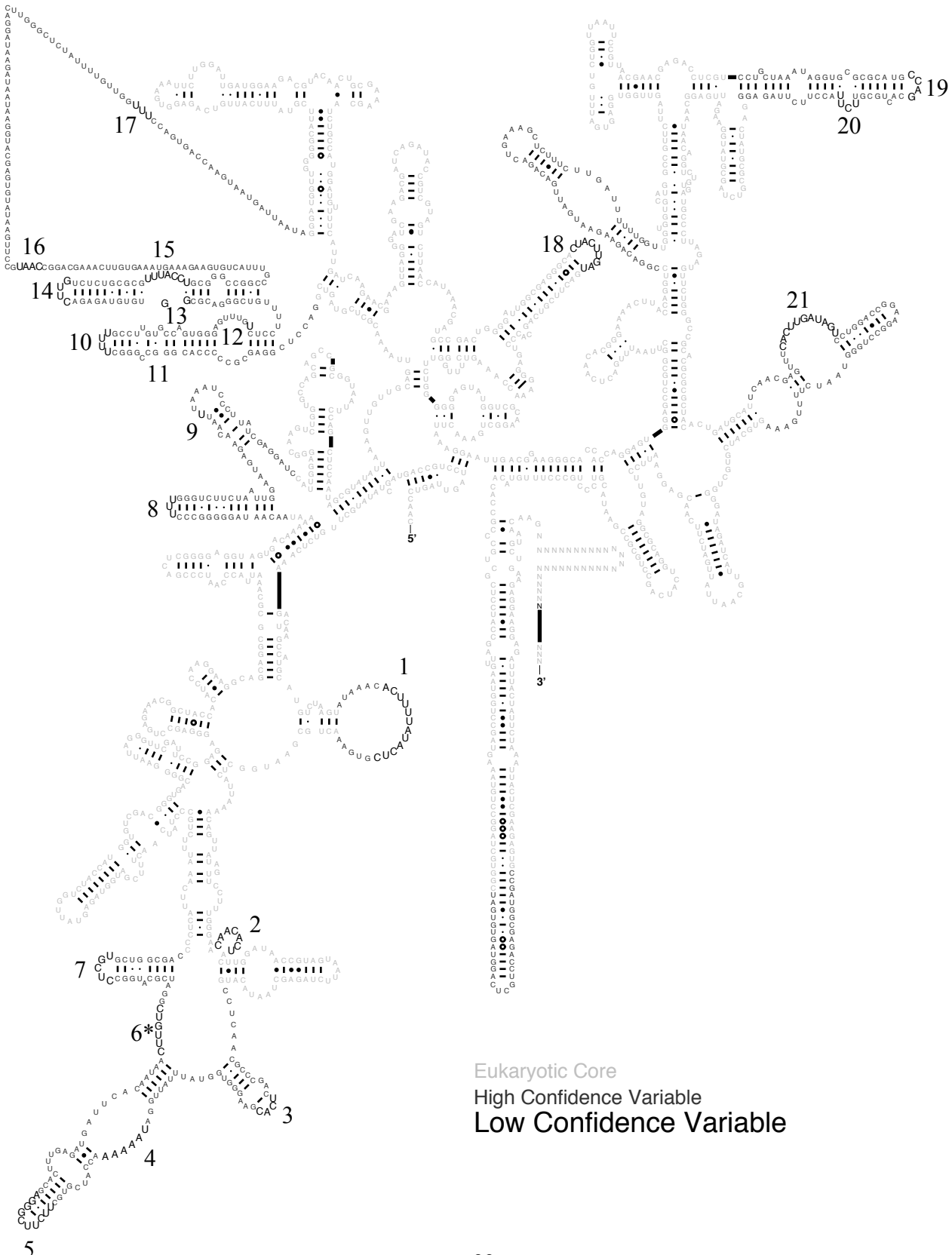
In order to further support phylogenetic results from nSSU rRNA gene sequence analyses and to investigate the utility of additional sequence information in the resolution of Bangiophyceae *s.l.* phylogeny, a two-gene sequence alignment was constructed. This alignment consisted of both the nSSU rRNA and the large subunit of RuBisCO (*rbc*L) for at least two Bangiophyceae *s.l.* species for each currently recognized order (Saunders and Hommersand, 2004; Yoon *et al.,* 2006a), dependent on sequence availability in GenBank. As the Bangiales contains the non-monophyletic genera *Bangia* (paraphyletic) and *Porphyra* (polyphyletic), taxa were selected representing known clades of these genera (Müller *et al.*, 2005; Lynch *et al.,* 2008). For each species, the *rbc*L nucleotide sequences were aligned using MUSCLE v.3.7 (Edgar, 2004) and then concatenated to the corresponding nSSU sequence from the previous structural alignment. In each case priority was given to sequences from the same individual or culture collection (noted in resulting phylogenies). In order to increase the numbers of included taxa, sequences were accepted if derived from either independent collections at the same geographic location or an algal culture collection that has been identified to the same species (i.e. nSSU rRNA and *rbc*L sequences from isolates of different culture collections). These instances are clearly noted in corresponding phylogenies. The resulting sequence alignment contained 30 Bangiophyceae *s.l.* taxa and

1028 sites more than the nSSU sequence alignment, for a total of 4331 sites (991 gap-only nSSU rRNA sites). No outgroup taxa were used in the multigene analyses due to the non-orthology of *rbc*L between the Rhodophyta and Chlorophyta (Keeling, 2004).

### 4.3.2 Phylogenetic analysis

The nSSU rRNA gene sequence alignment secondary structure model contained three categories of nucleotides (Figure 4-1): eukaryotic core (1323 sites), high confidence variable (777 sites) and low confidence variable (1203 sites). Gap only sites were distributed throughout all three categories, leaving 2306 nucleotide sites that were analyzed (1230 eukaryotic core, 658 high confidence and 418 low confidence variable). In general the low confidence sites corresponded to loop regions of the secondary structure, and due to the age of the lineage and the ambiguously aligned low confidence variable region, only high confidence and eukaryotic core regions were included in phylogenetic analyses.

**Figure 4-1.** A schematic representation of the secondary structure of the nuclear small subunit of ribosomal RNA showing regions of the eukaryotic core (light grey nucleotides), high confidence variable region (dark grey nucleotides, small font) and low confidence variable regions (black nucleotides, largest font, regions numbered 1-21 in the 5' to 3' direction). Structure is inferred and refined from the compensatory mutations observed across nucleotide sequence alignments and is available from the Comparative RNA Website (http://www.rna.ccbb.utexas.edu). Schematic is derived from the inferred structure of *Bangia fuscopurpurea* Northwest Territories (GenBank Accession #: AF043355). Note that the high and low confidence designations refer to quality of alignment sites and not confidence in the inferred structure.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20
21
5'
3'
Eukaryotic Core
High Confidence Variable
Low Confidence Variable

Maximum likelihood (ML) phylogenies were inferred using RAxML v.7.2.0 (Stamatakis, 2006) with the modified 16-state GTR model for paired nucleotides (16A) and the GTR+G model for non-interacting sites. The secondary structure of *Bangia fuscopurpurea* (NWT, GenBank Accession # AF043355) was used as the consensus secondary structure model for analyses. One hundred independent iterations of the RAxML v.7.2.0 algorithm were performed and the phylogeny with the best scoring likelihood was maintained. Default parameters were used as they gave better likelihood scores than manually set parameters. In order to provide support for nodes in the derived phylogeny, a combination of ML bootstrap (MLBS) and Bayesian posterior probabilities (BPP) were used. One thousand parametric bootstrap replicates were performed using RAxML v.7.2.0 using parameters as outlined above. Bayesian posterior probability support values were derived using a partitioned data set corresponding to paired and non-paired nucleotides. A total of 532 pairs of interacting nucleotides were derived from the structurally informed nSSU rRNA gene alignment and analyzed using the doublet model for paired sites implemented in MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001). This model considers that paired nucleotides in a stem region of RNA mutate to form another pair in a two-step process. Consequently, it is a 16-parameter model modeling the secondary structure of helical regions in RNA and is a slight minimization of the 16A model used in RAxML v.7.2.0 (see Savill et al., 2001) for review). Non-paired nucleotides were evaluated utilizing the GTR+G model of nucleotide sequence evolution. In order to facilitate convergence of independent runs the temperature parameter was increased to 0.22 and default values were used for all remaining parameters. Four independent chains for each of two independent runs were initiated and

allowed to run for 2,000,000 generations and trees were collected from the posterior distribution every 100 generations. To calculate the posterior probability of nodes, trees constructed before convergence of independent runs below a standard deviation of 0.01 of the independent runs were discarded. As this implementation is computationally expensive, 99% sequence identity groups were collapsed using Jalview 2.4 (Waterhouse *et al.,* 2009). Three deviations from this procedure were observed; i. sequences within the non-Bangiales Bangiophyceae *s.l.* were only removed if they were conspecific with the maintained sequence representative for the identity sequence group, ii. Bangiales sequences were only removed if they were congeneric with the maintained sequence representative for the sequence group and iii. due to the large number of *Porphyra* sequences belonging to *Porphyra* clade 2 (Müller *et al.*, 2005; Lynch *et al.,* 2008), these sequences were reduced as in ii., but based on 98% sequence identity. This procedure was observed to reduce duplication in the data set and facilitate analyses while maintaining unique taxonomic characteristics (e.g. observed paraphyly and polyphyly within the Bangiales). The final sequence alignment for Bayesian analyses contained 82 Bangiophyceae *s.l.* and three Chlorophyta outgroup sequences.

To account for the influence of the secondary structure nSSU rRNA model choice on phylogenetic analyses, all eight Bangiophyceae *s.l.* structural models currently available from the CRW website were each independently used in a single ML analysis as outlined above and phylogenetic tree topologies were compared. Similarly, as the choice of outgroups can significantly affect tree topology, nSSU rRNA gene phylogenetic analyses were duplicated with the three outgroup sequences removed (*Chlorella vulgaris*, *Parachlorella kessleri* and *Trentepohlia iolithus*).

For the multigene data set, sequences were partitioned into nSSU and *rbc*L character sets and analyzed concurrently. The nSSU rRNA partition was evaluated in the same manner as the full nSSU alignment, while the *rbc*L partition was evaluated using the GTR+G sequence model with the default search parameters in RAxML v.7.7.0. The highest scoring tree of 100 independent ML runs was taken as the most accurate. Support for nodes in the phylogeny was obtained by 1000 random Maximum Likelihood parametric bootstrap replicates and Bayesian posterior probabilities were derived by summarizing 2,000,000 iterations of two independent runs of four chains each. As above, only trees after convergence to 0.01 were included in the calculation of posterior probabilities. The nSSU rRNA partition was analyzed as above, while the *rbcL* partition was analyzed independently with the GTR+G model of sequence evolution. The temperature parameter was increased to 0.22 to facilitate convergence and default values for all other parameters were used. To characterize the influence of adding the *rbc*L gene on phylogenetic topology, this same taxa set was re-analyzed using only the nSSU rRNA gene using the same parameters as above. Similarly, the reduced taxa set was also analyzed using the GTR+G model for the nSSU rRNA gene to characterize the effect of secondary structural nucleotide models of evolution relative to common stochastic models.

All phylogenetic trees were visualized using FigTree v.1.2.2 (Rambaut).

#### 4.3.3 Characterization of Secondary Structure Elements

Full secondary structure models are available for only eight individuals corresponding to six of eight orders within the Bangiophyceae *s.l.*, making direct structural comparisons difficult. Consequently, surrogates for secondary structure including length of low confidence variable

regions (e.g. non-paired loops) as well as the conservation patterns of non-canonical base pairings were characterized as molecular signatures of evolutionary history. The length of each low confidence variable segment was deduced for each sequence in the nSSU rRNA gene sequence alignment and screened for length polymorphisms among major clades of bangiophytes. These were subsequently treated as stable characters for taxonomic inference and were mapped onto the inferred phylogeny to evaluate the evolutionary distribution of characters using maximum parsimony ancestral state reconstruction implemented in Mesquite v.2.6 (Maddison and Maddison).

The taxonomic distribution of non-canonical nucleotide pairings for each paired position in the nSSU rRNA gene sequence alignment was summarized from the structural sequence alignment. Only sites that contained a consensus non-canonical pair for at least one clade of Bangiophyceae *s.l.* were maintained and the congruence between the resulting set of paired sites and inferred phylogenies was further evaluated. All alignment and paired site processing was performed using PERL scripts, which are available upon request.

## 4.4 RESULTS

### 4.4.1 nSSU rRNA structural and multigene phylogenies

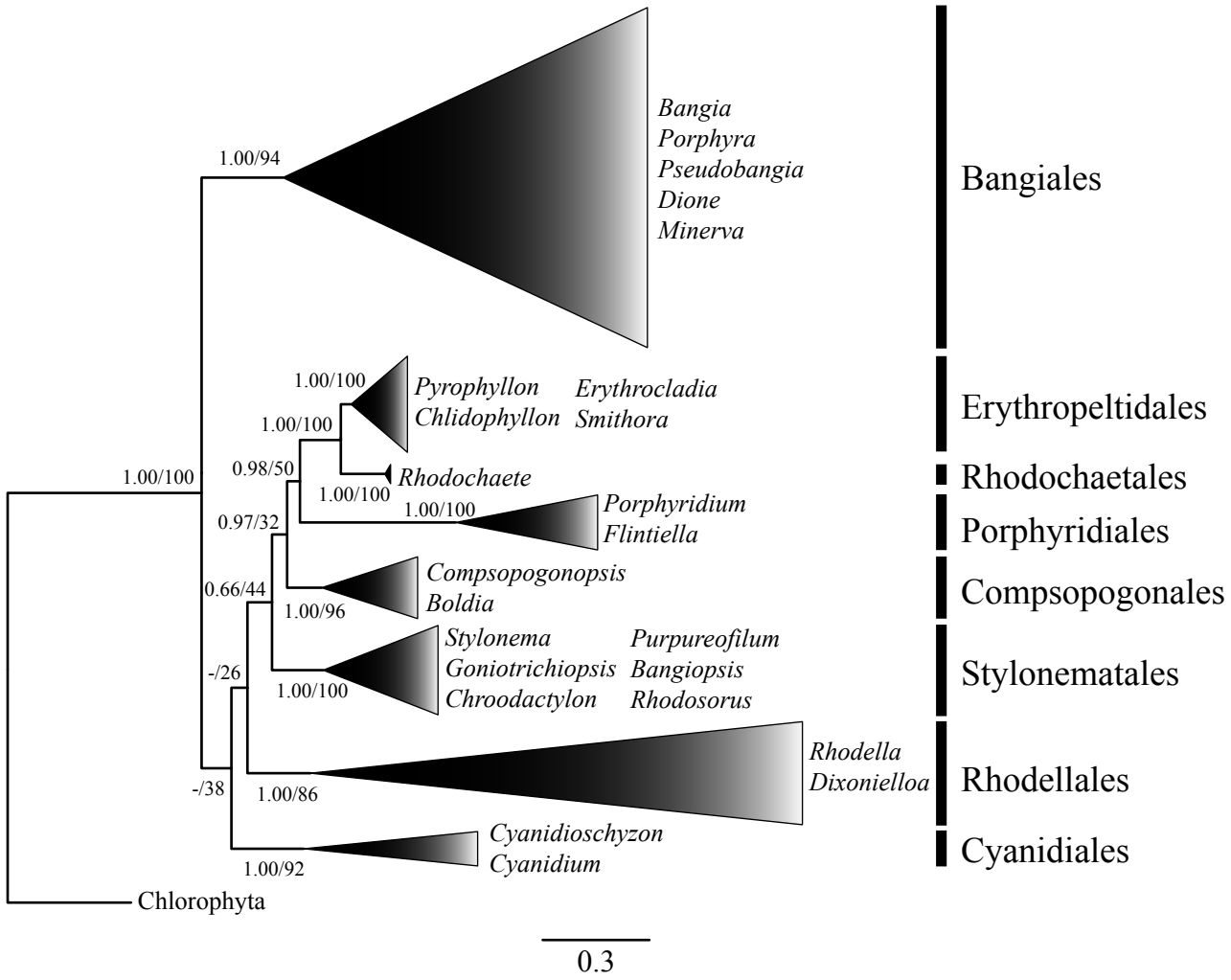
The nSSU rRNA gene sequence alignment contained representative taxa from all known lineages of Bangiophyceae *s.l.* (Yoon *et al.,* 2006a) as well as multiple lineages of the Bangiales (all known clades of marine *Bangia* and *Porphrya*, as well as the monotypic lineages of *Dione*, *Minerva*, *Pseudobangia* and freshwater *Bangia*). In these analyses, sequences that contained large (>100) runs of unknown nucleotides or did not cover the

entire gene region were excluded, as their phylogenetic placement was inconsistent. Furthermore, highly divergent sequences with large amounts of sequence data missing from the gene interior (e.g. nSSU rRNA gene of *Galdieria* sp.) were excluded to reduce phylogenetic artifacts (e.g. long-branch attraction, disparate GC content), which similarly tended to disrupt placement of the Cyanidiales.

In phylogenetic analyses of the nSSU rRNA gene using secondary structure models of sequence evolution each Bangiophyceae *s.l.* ordinal clade had Bayesian posterior probability (BPP) support values of 1.00 and strong support from ML bootstrap analysis (MLBS), each ≥ 86% (Figure 4-2). Similarly, some supraordinal phylogenetic relationships were well resolved in these analyses, although BPP support was considerably higher than MLBS support (Figure 4-2). In ML analysis, based on the Chlorophyta root there were two lineages of Bangiophyceae *s.l.*, the Bangiales and all remaining lineages including the cyanidiophytes. Although this was the best scoring phylogeny and the topology was recovered in each of the independent ML iterations it was not supported by MLBS. Conversely, in Bayesian analysis there were three independent lineages of bangiophytes forming a polytomy at the node defining the Chlorophyta/Rhodophyta split, the Bangiales, the Cyanidiales and Rhodellales together and the remaining Bangiophyceae *s.l.* This separation of the non-Bangiales taxa into two separate clades was not well supported (0.57 BPP for Cyanidiales-Rhodellales and 0.73 BPP for the remaining taxa). A monophyletic relationship for the Stylonematales, Compsopogonales, Porphyridiales, Rhodochaetales and Erythropeltidales that was observed in ML analyses (Figure 4-2) was weakly supported in Bayesian analyses (0.66 BPP). Phylogenetic support at the supraordinal level was strongest

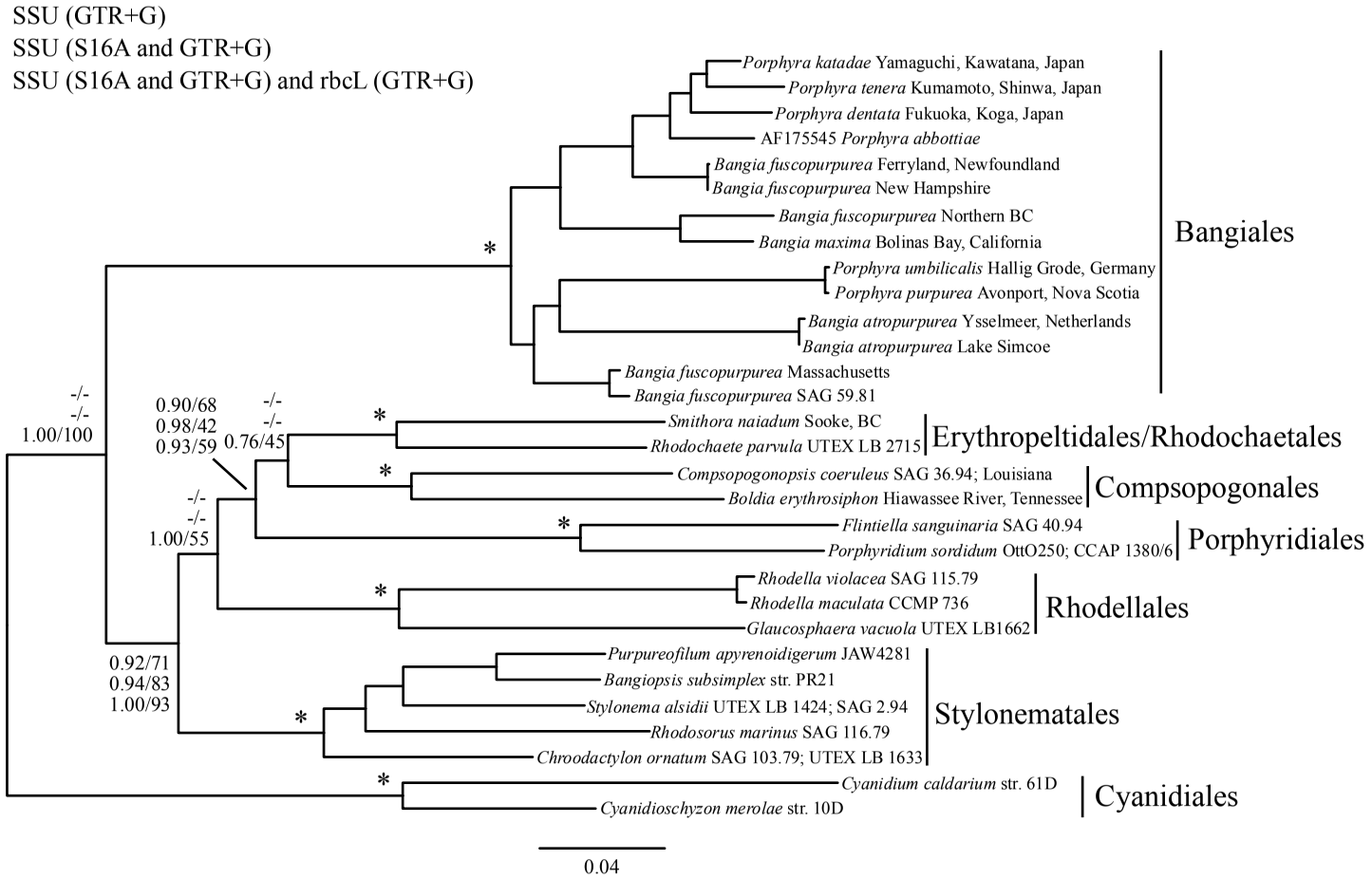
for the monophyly of the Porphyridiales with the Rhodochaetales, Erythropeltidales and Compsopogonales (0.97 BPP, Figure 4-2). This clade, however, was not supported by MLBS (32%). While there was clear support for *Porphyra* being polyphyletic with respect to *Bangia*, infra-ordinal taxonomy is beyond the focus of this study.

**Figure 4-2.** A schematic representation of the nuclear small subunit ribosomal RNA (nSSU rRNA) Bangiophyceae *s.l.* phylogeny derived from a structure-based model of sequence evolution S16A (GTR+G for non-paired sites). The phylogeny is a simplification of the highest scoring Maximum Likelihood tree across 100 independent iterations from 269 Bangiophyceae *s.l.* sequences. Support values represent Bayesian posterior probabilities and the results of 100 Maximum Likelihood parametric bootstrap replicates. Clade naming conventions follow Yoon *et al.* (2006a) or the revised taxonomy presented in this study.


Bangia
Porphyra
Pseudobangia
Dione
Minerva
Bangiales
1.00/94
1.00/100
Pyrophyllon
Chlidophyllon
Erythrocladia
Smithora
Erythropeltidales
1.00/100
0.98/50
Rhodochaete
Rhodochaetales
1.00/100
1.00/100
Porphyridium
Flintiella
Porphyridiales
0.97/32
Compsopogonopsis
Boldia
Compsopogonales
0.66/44
1.00/96
Stylonema
Goniotrichiopsis
Chroodactylon
Purpureofilum
Bangiopsis
Rhodosorus
Stylonematales
-/26
1.00/100
Rhodella
Dixonielloa
Rhodellales
-/38
1.00/86
Cyanidioschyzon
Cyanidium
Cyanidiales
1.00/92
Chlorophyta
0.3

Phylogenetic analyses of the reduced taxa set successfully demonstrated influences on phylogenetic resolution of structural (S16A/doublet) over non-structural (GTR+G) models of nucleotide evolution as well as the influence gene sequence information, in this case the *rbc*L (Figure 4-3). Similar to the strong ordinal support observed in the full complement nSSU rRNA gene phylogenetic analyses (Figure 4-2), each ordinal clade had full BPP and MLBS support (Figure 4-3). Similar to the species rich phylogeny, the majority of the supraordinal relationships were poorly supported by the MLBS metric, with notable exceptions of the monophyly of all Bangiophyceae *s.l.* (the Florideophyceae were not included in these analyses) and the clade containing all Bangiophyceae *s.l.* orders excluding the Bangiales and Cyanidiales. Although both structural and stochastic models gave the same tree topologies, the BPP support values tended to be higher with structural models. The relative phylogenetic positions of the Rhodellales and Stylonematales were variable among the different analyses of the reduced taxa data set. In the nSSU rRNA gene analyses the Stylonematales were monophyletic with the Porphyridiales, Compsopogonales, Rhodochaetales and Erythropeltidales with the Rhodellales branching from a basal position (phylogenetic topology not shown). While this is in agreement with the larger nSSU rRNA gene phylogeny (Figure 4-2), the Stylonematales was sister to the other orders in this clade when the *rbc*L gene was added to the analyses (Figure 4-3). In all analyses with the reduced taxa data set the Porphyridiales strongly resolved as monophyletic with the Erythropeltidales, Rhodochaetales and Compsopogonales. Similar to the larger nSSU rRNA gene phylogeny (Figure 4-2) this relationship was strongly supported by BPP (0.90-0.98) but not by MLBS (42-68%).

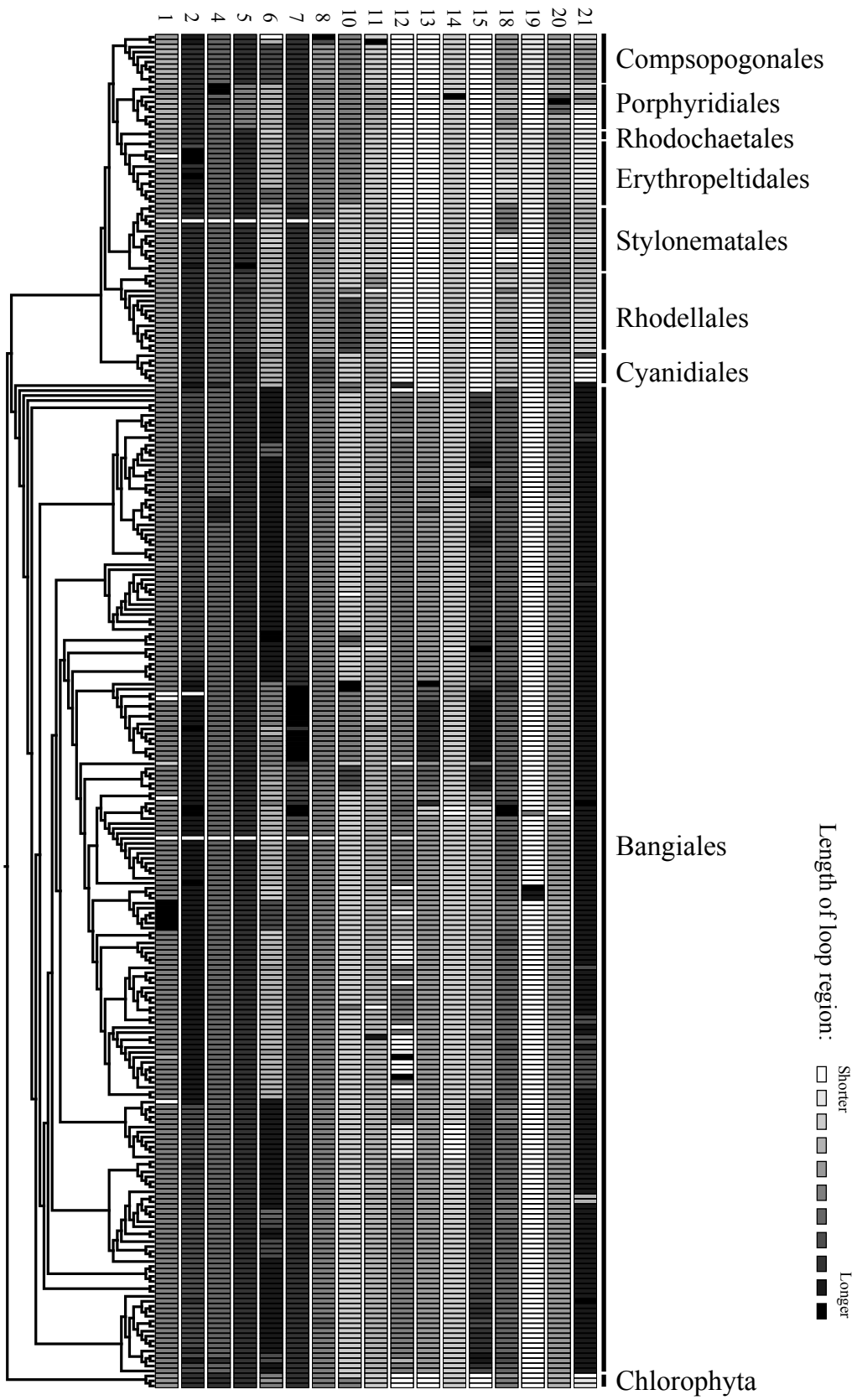
**Figure 4-3.** A midpoint rooted phylogeny demonstrating the influences of secondary structure models of evolution and additional gene sequence evolution on phylogenetic tree topology. The phylogeny represents the highest scoring Maximum Likelihood phylogeny derived from 100 independent random iterations, derived from a concatenated data set of nSSU rRNA and *rbc*L gene sequences. A secondary structure model of sequence evolution (S16A) was applied to paired nucleotide sites of the nSSU rRNA gene, while GTR+G model of nucleotide evolution was applied to unpaired sites of the nSSU rRNA gene and the *rbc*L gene. Support values represent the Bayesian posterior probability and 1000 iterations of a Maximum Likelihood parametric bootstrap for each of three cases. The first set of values corresponds to nSSU rRNA gene phylogeny with the GTR+G model of nucleotide evolution applied to all analyzed sites. The second set of values corresponds to the nSSU rRNA gene phylogeny with S16A applied to paired sites and GTR+G applied to non-paired nucleotide sites. The third set of values was derived using the same parameters as the presented phylogeny. Nodes not supported by analyses (either <0.50 BPP or 50% MLBS) are represented by a dash while full support for a node in all analyses (1.00 BPP and 100% MLBS) is represented by *. Clade naming conventions follow (Yoon *et al.,* 2006a) or the revised taxonomy presented in this study. Sequence identifiers indicate either collection location or culture collection identifier for the isolate (SAG = Sammlung von Algenkulturen, Culture Collection of Algae at the University of Göttingen; CCAP = Culture Collection of Algae and Protozoa).


SSU (GTR+G)
SSU (S16A and GTR+G)
SSU (S16A and GTR+G) and rbcL (GTR+G)
Porphyra katadae Yamaguchi, Kawatana, Japan
Porphyra tenera Kumamoto, Shinwa, Japan
Porphyra dentata Fukuoka, Koga, Japan
AF175545 Porphyra abbottiae
Bangia fuscopurpurea Ferryland, Newfoundland
Bangia fuscopurpurea New Hampshire
Bangia fuscopurpurea Northern BC
Bangia maxima Bolinas Bay, California
Porphyra umbilicalis Hallig Grode, Germany
Porphyra purpurea Avonport, Nova Scotia
Bangia atropurpurea Ysselmeer, Netherlands
Bangia atropurpurea Lake Simcoe
Bangia fuscopurpurea Massachusetts
Bangia fuscopurpurea SAG 59.81
Bangiales
Smithora naiadum Sooke, BC
Rhodochaete parvula UTEX LB 2715
Erythropeltidales/Rhodochaetales
Compsopogonopsis coeruleus SAG 36.94; Louisiana
Boldia erythrosiphon Hiawassee River, Tennessee
Compsopogonales
Flintiella sanguinaria SAG 40.94
Porphyridium sordidum OttO250; CCAP 1380/6
Porphyridiales
Rhodella violacea SAG 115.79
Rhodella maculata CCMP 736
Glaucosphaera vacuola UTEX LB1662
Rhodellales
Purpureofilum apyrenoidigerum JAW4281
Bangiopsis subsimplex str. PR21
Stylonema alsidii UTEX LB 1424; SAG 2.94
Rhodosorus marinus SAG 116.79
Chroodactylon ornatum SAG 103.79; UTEX LB 1633
Stylonematales
Cyanidium caldarium str. 61D
Cyanidioschyzon merolae str. 10D
Cyanidiales
-/-
-/-
1.00/100
0.90/68
0.98/42
0.93/59
-/-
-/-
0.76/45
-/-
-/-
1.00/55
0.92/71
0.94/83
1.00/93
*
0.04

### 4.4.2 Structural and Nucleotide Taxonomic Signatures

The large majority of structural variations among known resolved secondary structures of nSSU rRNA in the Bangiophyceae *s.l.* occurred in loop regions (Figure 4-1), which corresponded to low confidence sites in the nucleotide alignment. These variations were limited primarily to length polymorphisms of these variable regions, with no presence/absence of helices/loops apomorphic for specific lineages. A total of 17 low confidence regions with length polymorphisms were identified in the structural alignment and plotted on the optimal ML phylogeny (Figure 4-4). Overall, the length of variable regions was often consistent with taxonomic groupings, the most prominent discrimination being between the Bangiales and non-Bangiales taxa. For example there were 3 low-confidence regions (regions 12, 13 and 15, Figures 4-1, 4-4) that were only present in Bangiales sequences. Similarly, one loop was quite large in Bangiales sequences relative to non-Bangiales ones (region 21, Figure 4-1). Generally there was a minimization of loop lengths observed in non-Bangiales Bangiophyceae *s.l.* taxa indicating a minimization of the size of the nSSU rRNA within these lineages, at least for the sequences currently available.

**Figure 4-4.** Full nSSU phylogeny derived as in Figure 4-2 with nuclear small subunit ribosomal RNA (nSSU rRNA) low confidence length polymorphisms indicated. The horizontal boxes represent the lengths of individual low-confidence loops for each sequence, with darkness of shading directly proportional to the length of the region in that sequence (i.e. longer regions are darker), normalized to the longest length for that region observed. Numbering of horizontal boxes correspond to numbering of variable regions in Figure 4-1.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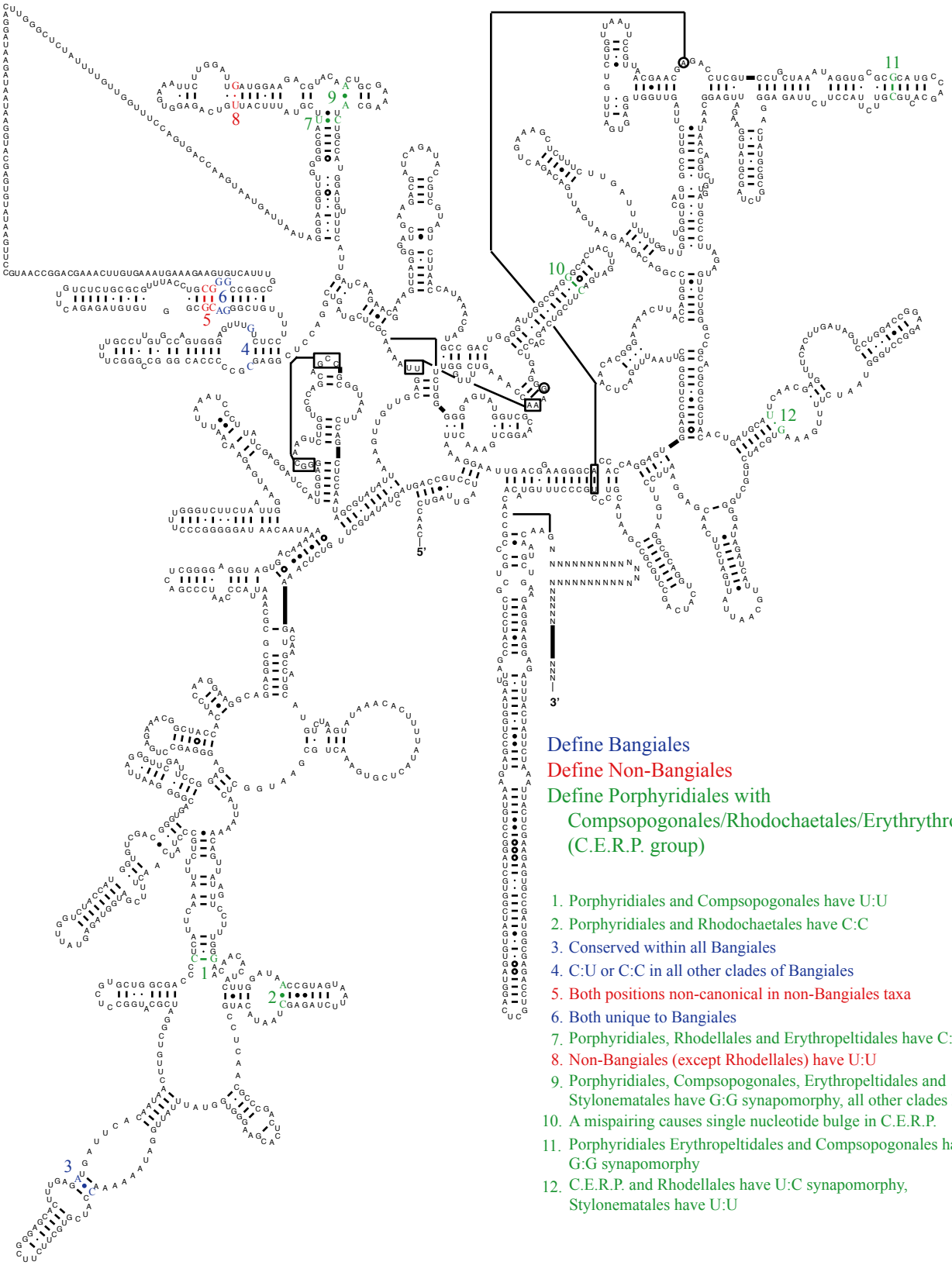
21
Compsopogonales
Porphyridiales
Rhodochaetales
Erythropeltidales
Stylonematales
Rhodellales
Cyanidiales
Bangiales
Chlorophyta
Length of loop region:
Shorter
Longer

In addition to loop length polymorphisms congruent with Bangiophyceae *s.l.* taxonomy, the distribution of non-canonical nucleotide pairs present in the nSSU rRNA secondary structures was informative. Canonical nucleotide pairings are common, structurally stable states and due to the ages of these lineages can be homoplasious. Consequently, only non-canonical pairings were investigated as possible taxonomic signatures suitable for reinforcing phylogenetic results. Intermediate or transitional forms were not weighted. The large majority of these pairs supported phylogenetic results (Figures 4-2, 4-3). Of the 532 pairs of interacting sites in the alignment, 99 pairs contained a non-canonical nucleotide pairing as the 90% majority consensus within at least one Bangiophyceae *s.l.* order. Of these 99 pairs, 75 showed some variation across the Bangiophyceae *s.l.* orders; 40 sites contained at least two different types of non-canonical pair and 35 contained at least one Bangiophyceae *s.l.* order with the same consensus canonical pair. Within the 75 variable non-canonical sites 29 sites were autapomorphic at the ordinal level and therefore not taxonomically informative within the scope of this study. Consequently, 46 remaining paired sites were useful as signatures of supraordinal taxonomy, only five of which were incongruent with inferred phylogenies (Figures 4-2, 4-3).

Non-canonical pairing signatures supported broad taxonomic delineations. For example, eight sites directly supported the separation of Bangiales and non-Bangiales taxa. Similarly, signatures were also useful in the discrimination of more derived ordinal relationships where seven pairs supported the monophyly of the Porphyridiales with the Compsopogonales, Erythropeltidales and Rhodochaetales clade. Furthermore, only a single site contradicts this phylogenetic relationship.

Non-canonical pairing site signatures also supported the placement of the Stylonematales with the non-Bangiales/Cyanidiales Bangiophyceae *s.l.* clade with a total of five non-canonical pairing sites that supported the relationship and none contradicting it. A structural summary of specific non-canonical sites consistent with Bangiales/non-Bangiales discrimination as well as the monophyly of the Porphyridiales with the Compsopogonales, Erythropeltidales and Rhodochaetales is presented in Figure 4-5.

**Figure 4-5**. Schematic of selected non-canonical nucleotide pairs supporting the separation of Bangiales and non-Bangiales taxa and the monophyly of the Porphyridiales with the Compsopogonales, Erythropeltidales and Rhodochaetales. Sites are relative to inferred structure of *Bangia fuscopurpurea* Northwest Territories (GenBank Accession #: AF043355). Taxonomically informative pairs are labeled numerically in the 5'-3' direction and summarized in the figure legend.




Define Bangiales

Define Non-Bangiales

Define Porphyridiales with Compsopogonales/Rhodochaetales/Erythrythropeltidales (C.E.R.P. group)

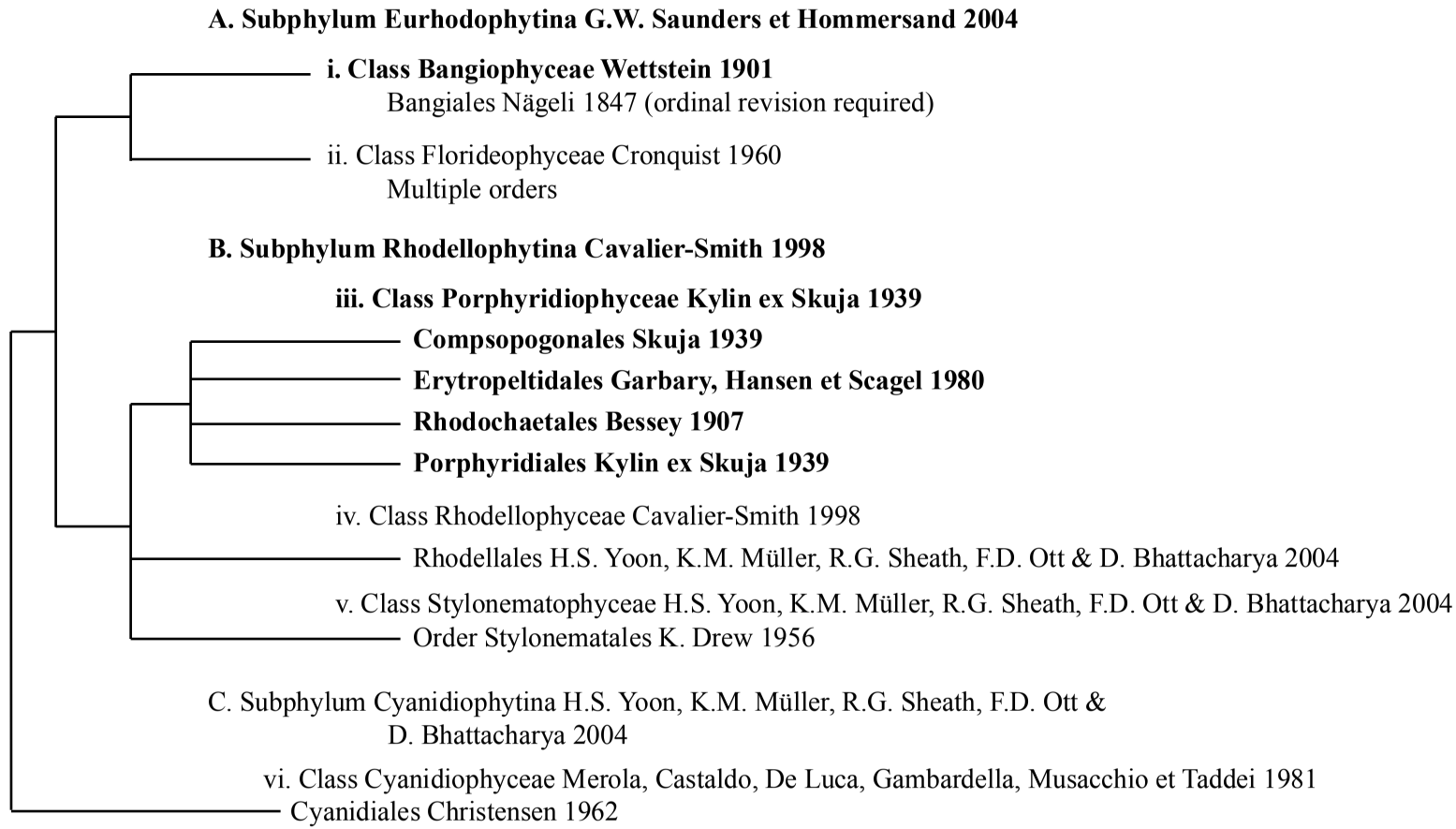
1. Porphyridiales and Compsopogonales have U:U
2. Porphyridiales and Rhodochaetales have C:C
3. Conserved within all Bangiales
4. C:U or C:C in all other clades of Bangiales
5. Both positions non-canonical in non-Bangiales taxa
6. Both unique to Bangiales
7. Porphyridiales, Rhodellales and Erythropeltidales have C:U
8. Non-Bangiales (except Rhodellales) have U:U
9. Porphyridiales, Compsopogonales, Erythropeltidales and Stylonematales have G:G synapomorphy, all other clades have A:A
10. A mispairing causes single nucleotide bulge in C.E.R.P.
11. Porphyridiales Erythropeltidales and Compsopogonales have G:G synapomorphy
12. C.E.R.P. and Rhodellales have U:C synapomorphy, Stylonematales have U:U

## 4.5 DISCUSSION

The results of the phylogenetic and structural signature analyses lead to the conclusion that the supraordinal classification of the traditional Bangiophyceae *sensu lato* (*s.l.*) required significant revision. Our treatment is summarized in Figure 4-6 and explained below. Unless otherwise stated, the delimitations of subphyla, classes, subclasses and orders in this section follow that presented in Figure 4-6.

**Figure 4-6.** Proposed revised taxonomy for the Bangiophyceae *s.l.* as suggested by nSSU rRNA gene phylogenetic analysis using structural models of nucleotide evolution. The taxonomy was further supported by combined nSSU rRNA and *rbc*L and non-canonical pair signature analyses. Bolded text represents deviations from (Yoon *et al.,* 2006a).

I. Phylum Rhodophyta Wettstein 1901



The incorporation of secondary structure information in large-scale phylogenies of the Bangiophyceae *s.l.* improved phylogenetic resolution of these taxa relative to previous studies (Müller *et al.,* 2001a; Saunders and Hommersand, 2004; Yoon *et al.,* 2006a). This highlights the importance of detailed, appropriate analyses of existing data as taxonomic studies migrate towards multigene phylogenies and phylogenomics in efforts to resolve taxonomic ambiguities (Dunn *et al.,* 2008). The increased phylogenetic resolution observed here relative to studies using stochastic models of sequence evolution (e.g. GTR) for nSSU rRNA gene sequence analysis stresses the significance of the application of biologically realistic sequence alignment and phylogenetic methods. Furthermore, the partitioning of data in multigene analyses, as performed here, allows the application of models that are appropriate for the different genes involved. It has been demonstrated that applying incorrect or unsuitable models of nucleotide evolution can strongly influence phylogenetic results (Buckley, 2002; Huelsenbeck and Rannala, 2004). This is especially relevant in studies involving rRNA molecules that conform to a distinct secondary structure, as applying a model suitable for genes with non-interacting sites (e.g. GTR) would result in under-parametrization of the RNA sequences in the analysis. Furthermore, as BPP values are more sensitive to under-parametrization of a model than over-parametrization (Huelsenbeck and Rannala, 2004), potential phylogenetic errors can be magnified when not incorporating structural information in the analysis of RNA sequences. Additionally, while model averaging may still be a suitable approach in many cases (e.g. where a suitably complex model such as GTR reflects the biology of the sequences involved), the use of such stochastic

models with RNA sequences discards a non-trivial amount of phylogenetic information present in the sequence alignment.

The improvements in phylogenetic resolution observed in this study relative to previous phylogenies can be further attributed to the improved alignment of nucleotides based on secondary structure of the nSSU rRNA and subsequent conservative selection of characters from the eukaryotic core or highly conserved variable regions for phylogenetic analyses. The Bangiophyceae *s.l.* span at least 1.2 billion years (Butterfield, 2000; Butterfield, 2001) and consequently demonstrate large sequence variation (Broom *et al.,* 2004; Müller *et al.*, 1998; Müller *et al.,* 2005; Lynch *et al.,* 2008) making reliable sequence alignment difficult. By aligning sequences based on known structures and pairing sites the ambiguity of the divergent sites in the nSSU rRNA gene sequence alignment was dramatically reduced, improving phylogenetic signal. The completed structural alignment subsequently clearly delineated between sites that were confidently aligned (eukaryotic core and high-confidence variable sites) and low confidence sites (Figure 4-1). The strong resolution of the analyses using stochastic models here (e.g. GTR+G of nSSU rRNA, Figure 4-3), indicated by the strong clade BPP and MLBS support values, can be attributed to an improved sequence alignment and the stringent exclusion of ambiguous sites (e.g. the complete exclusion of the low-confidence variable regions, which correspond to rapidly evolving loop sites). The combination of structural alignment and conservative inclusion of sequence sites was an effective first step in the resolution of some ambiguous phylogenetic relationships, especially for divergent sequences (i.e. either due to rapidly evolving lineages or ancestral sequences).

Both metrics of phylogenetic support used in this study, MLBS and BPP, demonstrated exceptionally strong support for ordinal clades, which is not surprising as this taxonomy is generally resolved (Saunders and Hommersand, 2004; Yoon *et al.,* 2006a); however, supraordinal relationships were not as clearly resolved by MLBS compared to BPP. For example, some relationships (e.g. Porphyridiophyceae as defined in Figure 4-1) were strongly supported by BPP but not MLBS. The calculation of each support metric differs significantly; however, specific to the results of this study BPP utilizes the entire sequence alignment in context (except for sites excluded from analysis), while bootstrapping resamples the alignment. Nucleotides involved in secondary structural interactions are paired and can be disproportionately removed from bootstrapping when sites are randomly resampled, leading to an increased representation of non-paired sites in the bootstrapping analyses. This further magnified the incidence of homoplasy in a bootstrap iteration making resolution of ancestral nodes in taxa as divergent as those in the Rhodophyta difficult. As clades with high BPP were recovered from each of the independent ML iterations regardless of the degree of MLBS support, the disparity of BPP and MLBS values for some supraordinal clades appeared to be artifacts of the bootstrapping procedure.

In this study, the increased phylogenetic resolution that came with the addition of a second sequence (*rbc*L) was present, though minimal (Figure 4-3), primarily resolving the position of the Rhodellales, moderately resolving the Cyanidiales position and increasing some MLBS values. Despite the rising use of multigene sequence information in phylogenetics there is still much taxonomic information in nSSU rRNA gene sequence data that has yet to be fully utilized. Consequently, a re-analysis of data already available is an

effective first step in both addressing outstanding taxonomic issues and accurately directing effort in future multigene and phylogenomic studies. Additionally, the use of multiple gene sequences in taxonomic research of algae can potentially be misleading, especially when mining existing sequence data from public databases, as morphological simplicity has led to a large number of cryptic species. Consequently, care must be taken to ensure each gene sequence in a multigene analysis is derived from the same species (i.e. by utilizing the same individual or culture collection). The considerable taxonomic reduction between nSSU rRNA (Figure 4-2) and multigene (Figure 4-3) analyses is indicative of this challenge.

### 4.5.1 Signatures in Secondary Structure

Molecular structure evolves at a much slower rate than the constituent sequence, consistent with the uniformity of all known nSSU rRNA structures across the Bangiophyceae *s.l.* taxa. For example, there were no novel nSSU rRNA loop signatures defining a major (e.g., ordinal) lineage other than the Bangiales. While there is evidence of unique signatures defining other lineages of Rhodophyta (Müller *et al.,* 2002), the monophyly and taxonomic position of the Bangiales is already generally accepted muting the significance of these unique morphometric sequence signatures. Alternatively, there were several less prominent structural signatures that helped characterize the different lineages of Bangiophyceae *s.l.* that further supported derived phylogenies.

Phylogenetically informative variation in the length of loop regions in the nSSU rRNA, an abstraction for secondary structure (Figure 4-4), demonstrated a minimization or simplification of the nSSU structures in the non-Eurhodophytina Bangiophyceae *s.l.* (i.e. lineages other than the Bangiales). It is possible that smaller nSSU rRNA structures are the

ancestral state and the larger structures within the Eurhodophytina reflect derived increases in size. This hypothesis needs further investigation. Simplified or minimized rRNA structures, especially in variable regions, has been previously noted in the literature (Caetano-Anollés, 2002a, b); although this has only been characterized in prokaryote taxa for which more taxonomically diverse complete structural information is currently available. In that case, streamlining of rapidly replicating genomes was suggested as one proposal for such minimization. Another related contributing factor in the Bangiophyceae *s.l.* may be asexuality as the lineages with minimized structures tended to be from known or putative asexual taxa. Furthermore, the structures of the putatively asexual *Bangia atropurpurea* are minimized relative to other Bangiales taxa, further supporting this trend; however, the mechanism for such patterns is unknown. Interestingly, the cryptically sexual Cyanidiales, which would have a higher likelihood of mutational changes due to environmental conditions, had remarkably stable loop lengths among the taxa studied (Figure 4-4). This stability is more notable when considering the high sequence variation across the Cyanidiales taxa here (Figures 4-2, 4-3) and in previous studies (Toplin et al., 2008).

### 4.5.2 Revisions to Rhodophyta taxonomy

The current taxonomy of the Bangiophyceae *s.l.* is somewhat well resolved at the ordinal level (Saunders and Hommersand, 2004; Yoon *et al.,* 2006a), further corroborated in this study with notably higher phylogenetic support in the majority of cases (Figures 4-2, 4-3). Resolving supraordinal relationships, on the other hand, has proven difficult. For example, one recent taxonomic scheme of the Bangiophyceae *s.l.* (Yoon *et al.,* 2006a) indicated a strong resolution of two lineages, the Bangiophyceae *sensu stricto* (*s.s.*) sister to the

Florideophyceae and the Cyanidiophyceae (Cyanidiales). All other orders of Rhodophyta were unresolved, forming a polytomous sister to the Bangiophyceae *s.s.* and Florideophyceae clade. While that study was a significant and necessary improvement in the taxonomic status of the Rhodophyta, this current study demonstrated novel phylogenetic resolution in the Bangiophyceae *s.l.* tree (Figures 4-2, 4-3). Here there was a distinct monophyly of Bangiophyceae *s.l.* taxa not belonging to the Bangiales or Cyanidiales (Figures 4-2, 4-3). Consequently, we suggest the resurrection of the subphylum Rhodellophytina *sensu* Cavalier-Smith (Cavalier-Smith, 1998) to represent the lineage (Figure 4-6), separating these taxa from the Rhodophytina *sensu* (Yoon *et al.,* 2006a), which contained all Rhodophyta except the Cyanidiales (Cyanidiophytina).

The second significant deviation from previous taxonomic schemes concerned the unicellular Porphyridiales. All phylogenies in this current study provided strong phylogenetic and nucleotide signature support for the monophyly of the Porphyridiophyceae (constituent orders include the Compsopogonales, Erythropeltidales, Rhodochaetales and Porphyridiales). The strong BPP support for this clade as well as the reproducibility of the relationship in ML iterations and the consensus support of non-canonical nucleotide pair signatures provide strong evidence that the taxonomic change is warranted. The taxonomic association of the unicellular Porphyridiales with the three multicellular orders is reflected in Figure 4-6 and provides insight into the development of multicellularity in these groups. For example, if clade branching of the orders in the multigene phylogeny (Figure 4-3) is reflective of the evolution of the Rhodophyta and the ancestral growth form is unicellular, it suggests that multicellularity evolved three times independently within the Bangiophyceae *s.l.*, once each

along the branches leading to the Bangiales and the Stylonematales and once after the split between the Porphyridiales and the other three orders Compsopogonales, Rhodochaetales and Erythropeltidales (Figure 4-3). This is a more parsimonious development of multicellularity than would be suggested by recent phylogenetic work in the Bangiophyceae *s.l.* (Yoon *et al.,* 2006a); however, this hypothesis requires significantly more research.

Supraordinal organization was not well supported in any of the individual phylogenies for the remaining bangiophyte ordinal clades, Stylonematales and Rhodellales. The inclusion of the *rbc*L gene in the reduced taxa set did demonstrate strong BPP resolution for the Rhodellales as sister to the Porphyridiophyceae clade. As this relationship was not resolved in either the inclusive taxa phylogeny or non-canonical nucleotide signatures, taxonomic changes reflecting this relationship have not been suggested here. As the resolution of ambiguous clades increased with the addition of a second gene sequence, the verification of the phylogenetic positions of the Rhodellales and Stylonematales will likely be accomplished with the addition of novel gene sequence information in future taxonomic studies; however, as the taxonomy was not resolved in phylogenies with a large number of chloroplast genes (Yoon *et al.,* 2006a), an increase in nuclear or mitochondrial genome sampling may be required. Due to this ambiguity, the Rhodellales and Stylonematales orders are maintained as a polytomy within the Rhodellophytina in the proposed taxonomy (Figure 4-6).

The Porphyridiales *sensu* Garbary and Gabrielson (1990) and van den Hoek *et al.,* (1995) were spread across at least three clades as was observed in other studies (Oliveira and Bhattacharya, 2000; Müller *et al.,* 2001; Yoon *et al.,* 2006a) and consistent with the most

recent taxonomic revisions in the Bangiophyceae *s.l.* (Saunders and Hommersand, 2004; Yoon *et al.,* 2006a). The orders Bangiales and Cyanidiales were phylogenetically distinct in multigene analysis (Figure 4-3), and are therefore placed here in the class Bangiophyceae (subphylum Eurhodophytina) and Cyanidiophyceae (subphylum Cyanidiophytina) respectively. The remaining red algae orders are more phylogenetically aligned (occupy the same depth within the tree) relative to the Bangiales and Cyanidiales. Consequently, the Porphyridiophyceae (orders Porphyridiales, Compsopogonales, Rhodochaetales, Erythropeltidales), Stylonematophyceae (order Stylonematales) and Rhodellophyceae (order Rhodellales) are placed in a separate subphylum, the Rhodellophytina.

Based on phylogenetic evidence presented here (Figures 4-2, 4-3), there are three lineages of Rhodophyta, in contrast to the two presented in Yoon *et al.,* (Yoon *et al.,* 2006a). In that study, the separation of the cyanidiophytes from the remaining Rhodophyta at the subphylum Cyanidiophytina is further supported by analyses here, most evident in combined sequence analyses (Figure 4-3). The further clear distinction between the Bangiales and the remaining Bangiophyceae *s.l.*, however, suggests the remaining Bangiophyceae *s.l.* be elevated to the same taxonomic depth, suggested here as the subphylum Rhodellophytina (Figure 4-6). As with Figure 4-6, taxonomic revisions suggested below are organized to be congruent with phylogenetic analyses (Figures 4-2, 4-3). Formal taxonomic descriptions follow for only those groups whose taxonomy deviates from the primary reference. Comments are presented where taxonomy suggested by this research warrants further clarification of the primary reference for the group.

**I. Phylum Rhodophyta Wettstein 1901**

**A. Subphylum Eurhodophytina G.W. Saunders et Hommersand 2004**

**i. Class Bangiophyceae Wettstein 1901**

Order Bangiales Nägeli 1847

Family Bangiaceae Engler 1892

Genera *Bangia, Dione, Minerva, Porphyra, Pseudobangia*

**ii. Class Florideophyceae Cronquist 1960** (multiple orders)

**B. Subphylum Rhodellophytina Cavalier-Smith 1998**

*Comments* – This subphylum is congruent with the description of Cavalier-Smith (Cavalier-Smith 1998) with a single exception; the inclusion of the Rhodochaetales, which were ascribed to the Bangiophyceae in that treatment.

**iii. Class Porphyridiophyceae Kylin ex Skuja 1939**

*Thalli filamentis simplicis uniseriatis, crustosis, foliosis, saccatis vel filamentosis ramosis aut uni-cellulibus. Thalli et uni-celluli cum chloroplastis stellatis singularis et pyrenoidbus centralis singularis aut cum chloroplastis parietalis singularis vel pluralis et cum vel sine pyrenoidis. Reproductio a divisione cellulis et a monosporangiis et spermatangiis aut reproductio sexualis non cognitus. Dulcis aut marinis.*

Plants of simple uniseriate filaments, crustose, foliose, saccate or branched filamentous thalli or unicells. Thalli and unicells with a single stellate plastid and central pyrenoid

per cell or single to multiple and parietal plastids with or without pyrenoids. Reproduction by cell division and by monosporangia and spermatangia or sexual reproduction unknown. Freshwater and marine.

Freshwater and marine.

Order Compsopogonales Skuja 1939

Family Boldiaceae Herndon 1964

Genus *Boldia*

Family Compsopogonaceae Schmitz in Engler et Prantl 1896

Genus *Compsopogon*

Order Erytropeltidales Garbary, Hansen et Scagel 1980

Family Erythrotrichiaceae G. M. Smith 1933

Genera *Erythrotricia, Chlidophyllon, Erythrocladia, Pyrophyllon, Sahlingia, Smithora*

Order Rhodochaetales Bessey 1907

Family Rhodochaetaceae Schmitz in Engler et Prantl 1896

Genus *Rhodochaete*

Order Porphyridiales Kylin ex Skuja 1939

Family Porphyridiaceae Skuja 1939

Genera *Porphyridium, Erythrobolus, Flintiella*

*Comments* - The class Porphyridiophyceae was well supported in all phylogenetic (Figures 4-2, 4-3) and non-canonical pair signature analyses, providing supraordinal organization for four orders within the Rhodophyta that have been previously grouped in a polytomy based on inconsistent phylogenetic results (Yoon *et al.,* 2006). Infraclass taxonomy was inconsistent in these analyses.

**iv. Class Rhodellophyceae Cavalier-Smith 1998**

Order Rhodellales H.S. Yoon, K.M. Müller, R.G. Sheath, F.D. Ott & D. Bhattacharya 2004

Family Rhodellaceae H. S. Yoon, K. M. Müller, R. G. Sheath, F. D. Ott et D. Bhattacharya 2004

Genera *Rhodella, Dixoniella, Glaucosphaera*

*Comments* - The Rhodellales contains the genera *Rhodella*, *Dixoniella*, and *Glaucosphaera*. Notably, *Glaucosphaera*, a glaucophyte and not traditionally considered within the Rhodophyceae, appears to be a reduced red algae lacking phycoerythrin and lacking the pyrenoid (Broadwater *et al.,* 1995), which it may have lost (Yokoyama et al., 2004). The Rhodellophyceae is clearly distinct from the Porphyridiales *s.l.* in phylogenetic analyses (Figures 4-2, 4-3). The Rhodellales clade is incomplete in that members of the Phragmonemataceae (*Phragmonema*) are not

included in phylogenetic analyses. It has been suggested (Saunders and Hommersand, 2004) that as *Phragmonema* has yet to be included the order should be considered Porphyridiales 1 to reduce "unnecessary taxonomic congestion" in the literature. Although *Phragmonema* is of historical precedence relative to *Rhodella*, those rules of precedence do not apply above the familial level (ICBN Art. 11.9). Consequently, due to the unique and recognizable ultrastructure of *Rhodella* (Patrone et al., 1991; Scott *et al.,* 1992) we are confident in supporting the order Rhodellales.

**v. Class Stylonematophyceae H.S. Yoon, K.M. Müller, R.G. Sheath, F.D. Ott & D. Bhattacharya 2004**

Order Stylonematales K. Drew 1956

Family Stylonemataceae K. Drew 1956

Genera *Stylonema, Bangiopsis, Chroodactylon, Chroothece, Purpureofilum, Rhodosorus, Rhodopsora, Rufusia*

**C. Subphylum Cyanidiophytina H.S. Yoon, K.M. Müller, R.G. Sheath, F.D. Ott & D. Bhattacharya 2004**

**vi. Class Cyanidiophyceae Merola, Castaldo, De Luca, Gambardella, Musacchio et Taddei 1981**

Order Cyanidiales Christensen 1962

Family Cyanidiaceae Geitler 1935

Genera *Cyanidium*, *Cyanidioschyzon*

Family Galdieriaceae Merola, Castaldo, De Luca, Gambardella, Musacchio et Taddei 1981

Genus *Galdieria*

## 4.6 CONCLUSIONS

These analyses did not represent the totality of species diversity and relationships within the Bangiophyceae *s.l.*, especially within the Cyanidiales and Bangiales as indicated by genetic diversity and paucity of morphological characters for identification. With the addition of sequences from more red algae the resolution of these phylogenies can be increased. It is clear that the evolutionary information in gene sequences currently available has not been fully utilized. Due to advancements in computational power, including novel algorithmic approaches, a re-evaluation of unresolved taxonomic relationships is advisable. Not only will this effectively resolve many difficult phylogenetic relationships, it will also help direct future efforts in multigene or phylogenomic taxonomic research within phycology.

# Chapter 5
# Ordinal and infraordinal taxonomy of the subphylum Eurhodophytina (Rhodophyta)

## 5.1 OVERVIEW

The Eurhodophytina is a subphylum recently erected to accommodate the well-recognized monophyly of the Bangiophyceae *sensu stricto* and Florideophyceae, a lineage excluding more ancestral-like Rhodophyta taxa. Despite considerable research within the subphylum, many taxonomic relationships are not reconciled with phylogeny. The largest and most taxonomically mature phylogenetic data set for the Eurhodophytina is the nuclear small subunit ribosomal RNA. Until recently, computational constraints have limited the robust concurrent evaluation of this large sequence data set. Furthermore, as patterns of nucleotide variation in RNA are constrained by their secondary structure, much of the phylogenetic information within these data has not been fully utilized.

The objectives of this study were to apply parametric structural models of RNA sequence evolution to a taxonomically diverse set of nSSU rRNA gene sequences from the Eurhodophytina in order to resolve outstanding taxonomic inconsistencies present at the ordinal and infraordinal level as well as identify poorly characterized areas of the species phylogeny, which require the development of robust multigene data sets.

The incorporation of parametric structural models of RNA sequence evolution increased the resolution of both the Bangiophyceae *s.s.* and Florideophyceae phylogenies relative to previous studies. The Bangiophyceae *sensu stricto* were very strongly

characterized as having a minimum of 16 independent generic lineages. In order to accommodate this taxonomic reassessment, isolates previously characterized as *Bangia* require at least three new genera and eight new genera should be erected to accommodate *Porphyra* species.

Taxonomic changes within the Florideophyceae suggested by these results are more subtle, including the resolution of topologies that already have precedence in the literature; however, these topologies were recovered with fewer numbers of genes and typically with a higher degree of confidence. These analyses also clearly identified groups of the Florideophyceae that require further taxonomic research, effectively directing the generation of novel data and increased sampling intensity.

## 5.2 INTRODUCTION

The sister evolutionary relationship between the Florideophyceae and Bangiophyceae *sensu stricto* (*s.s.*), which contains only the Bangiales, first proposed as the subclass Eurodophycidae (Magne, 1989) has been more generally recognized since the first large-scale phylogenetic treatments of the Rhodophyta using molecular sequences (Freshwater et al., 1994; Ragan et al., 1994). However, it wasn't until recently that taxonomies reflecting this relationship were proposed (Saunders and Hommersand, 2004; Yoon et al., 2006a). This hesitation was understandable as the relatively simple morphologies of species within the Bangiales share distinct similarities with simple, ancestral-like Rhodophyta rather than their sister class the Florideophyceae. For example, the Bangiophyceae *sensu lato* (*s.l.*) tend to be uninucleate while the Flordieophyceae tend to be multinucliate. Additionally, plastid morphology and position, cellular morphology (pit connections) and gametophyte

complexity tend to distinguish the Bangiophyceae *s.l.* and the Florideophyceae (see Ragan et al., 1994). These patterns in morphology explain the traditional classification of the Bangiophyceae *sensu lato* (*s.l.*); however, in light of a great deal of molecular phylogenetic evidence, the erection of the subphylum Eurhodophytina *sensu* Saunders and Hommersand (2004), containing the classes Florideophyceae and Bangiophyceae *s.s.*, was warranted.

The Eurhodophytina is the most morphologically diverse, conspicuous and species-rich group of red algae (Rhodophyta); however, existing phylogenetic treatments for these organisms have limited taxonomic breadth and many phylogenetic relationships are not well resolved. The nSSU rRNA gene region is, by a large margin, the most taxonomically comprehensive sequence data set currently available for the Eurhodophytina. This gene has a long history in Rhodophyta phylogenetics (Ragan et al., 1994); however, a large proportion of the phylogenetic information contained within the molecule has not yet been utilized. The nSSU rRNA molecule has a highly conserved secondary structure (Cannone et al., 2002), allowing for the inference of interacting pairs of nucleotide positions (helices) and non-interacting nucleotides (loops). By aligning sequences to an inferred model of secondary structure, patterns of interacting pairs can be inferred for a very large number of sequences, avoiding the time-intensive inference of structural models for each sequence. Traditional models of nucleotide evolution do not use the evolutionary information contained within these interactions, and by extension all published RNA phylogenetic treatments of the Rhodophyta using RNA molecules are simplifications of the underlying biology. The degree to which this simplification influences phylogenetic resolution, however, is not known.

The increase in biological accuracy and computational complexity associated with recently derived RNA-specific models of sequence evolution provide novel research potential for the taxonomically mature nSSU rRNA data set. In general, widely used parametric models (e.g. General Time Reversible (GTR), Jukes-Cantor) fail to account for structural interactions inherent in the RNA molecules. Models that parameterize these interactions, on the other hand, can increase phylogenetic signal and resolve taxonomic ambiguities. Furthermore, until recently, parametric structural models of nucleotide evolution have been difficult to implement for non-trivial phylogenies primarily due to the high number of rate parameters involved, e.g., 21 for the structural GTR seven-state model (Higgs, 2000) compared to six for the non-structural GTR model (Tavaré, 1986). The development of much more efficient Maximum Likelihood implementations, such as PhyML (Guindon and Gascuel, 2003) and RAxML (Stamatakis, 2006), now allow for the large-scale application of structural models to taxonomically diverse and complex data sets. The application of such models has been effective in resolving some ambiguous ancestral phylogenetic relationships within the Rhodophyta (Chapter 4). In that case, taxon sampling within the Bangiophyceae *s.l.* was low (i.e. relatively small number of taxa within each ordinal lineage). The more robust taxonomic sampling present in the Bangiophyceae *s.s* and the Florideophyceae should provide a data set appropriately complex for the strong resolution of the majority of infraordinal phylogenetic relationships.

The objectives of this study were to apply parametric structural models of RNA sequence evolution to a taxonomically diverse set of nSSU rRNA gene sequences from the subphylum Eurhodophytina in order to resolve outstanding taxonomic inconsistencies

present at the familial, ordinal and supraordinal level. Furthermore, these analyses will be used to identify taxonomic clades within the Eurhodophytina that remain unresolved or poorly resolved in nSSU rRNA gene phylogenies. Such clades therefore require the development of robust, multigene data sets in order to establish reliable taxonomy. The results of this study will efficiently direct future taxonomic research in the Rhodophyta.

## 5.3 METHODS

The sister-relationship between the two classes within the Eurhodophytina, the Bangiophyceae *s.s.* and Florideophyceae, is well established in the taxonomic literature (Magne, 1989; Freshwater et al., 1994; Ragan et al., 1994; Saunders and Hommersand, 2004; Yoon et al., 2006a). Consequently, due to the taxonomic complexity of the nSSU rRNA gene sequence data set and the computationally intensive structural models of evolution the Bangiophyceae *s.s.* and Florideophyceae were analyzed independently in this study.

### 5.3.1 Sequence alignment construction

Sequence alignments of the nSSU rRNA gene using all sequences available in GenBank were constructed based on evaluations of compensatory substitutions using comparative sequence analysis of the eukaryotic nSSU rRNA gene and were provided by the Comparative RNA Website (Cannone et al., 2002). Short sequences (< 800 nucleotides) were removed and the remaining sequences were verified against GenBank to ensure sequence annotations were current. See Appendix A for sequence information, including GenBank accession number and collection information. Taxonomically important *Bangia* and *Porphyra* sequences not publicly available were provided by members of the Bangiales Working Group, an

international panel of researchers constructed to facilitate taxonomic research within the Bangiophyceae *s.s.* (Broom et al, *in press*). These sequences were compared manually against the consensus Rhodophyta nSSU rRNA secondary structure model (Cannone et al., 2002) and added to the Bangiophyceae *s.s.* sequence alignment. The analyzed Bangiophyceae *s.s.* sequence alignment contained 157 ingroup sequences taxonomically verified by Bangiales Working Group members and three outgroup sequences from sister Rhodophyta clades (*Chlidophyllon kaspar*, *Pyrophyllon subtumens* and *Smithora naiadum*). The Florideophyceae sequence alignment contained 857 sequences with five Bangiophyceae *s.s.* outgroup sequences. In order to limit taxonomic redundancy and facilitate phylogenetic analysis, the Florideophyceae sequence alignment was reduced based on 100% sequence identity clustering using Jalview v.2.4 (Waterhouse et al., 2009), resulting in 681 ingroup and five outgroup sequences.

The Bangiophyceae *s.s.* are species-poor and sequence information for this class is typically more taxonomically mature relative to the Florideophyceae (Verbruggen et al., 2010), enabling multiple gene sequence information to be included in these analyses. Therefore sequence information from the large subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase gene (*rbc*L) was also used in Bangiophyceae *s.s*. phylogenetic analyses. In each case, sequences of *rbc*L used in these analyses corresponded to the same individual or collection as the nSSU rRNA gene sequence to maintain taxonomic provenance (Appendix A). Corresponding *rbc*L sequences for each nSSU rRNA gene sequence of the Bangiophyceae *s.s.* included in single-gene nSSU rRNA analysis were downloaded from NCBI GenBank. Alternatively, sequences not publicly available were procured from research

labs attributed to those collections. Sequences were aligned using MUSCLE v.3.7 (Edgar, 2004), and concatenated to the corresponding nSSU rRNA gene sequence previously aligned using consensus secondary structure models.

### 5.3.2 Phylogenetic analysis

The nSSU rRNA gene sequence alignment secondary structure model contained three categories of nucleotides (Figure 4-1): eukaryotic core (1323 alignment sites), high confidence variable (777 alignment sites) and low confidence variable (1203 alignment sites). In general the low confidence sites corresponded to loop regions of the secondary structure and contained a large number of gaps. Due to the age of the lineage and the ambiguously aligned low confidence variable region, only high confidence and eukaryotic core regions were included in their entirety in all phylogenetic analyses. A subset of sites from the low confidence variable regions was excluded representing cases where homology of alignment sites could not be reasonably assumed. Sequences within the Florideophyceae were more highly conserved than the Bangiophyceae *s.s.* within the low confidence variable region. Consequently, phylogenetic analysis for this group was repeated with no excluded sites in order to contrast the affect on phylogenetic topology.

Maximum likelihood (ML) phylogenies were inferred with RAxML v.7.2.2 (Stamatakis, 2006) using the modified 16-state GTR model for paired nucleotides (16A) and the GTR+G model for non-interacting (Bangiophyceae *s.s.* and Florideophyceae) and *rbc*L sites (Bangiophyceae only). Sequence data were analyzed as a series of independent partitions with two partitions for the nSSU rRNA gene (structurally paired and non-

interacting sites) and three partitions for the *rbc*L gene (codon 1, codon 2, codon 3). Other partitioning strategies were attempted (e.g. single partition for the rbcL gene sequence data) but did not affect resolution of major clades in ML analysis and therefore are not presented. The secondary structures of *Bangia fuscopurpurea* (NWT, GenBank Accession AF043355) and *Palmaria palmata* (GenBank Accession Z14142) were used as the consensus secondary structure model for inference of paired interacting sites in the Bangiophyceae and Florideophyceae respectively. For each data set, one hundred independent ML iterations were performed and the phylogeny with the highest likelihood was maintained. Default parameters were used as they outperformed a collection of manually set parameters in preliminary testing.

In order to provide support for nodes in derived phylogenies a combination of ML bootstrap (MLBS) and Bayesian posterior probabilities (BPP) were used. For each set of sequences one thousand parametric MLBS replicates were performed using RAxML v.7.2.2 with the partitioning and parameter strategy outlined above. As Bayesian analysis with secondary structure models is computationally prohibitive, only the Bangiophyceae *s.s.* was evaluated using the full sequence set. Bayesian posterior probability support values were derived using the partitioning strategy outlined above, except paired and non-paired nSSU rRNA nucleotides were based on 532 pairs of interacting nucleotides derived from the consensus Rhodophyta secondary structure. Paired structural sites were analyzed using the doublet model implemented in MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001). This model considers that paired nucleotides in a stem region of RNA mutate to form another pair in a two-step process. Consequently, it is a 16-parameter model modeling the secondary

structure of helical regions in RNA and is a slight minimization of the 16A model used in RAxML v.7.2.2 (see Savill *et al.* (2001) for review). All non-paired nucleotides were evaluated utilizing the GTR+G model of nucleotide evolution. In order to facilitate convergence of independent runs, the temperature parameter was increased to 0.22 and default values were used for all remaining parameters, including priors. Four independent chains for each of two independent runs were initiated and allowed to run for 4,000,000 generations and trees were collected from the posterior distribution every 100 generations. To calculate the posterior probability of nodes, trees constructed before convergence of independent runs below a standard deviation of 0.01 were discarded.

Due to the number of parameters involved, phylogenetic analyses using structural models of sequence evolution tend to be computationally expensive, especially for Bayesian analysis of large taxonomic data sets. A subset sequence alignment consisting of representatives of each independent familial clade within the Florideophyceae based on the ML tree was therefore constructed. The resulting sequence alignment contained nSSU rRNA genes for 114 Florideophyceae taxa and one outgroup Bangiophyceae taxon. ML, MLBS and BPP analyses were performed as above. This approach to parsing a sequence alignment differs from the sequence identity-based filtering typically performed on large sequence data sets in that it maintains taxonomic diversity independent of genetic divergence. This approach ensured the inclusion of representative taxa from all non-monophyletic lineages noted in analysis of the complete sequence set regardless of pairwise nucleotide distance.

All phylogenetic trees were visualized using FigTree v.1.2.2 (Rambaut).

## 5.4 RESULTS

The inclusion of consensus secondary structure information substantially improved the resolution of homologous nucleotide sites within each nSSU rRNA gene sequence alignment. Furthermore, the start and end points of variable regions within the nSSU rRNA gene region were easily resolved, facilitating the exclusion of ambiguous sites (sites for which homology of nucleotide character could not reasonably be assumed). These sites accounted for approximately seven and five percent of nucleotide sites within the Bangiophyceae and Florideophyceae alignments respectively. Structurally informed multiple sequence alignments increased alignment quality such that when using stochastic models for all sites (e.g. GTR), phylogenetic analyses demonstrated increased resolution over nSSU rRNA gene phylogenies using alignments derived from non-structural but widely used algorithms such as MUSCLE (Edgar, 2004) (results not shown). Generally, the use of structural models further improved phylogenetic resolution of ordinal and familial clades within both the Bangiophyceae *s.s.* and Florideophyceae, supporting some current relationships and resolving ambiguities within the taxonomy of these classes of Rhodophyta.

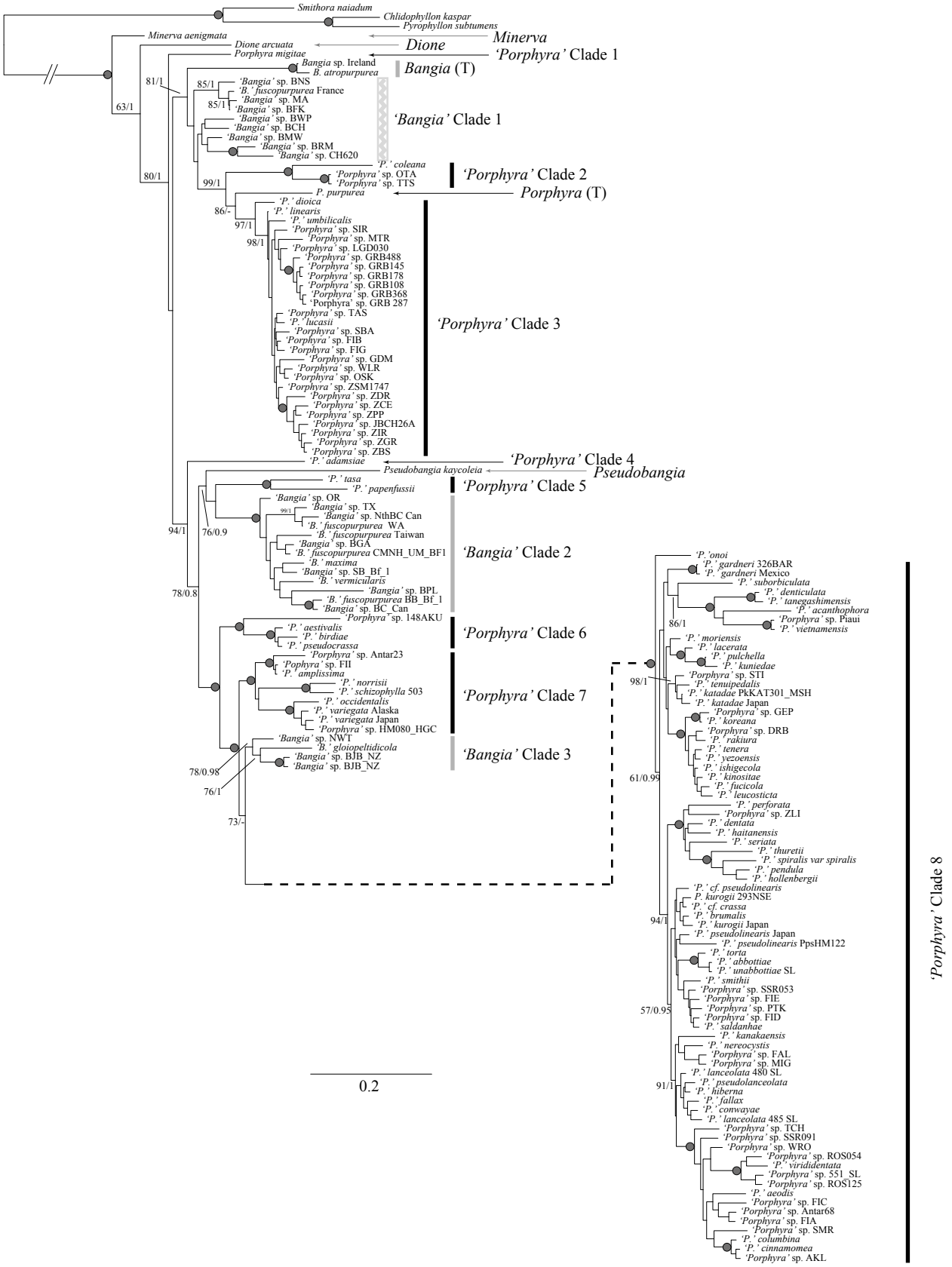
### 5.4.1 Bangiophyceae *sensu stricto*

The phylogenetic treatment of the Bangiophyceae *s.s.* presented here suggests a significant taxonomic revision for the class is required. A formal revision is not proposed, however, as this has been completed by the Bangiales Working Group (Broom et al., *in press*). The results pertaining to the Bangiophyceae presented here are part of my contribution to that effort.

There was clear evidence of Bangiales paraphyly (Figure 5-1), a previously recognized result (Müller et al., 1998, 2001a, 2005; Broom et al., 2004; Yoon et al., 2006a; Lynch et al., 2008); however, this study is the most taxonomically rich treatment of these taxa. The Bangiophyceae *s.s.* contains five recognized genera, three of which are monotypic (*Pseudobangia, Dione, Minerva*). In current taxonomic schemes, the remaining 126 species (Guiry and Dhonncha, 2002) are distributed between two genera based on homoplasious gametophyte morphology, *Bangia* (filamentous) and *Porphyra* (sheet).

In phylogenetic analyses, there were 17 clades within the Bangiophyceae *s.s.*, 15 of which had full or nearly full phylogenetic support or were monotypic (Figure 5-1). Three clades corresponded to the monotypic genera of filamentous bangiophytes *Dione*, *Minerva* and *Pseudobangia*. The remaining 14 clades corresponded to currently recognized *Bangia* (five clades) and *Porphyra* (nine clades). The two clades within *'Bangia'* 'Clade' 1 were not strongly resolved and occupied a similar area in the phylogeny. Therefore they were treated as a single paraphyletic entity. The clades corresponding to the type species of the genera *Bangia* (*Bangia atropurpurea* (Mert. ex Roth) C. Agardh 1824: 76) and *Porphyra* (*Porphyra purpurea* (Roth) C. Agardh (1824), p. 191) were maintained as *Bangia* and *Porphyra* respectively. The remaining clades are designated '*Bangia*' Clade 1-3 and '*Porphyra*' Clade 1-8 corresponding to the order in which they are encountered in the phylogeny (Figure 5-1). Nine of the 16 taxonomic lineages contained multiple sequences, while there were four monotypic filamentous clades (*Minverva, Dione, Pseudobangia* and *Bangia atropurpurea*) and three monotypic sheet clades (*'Porphyra'* Clades 1 and 4 and *Porphyra purpurea*).

**Figure 5-1**. Maximum Likelihood phylogeny inferred using the nSSU rRNA and *rbc*L genes of the Bangiophyceae derived using RAxML v.7.2.2 (Stamatakis, 2006). Data were analyzed in five partitions corresponding to structural and non-structural sites (nSSU rRNA) and the three codon positions (*rbc*L). Support values correspond to Maximum Likelihood bootstrap and Bayesian posterior probabilities for each node. Grey circles represent full support (100/1) and values below 60 % (bootstrap) and 0.9 (posterior probabilities) are not shown. Bars representing major clades correspond to filamentous (grey) and sheet (black) gametophyte morphologies. Adapted from Broom et al. (*in press*)


Smithora naiadum
Chlidophyllon kaspar
Pyrophyllon subtumens
Minerva aenigmata
Dione arcuata
Porphyra migitae
Minerva
Dione
'Porphyra' Clade 1
Bangia sp. Ireland
B. atropurpurea
Bangia (T)
'Bangia' sp. BNS
'B.' fuscopurpurea France
'Bangia' sp. MA
'Bangia' sp. BFK
'Bangia' sp. BWP
'Bangia' sp. BCH
'Bangia' sp. BMW
'Bangia' sp. BRM
'Bangia' sp. CH620
'Bangia' Clade 1
'P.' coleana
'Porphyra' sp. OTA
'Porphyra' sp. TTS
'Porphyra' Clade 2
Porphyra (T)
P. purpurea
'P.' dioica
'P.' linearis
'P.' umbilicalis
'Porphyra' sp. SIR
'Porphyra' sp. MTR
'Porphyra' sp. LGD030
'Porphyra' sp. GRB488
'Porphyra' sp. GRB145
'Porphyra' sp. GRB178
'Porphyra' sp. GRB108
'Porphyra' sp. GRB368
'Porphyra' sp. GRB 287
'Porphyra' sp. TAS
'P.' lucasii
'Porphyra' sp. SBA
'Porphyra' sp. FIB
'Porphyra' sp. FIG
'Porphyra' sp. GDM
'Porphyra' sp. WLR
'Porphyra' sp. OSK
'Porphyra' sp. ZSM1747
'Porphyra' sp. ZDR
'Porphyra' sp. ZCE
'Porphyra' sp. ZPP
'Porphyra' sp. JBCH26A
'Porphyra' sp. ZIR
'Porphyra' sp. ZGR
'Porphyra' sp. ZBS
'Porphyra' Clade 3
'P.' adamsiae
'Porphyra' Clade 4
Pseudobangia kaycoleia
Pseudobangia
'P.' tasa
'P.' papenfussii
'Porphyra' Clade 5
'Bangia' sp. OR
'Bangia' sp. TX
'Bangia' sp. NthBC Can
'B.' fuscopurpurea WA
'B.' fuscopurpurea Taiwan
'Bangia' sp. BGA
'B.' fuscopurpurea CMNH_UM_BF1
'B.' maxima
'Bangia' sp. SB_Bf_1
'B.' vermicularis
'Bangia' sp. BPL
'B.' fuscopurpurea BB_Bf_1
'Bangia' sp. BC_Can
'Bangia' Clade 2
'Porphyra' sp. 148AKU
'Porphyra' Clade 6
'P.' aestivalis
'P.' hirdiae
'P.' pseudocrassa
'Porphyra' sp. Antar23
'Pophyra' sp. FII
'P.' amplissima
'P.' norrisii
'P.' schizophylla 503
'P.' occidentalis
'P.' variegata Alaska
'P.' variegata Japan
'Porphyra' sp. HM080_HGC
'Porphyra' Clade 7
'Bangia' sp. NWT
'B.' gloiopeltidicola
'Bangia' sp. BJB_NZ
'Bangia' sp. BJB_NZ
'Bangia' Clade 3
'P.' onoi
'P.' gardneri 326BAR
'P.' gardneri Mexico
'P.' suborbiculata
'P.' denticulata
'P.' tanegashimensis
'P.' acanthophora
'Porphyra' sp. Piaui
'P.' vietnamensis
'P.' moriensis
'P.' lacerata
'P.' pulchella
'P.' kuniedae
'Porphyra' sp. STI
'P.' tenuipedalis
'P.' katadae PkKAT301_MSH
'P.' katadae Japan
'Porphyra' sp. GEP
'P.' koreana
'Porphyra' sp. DRB
'P.' rakiura
'P.' tenera
'P.' yezoensis
'P.' ishigecola
'P.' kinositae
'P.' fucicola
'P.' leucosticta
'P.' perforata
'Porphyra' sp. ZLI
'P.' dentata
'P.' haitanensis
'P.' seriata
'P.' thuretii
'P.' spiralis var spiralis
'P.' pendula
'P.' hollenbergii
'P.' cf. pseudolinearis
P. kurogii 293NSE
'P.' cf. crassa
'P.' brumalis
'P.' kurogii Japan
'P.' pseudolinearis Japan
'P.' pseudolinearis PpsHM122
'P.' torta
'P.' abbottiae
'P.' unabbottiae SL
'P.' smithii
'Porphyra' sp. SSR053
'Porphyra' sp. FIE
'Porphyra' sp. PTK
'Porphyra' sp. FID
'P.' saldanhae
'P.' kanakaensis
'P.' nereocystis
'Porphyra' sp. FAL
'Porphyra' sp. MIG
'P.' lanceolata 480 SL
'P.' pseudolanceolata
'P.' hiberna
'P.' fallax
'P.' conwayae
'P.' lanceolata 485 SL
'Porphyra' sp. TCH
'Porphyra' sp. SSR091
'Porphyra' sp. WRO
'Porphyra' sp. ROS054
'P.' virididentata
'Porphyra' sp. 551_SL
'Porphyra' sp. ROS125
'P.' aeodis
'Porphyra' sp. FIC
'Porphyra' sp. Antar68
'Porphyra' sp. FIA
'Porphyra' sp. SMR
'P.' columbina
'P.' cinnamomea
'Porphyra' sp. AKL
'Porphyra' Clade 8
0.2

### 5.4.2 Florideophyceae

The clear distinction between variable (loop) and conserved (helical) regions due to alignment to secondary structural models of the nSSU rRNA resulted in reliable alignment of characters, even within the loop regions. As a result, only a limited number of alignment sites were ambiguous enough to necessitate removal from phylogenetic analyses (<100 alignment positions that contained a majority rule consensus nucleotide). Furthermore, the exclusion of variable sites only marginally increased the resolution of MLBS within the scope of these analyses (supra-familial), and typically in clades that were highly resolved using all nucleotide sites (Figures 5-2, 5-3, 5-4). The use of secondary structure models of nucleotide evolution in phylogenetic analysis resulted in significant improvements relative to existing large-scale phylogenetic treatments of the class (Freshwater et al., 1994; Ragan, 1998; Harper and Saunders, 2001a; Saunders, 2005; Verbruggen et al., 2010). Notably, MLBS support for supraordinal nodes was typically poor in these analyses; however, MLBS values tended to skew towards full support (100%) when greater than 50 %.

Sequences corresponding to taxa from 24 orders and 77 families of Florideophyceae as currently recognized (Schneider and Wynne, 2007; Guiry and Dhonncha, 2002) were included in these analyses. Broad phylogenetic topology (Figure 5-2) mirrored existing lineage (supra-ordinal) characterizations of the Florideophyceae (Saunders and Hommersand, 2004) and lineage names are maintained here. Lineage 1, the Hildenbrandiophycidae, is generally accepted as sister to the remaining Florideophyceae (Ragan et al., 1994; Saunders

and Hommersand, 2004; Harper and Saunders, 2001b). This placement was fully supported in these analyses (Figures 5-3) as the Hildenbrandiophycidae contained a single, fully supported clade on a long branch sister to remaining Florideophyceae. Lineage 2 was resolved in these analyses as two distinct groupings (Figure 5-2). Lineage 2a contained seven of the constituent nine orders (Figures 5-2 to 5-4), while the Corallinales and Rhodogorgonales strongly resolved separately as Lineage 2b in all phylogenies. Lineage 3 similarly had full phylogenetic support in all analyses, containing the closely related orders Ahnfeltiales and Pihiellales. Lineage 4 contained the majority of orders (12/24, as well as two of uncertain taxonomic placement, *Incertae sedis*) and families (48/76, as well as two *Incertae sedis*) of Florideophyceae. The lineage was nearly fully supported in all analyses. Interestingly, the Plocamiales was monophyletic in full taxon analyses; however, in the reduced taxon set (Figure 5-5), the two constituent families, the Sarcodiaceae and Plocamiaceae, rendered the Plocamiales polyphyletic. Notably, neither phylogenetic topology was supported.

In general, metrics of phylogenetic support tended towards either high or low support for nodes, and the large majority of currently established orders and families were reinforced by these analyses. In the majority of cases when ordinal clades were monophyletic they were well supported by MLBS (Figures 5-2, 5-6) and to a marginally larger extent by BPP (Figures 5-5, 5-6). In the few cases where monophyletic ordinal clades were not supported by MLBS, they tended to be strongly supported by BPP. For example, two monophyletic ordinal clades were not supported by MLBS in either full or reduced taxon analyses (Gigartinales

and Batrachospermales); however, in the reduced data set analysis BPP support was very strong for both the Gigartinales (1.00) and Batrachospermales (0.98).

In full taxon analyses, only two of 24 orders were not monophyletic, the paraphyletic Acrochaetiales (Figure 5-3) and the polyphyletic Halymeniales (Figure 5-2). For each of these orders, taxa traditionally assigned to the order were distributed between two clades, and in each case only one of these infraordinal clades was strongly supported. In each of these orders the phylogenetic position of the unsupported clades were not strongly resolved and the orders could be monophyletic in future analyses; however, the more robust reduced taxon analyses (Figure 5-5) did not resolve the Acrochaetiales and Halymeniales as monophyletic.

Seventeen of the 77 familial clades were not monophyletic in full taxon phylogenetic analyses (Figures 5-2, 5-4); however, paraphyletic and polyphyletic relationships among these families typically manifested as familial clades containing entire other families. Fourteen of the 17 families were ambiguously paraphyletic/polyphyletic (weakly supported as such), while the Bonnemaisoniaceae (paraphyletic, Figure 5-2), Dumontiaceae (polyphyletic, Figure 5-4) and Gracilariaceae (paraphyletic, Figure 5-2) were each strongly supported and were the most significant departures from currently recognized Florideophyceae taxonomy. Reduced taxon analyses reflected the paraphyly and polyphyly observed in the full taxon set (Figures 5-5, 5-6), maintaining 11 of 17 non-monophyletic families. Seven of these 11 families were strongly supported as paraphyletic/polyphyletic (Figure 5-5: Bonnemaisoniaceae, Dumontiaceae, Faucheaceae, Gigartinaceae and Phyllophoraceae, Figure 5-6: Dasyaceae and Delesseriaceae). Notably, Gigartinaceae and Phyllophoraceae were intercalated with each other. For example, *Chondrus* sp.

(Gigartinaceae) and *Mastocarpus stellatus* (Phyllophoraceae) strongly resolved as sister taxa to the exclusion of other Gigartinaceae and Phyllophoraceae taxa (Figure 5-5). The four remaining non-monophyletic families occupied a polytomic relationship with other taxa. One family, the Cystocloniaceae, which was monophyletic in full taxon analyses, was moderately supported as polyphyletic in two clades in reduced taxonomic analyses. In this case, *Calliblepharis planicaulis*, taxonomically a member of the Cystocloniaceae, resolved as sister to the Solieriaceae, distinct from the strongly supported remaining members of the Cystocloniaceae family. Previously paraphyletic families Lemaneaceae and Batrachospermaceae, both of the Batrachospermales, and the Rhodophysemataceae were only represented by a single taxon each and were therefore trivially monophyletic (monotypic) in reduced analyses. Three families that were paraphyletic in full taxon analyses were resolved as monophyletic in the reduced set analyses (Figure 5-5), the Areschougiaceae, Hapalidiaceae and Rhodymeniaceae; however, only the monophyly of the Hapalidiaceae was strongly supported.

Of the 60 monophyletic familial clades, eight were not supported by MLBS in full taxon analyses. These clades were only represented by a single isolate in reduced taxon analyses and therefore were trivially supported.

**Figure 5-2**. Maximum Likelihood phylogeny inferred using the nSSU rRNA gene of the Florideophyceae derived using RAxML v.7.2.2 (Stamatakis, 2006). Data were analyzed in two partitions corresponding to structural and non-structural sites (nSSU rRNA). Nodal support values correspond to Maximum Likelihood bootstrap for all alignment sites as well as with ambiguous alignment sites removed from analyses. Grey circles correspond to full support (100/100). Values below 60 % (bootstrap) are not presented. Independent lineages of Florideophyceae consistent with Saunders and Hommersand (2004) are indicated numerically (1-4).

Figure 5-2 part 2

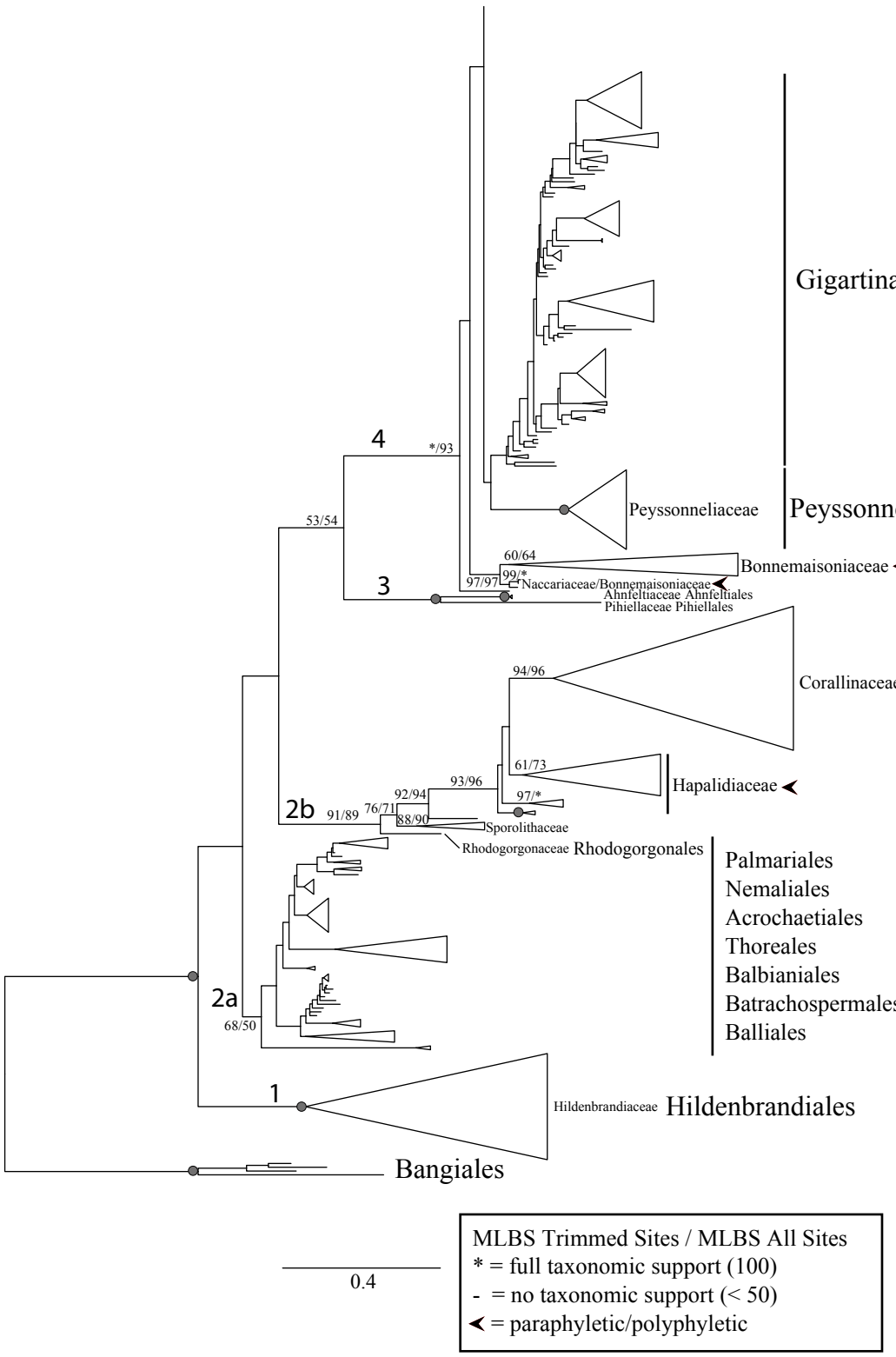



Figure 5-4

Figure 5-3

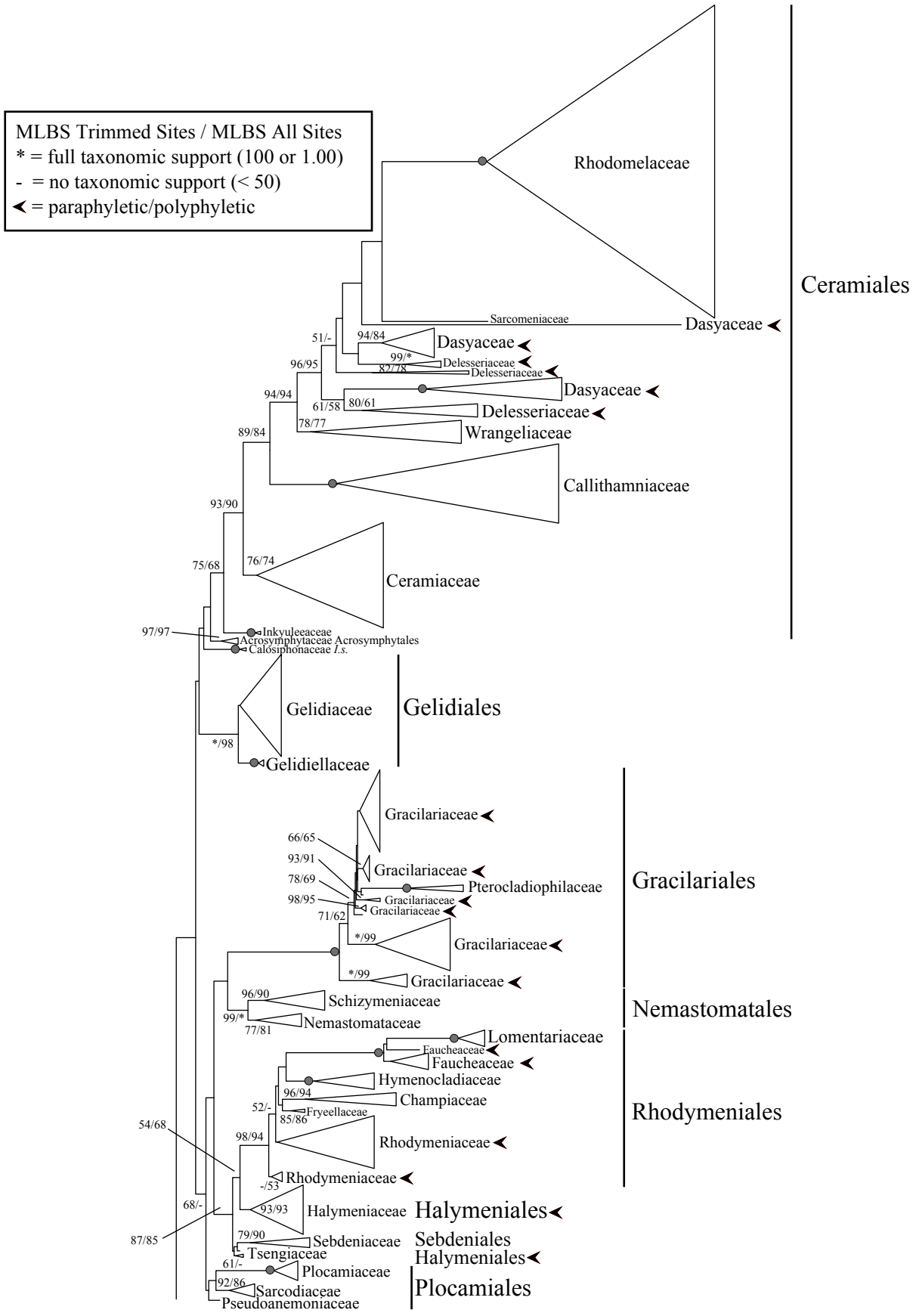

MLBS Trimmed Sites / MLBS All Sites
* = full taxonomic support (100 or 1.00)
- = no taxonomic support (< 50)
➤ = paraphyletic/polyphyletic
Rhodomelaceae
Ceramiales
Sarcomeniaceae
Dasyaceae ➤
51/-
94/84
Dasyaceae ➤
99/*
Delesseriaceae ➤
96/95
82/78
Delesseriaceae
Dasyaceae ➤
94/94
61/58
80/61
Delesseriaceae ➤
78/77
Wrangeliaceae
89/84
Callithamniaceae
93/90
75/68
76/74
Ceramiaceae
97/97
Inkyuleeaceae
Acrosymphytaceae Acrosymphytales
Calosiphoniaceae *l.s.*
Gelidiaceae
Gelidiales
*/98
Gelidiellaceae
Gracilariaceae ➤
66/65
93/91
Gracilariaceae ➤
78/69
Pterocladiophilaceae
98/95
Gracilariaceae ➤
71/62
Gracilariaceae ➤
Gracilariales
*/99
Gracilariaceae ➤
*/99
Gracilariaceae ➤
96/90
Schizymeniaceae
99/*
77/81
Nemastomataceae
Nemastomatales
Lomentariaceae
Faucheaceae ➤
Faucheaceae ➤
Hymenocladiaceae
96/94
Champiaceae
52/-
Fryeellaceae
85/86
Rhodymeniales
54/68
98/94
Rhodymeniaceae ➤
Rhodymeniaceae ➤
-/53
68/-
93/93
Halymeniaceae
Halymeniales ➤
87/85
79/90
Sebdeniaceae
Sebdeniales
Tsengiaceae
Halymeniales ➤
61/-
Plocamiaceae
92/86
Sarcodiaceae
Plocamiales
Pseudoanemoniaceae
0.4

**Figure 5-3.** Maximum Likelihood phylogeny inferred using the nSSU rRNA gene of the Florideophyceae derived using RAxML v.7.2.2 (Stamatakis, 2006). Focus is on the Nemaliophycidae subset of Figure 5-2 (Lineage 2a). All phylogenetic and topological characteristics are consistent with Figure 5-2.

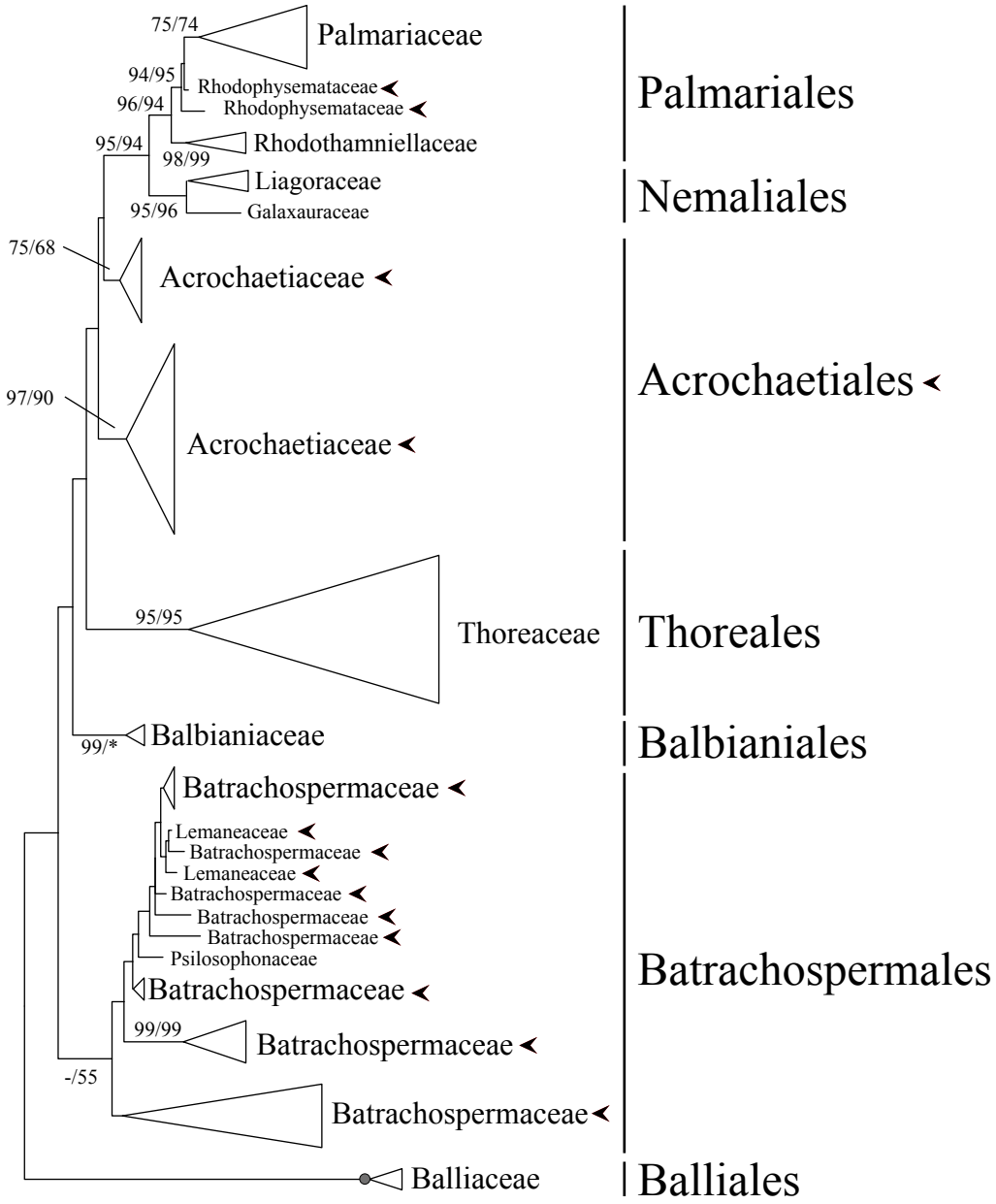

75/74
Palmariaceae
94/95
Rhodophysemataceae
96/94
Rhodophysemataceae
95/94
Rhodothamniellaceae
98/99
Liagoraceae
95/96
Galaxauraceae
Palmariales
Nemaliales
75/68
Acrochaetiaceae
97/90
Acrochaetiaceae
Acrochaetiales
95/95
Thoreaceae
Thoreales
99/*
Balbianiaceae
Balbianiales
Batrachospermaceae
Lemaneaceae
Batrachospermaceae
Lemaneaceae
Batrachospermaceae
Batrachospermaceae
Batrachospermaceae
Psilosophonaceae
Batrachospermaceae
99/99
Batrachospermaceae
-/55
Batrachospermaceae
Batrachospermales
Balliaceae
Balliales
0.4
MLBS Trimmed Sites / MLBS All Sites
* = full taxonomic support (100 or 1.00)
- = no taxonomic support (< 50)
= paraphyletic/polyphyletic

**Figure 5-4.** Maximum Likelihood phylogeny inferred using the nSSU rRNA gene of the Florideophyceae derived using RAxML v.7.2.2 (Stamatakis, 2006). Focus is on the Gigartinales subset of Figure 5-2 (largest order within the Rhodymeniophycidae, Lineage 4). All phylogenetic and topological characteristics are consistent with Figure 5-2.

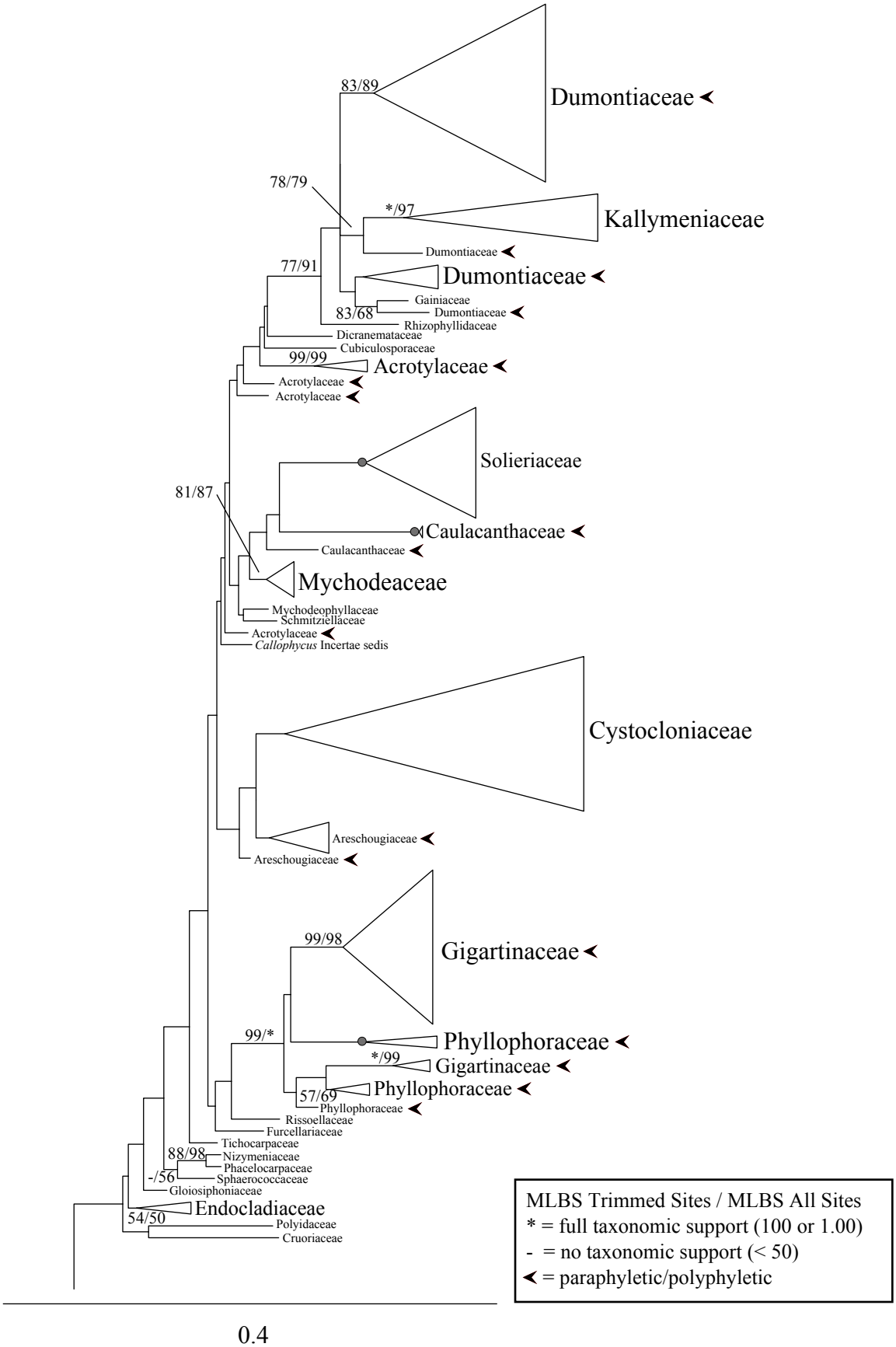

83/89
Dumontiaceae
78/79
*/97
Kallymeniaceae
Dumontiaceae
77/91
Dumontiaceae
Gainiaceae
83/68
Dumontiaceae
Rhizophyllidaceae
Dicranemataceae
Cubiculosporaceae
99/99
Acrotylaceae
Acrotylaceae
Acrotylaceae
Solieriaceae
81/87
Caulacanthaceae
Caulacanthaceae
Mychodeaceae
Mychodeophyllaceae
Schmitziellaceae
Acrotylaceae
Callophycus Incertae sedis
Cystocloniaceae
Areschougiaceae
Areschougiaceae
99/98
Gigartinaceae
99/*
Phyllophoraceae
*/99
Gigartinaceae
57/69
Phyllophoraceae
Phyllophoraceae
Rissoellaceae
Furcellariaceae
Tichocarpaceae
88/98
Nizymeniaceae
Phacelocarpaceae
-/56
Sphaerococcaceae
Gloiosiphoniaceae
Endocladiaceae
54/50
Polyidaceae
Cruoriaceae
MLBS Trimmed Sites / MLBS All Sites
* = full taxonomic support (100 or 1.00)
- = no taxonomic support (< 50)
◄ = paraphyletic/polyphyletic
0.4

**Figure 5-5.** Reduced taxa subset Maximum Likelihood phylogeny inferred using the nSSU rRNA gene of the Florideophyceae derived using RAxML v.7.2.2. (Stamatakis 2006). One taxon representing each familial lineage (including paraphyly/polyphyly) was selected from full taxa analysis (Figure 5-2). Data was analyzed in two partitions corresponding to structural and non-structural sites (nSSU rRNA). Nodal support values correspond to Bayesian Posterior Probabilities, BPP, and Maximum Likelihood Bootstrap, MLBS (BPP/MLBS). Grey circles correspond to full support (100/100). Values below 60 % (bootstrap) are not presented. Independent lineages of Florideophyceae consistent with Saunders and Hommersand (2004) are indicated numerically (1-4). Bolded text corresponds to paraphyletic or polyphyletic taxa.

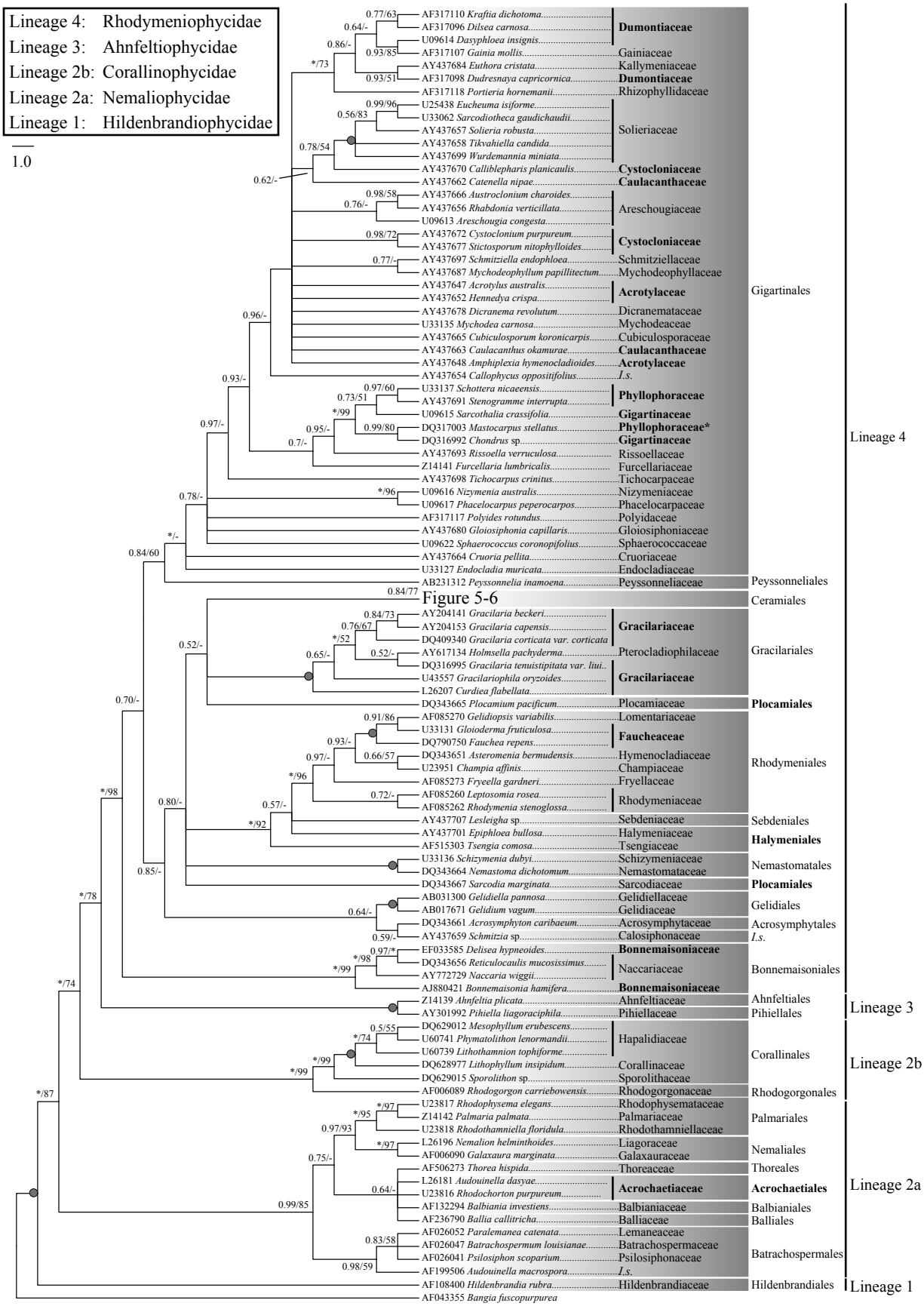
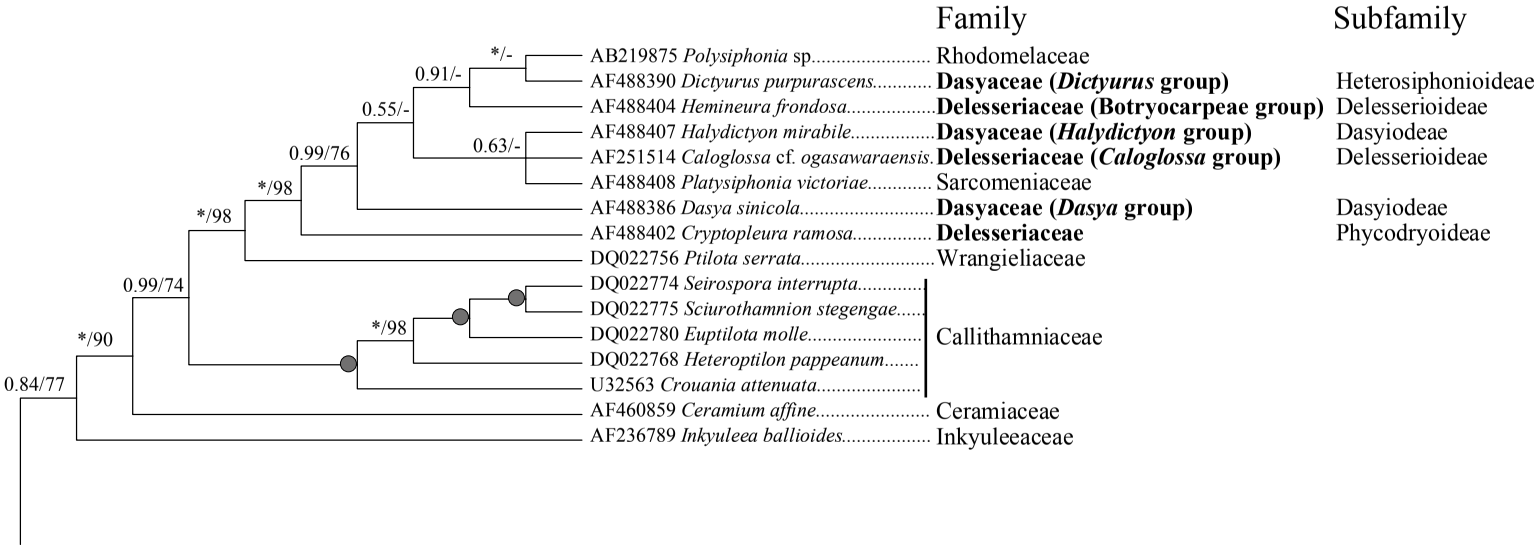

Lineage 4: Rhodymeniophycidae
Lineage 3: Ahnfeltiophycidae
Lineage 2b: Corallinophycidae
Lineage 2a: Nemaliophycidae
Lineage 1: Hildenbrandiophycidae
1.0
AF317110 Kraftia dichotoma
AF317096 Dilsea carnosa
U09614 Dasyphloea insignis
Dumontiaceae
AF317107 Gainia mollis
Gainiaceae
AY437684 Euthora cristata
Kallymeniaceae
AF317098 Dudresnaya capricornica
Dumontiaceae
AF317118 Portieria hornemanii
Rhizophyllidaceae
U25438 Eucheuma isiforme
U33062 Sarcodiotheca gaudichaudii
AY437657 Solieria robusta
Solieriaceae
AY437658 Tikvahiella candida
AY437699 Wurdemannia miniata
AY437670 Calliblepharis planicaulis
Cystocloniaceae
AY437662 Catenella nipae
Caulacanthaceae
AY437666 Austroclonium charoides
AY437656 Rhabdonia verticillata
U09613 Areschougia congesta
Areschougiaceae
AY437672 Cystoclonium purpureum
Cystocloniaceae
AY437677 Stictosporum nitophylloides
AY437697 Schmitziella endophloea
Schmitziellaceae
AY437687 Mychodeophyllum papillitectum
Mychodeophyllaceae
AY437647 Acrotylus australis
Acrotylaceae
AY437652 Hennedya crispa
Gigartinales
AY437678 Dicranema revolutum
Dicranemataceae
U33135 Mychodea carnosa
Mychodeaceae
AY437665 Cubiculosporum koronicarpis
Cubiculosporaceae
AY437663 Caulacanthus okamurae
Caulacanthaceae
AY437648 Amphiplexia hymenocladioides
Acrotylaceae
AY437654 Callophycus oppositifolius
I.s.
U33137 Schottera nicaeensis
Phyllophoraceae
AY437691 Stenogramme interrupta
U09615 Sarcothalia crassifolia
Gigartinaceae
DQ317003 Mastocarpus stellatus
Phyllophoraceae*
DQ316992 Chondrus sp
Gigartinaceae
AY437693 Rissoella verruculosa
Rissoellaceae
Z14141 Furcellaria lumbricalis
Furcellariaceae
AY437698 Tichocarpus crinitus
Tichocarpaceae
U09616 Nizymenia australis
Nizymeniaceae
U09617 Phacelocarpus peperocarpos
Phacelocarpaceae
AF317117 Polyides rotundus
Polyidaceae
AY437680 Gloiosiphonia capillaris
Gloiosiphoniaceae
U09622 Sphaerococcus coronopifolius
Sphaerococcaceae
AY437664 Cruoria pellita
Cruoriaceae
U33127 Endocladia muricata
Endocladiaceae
AB231312 Peyssonnelia inamoena
Peyssonneliaceae
Peyssonneliales
Figure 5-6
Ceramiales
AY204141 Gracilaria beckeri
AY204153 Gracilaria capensis
DQ409340 Gracilaria corticata var. corticata
Gracilariaceae
AY617134 Holmsella pachyderma
Pterocladiophilaceae
Gracilariales
DQ316995 Gracilaria tenuistipitata var. liui..
U43557 Gracilariophila oryzoides
Gracilariaceae
L26207 Curdiea flabellata
DQ343665 Plocamium pacificum
Plocamiaceae
Plocamiales
AF085270 Gelidiopsis variabilis
Lomentariaceae
U33131 Glioderma fruticulosa
Faucheaceae
DQ790750 Fauchea repens
DQ343651 Asteromenia bermudensis
Hymenocladiaceae
U23951 Champia affinis
Champiaceae
AF085273 Fryeella gardneri
Fryellaceae
AF085260 Leptosomia rosea
AF085262 Rhodymenia stenoglossa
Rhodymeniaceae
Rhodymeniales
AY437707 Lesleigha sp
Sebdeniaceae
Sebdeniales
AY437701 Epiphloea bullosa
Halymeniaceae
AF515303 Tsengia comosa
Tsengiaceae
Halymeniales
U33136 Schizymenia dubyi
Schizymeniaceae
DQ343664 Nemastoma dichotomum
Nemastomataceae
Nemastomatales
DQ343667 Sarcodia marginata
Sarcodiaceae
Plocamiales
AB031300 Gelidiella pannosa
Gelidiellaceae
AB017671 Gelidium vagum
Gelidiaceae
Gelidiales
DQ343661 Acrosymphyton caribaeum
Acrosymphytaceae
Acrosymphytales
AY437659 Schmitzia sp
Calosiphonaceae
I.s.
EF033585 Delisea hypneoides
Bonnemaisoniaceae
DQ343656 Reticulocaulis mucosissimus
AY772729 Naccaria wiggii
Naccariaceae
Bonnemaisoniales
AJ880421 Bonnemaisonia hamifera
Bonnemaisoniaceae
Z14139 Ahnfeltia plicata
Ahnfeltiaceae
Ahnfeltiales
AY301992 Pihiella liagoraciphila
Pihiellaceae
Pihiellales
DQ629012 Mesophyllum erubescens
U60741 Phymatolithon lenormandii
Hapalidiaceae
U60739 Lithothamnion tophiforme
Corallinales
DQ628977 Lithophyllum insipidum
Corallinaceae
DQ629015 Sporolithon sp
Sporolithaceae
AF006089 Rhodogorgon carriebowensis
Rhodogorgonaceae
Rhodogorgonales
U23817 Rhodophysema elegans
Rhodophysemataceae
Z14142 Palmaria palmata
Palmariaceae
U23818 Rhodothamniella floridula
Rhodothamniellaceae
Palmariales
L26196 Nemalion helminthoides
Liagoraceae
AF006090 Galaxaura marginata
Galaxauraceae
Nemaliales
AF506273 Thorea hispida
Thoreaceae
Thoreales
L26181 Audouinella dasyae
U23816 Rhodochorton purpureum
Acrochaetiaceae
Acrochaetiales
AF132294 Balbiania investiens
Balbianiaceae
Balbianiales
AF236790 Ballia callitricha
Balliaceae
Balliales
AF026052 Paralemanea catenata
Lemaneaceae
AF026047 Batrachospermum louisianae
Batrachospermaceae
AF026041 Psilosiphon scoparium
Psilosiphonaceae
Batrachospermales
AF199506 Audouinella macrospora
I.s.
AF108400 Hildenbrandia rubra
Hildenbrandiaceae
Hildenbrandiales
AF043355 Bangia fuscopurpurea
Lineage 4
Lineage 3
Lineage 2b
Lineage 2a
Lineage 1

**Figure 5-6.** Reduced taxa subset Maximum Likelihood phylogeny inferred using the nSSU rRNA gene of the Florideophyceae derived using RAxML v.7.2.2. (Stamatakis 2006). Focus on the Ceramiales subset of Figure 5-5. All phylogenetic and topological characteristics are consistent with Figure 5-5. Bolded text corresponds to paraphyletic or polyphyletic taxa.


Family
Subfamily
*/-
0.91/-
0.55/-
0.63/-
0.99/76
*/98
*/98
0.99/74
*/98
*/90
0.84/77
AB219875 Polysiphonia sp..... Rhodomelaceae
AF488390 Dictyurus purpurascens..... Dasyaceae (Dictyurus group) Heterosiphonioideae
AF488404 Hemineura frondosa..... Delesseriaceae (Botryocarpeae group) Delesserioideae
AF488407 Halydictyon mirabile..... Dasyaceae (Halydictyon group) Dasyiodeae
AF251514 Caloglossa cf. ogasawaraensis. Delesseriaceae (Caloglossa group) Delesserioideae
AF488408 Platysiphonia victoriae..... Sarcomeniaceae
AF488386 Dasya sinicola..... Dasyaceae (Dasya group) Dasyiodeae
AF488402 Cryptopleura ramosa..... Delesseriaceae Phycodryoideae
DQ022756 Ptilota serrata..... Wrangieliaceae
DQ022774 Seirospora interrupta.....
DQ022775 Sciurothamnion stegengae.....
DQ022780 Euptilota molle..... Callithamniaceae
DQ022768 Heteroptilon pappeanum.....
U32563 Crouania attenuata.....
AF460859 Ceramium affine..... Ceramiaceae
AF236789 Inkyuleea ballioides..... Inkyuleeaceae

## 5.5 DISCUSSION

Recent decreases in the cost of DNA sequencing have facilitated a shift away from single-gene phylogenies in taxonomic treatments. While these developments may effectively resolve, and in some cases uncover, much incorrect taxonomy, a concurrent focus on the use of recent advances in data analysis (e.g. phylogenetic algorithms) has been lacking in the taxonomic literature. The largest available pool of taxonomically useful sequence data is for the nSSU rRNA and much of the evolutionary information contained within these genes has not been sufficiently explored. By using more biologically realistic models of sequence evolution, phylogenetic resolution relative to existing phylogenies can be improved without additional sequencing effort. This is desirable as sequencing and computational barriers have largely been overcome. As a result, the limiting factors in robust taxonomic studies are now the acquisition and processing of specimens with known and reliable provenance. By revisiting currently available data pools and improving phylogenetic resolution, researchers can take advantage of the significant research investment reflected by these existing data. Furthermore, a robust focus on existing data will efficiently act as a 'road map' for future research efforts, identifying taxonomic priorities.

The increase in phylogenetic resolution in the Eurhodophytina observed here likely had two contributing factors, the increased quality of sequence alignments when utilizing secondary structure information and the use of biologically realistic structural models of sequence evolution. Notably, when a node was supported it was usually by relatively high

values (>95 MLBS/0.95 BPP), while lack of support for a topology tended to skew towards low values. In MLBS this pattern was more exaggerated and is likely due to the bootstrapping strategy employed by the RAxML algorithm. Based on evaluations of the source code, the resampling strategy of the algorithm is not fully structurally informed and pairs can therefore be disproportionately removed from resampled alignments. For example, if a paired nucleotide is not included in the resampled alignment its partner is automatically removed; however, there is no differential weighting of the resampling for paired vs. non-paired sites. This results in the composition of resampled alignments skewed towards less conserved (non-paired) sites relative to the original sequence alignment.

### 5.5.1 Bangiophyceae *sensu stricto*

The Bangiophyceae *s.s.* taxa represent an evolutionary history greater than 1.2 billion years (Butterfield, 2000, 2001; Butterfield et al., 1990), consistent with difficulties identifying homologous sites in some nucleotide sequence alignments. This was especially problematic within the eukaryotic variable regions corresponding to large loops of the nSSU rRNA structure. The aggressive exclusion of sites almost exclusively within these variable regions eliminated ambiguous sites in downstream analyses. There exists the risk of excluding too much information with such an approach; however, in parallel comparison of resulting phylogenies the removal of these sites did not affect the phylogenetic topology above the 'genus' level (i.e. topology was only affected within clades that define a genus or proposed genus). Rather, it was phylogenetic support metrics such as MLBS that were influenced by these variable sites, likely indicative of mutational saturation in the removed sites (i.e. homoplaseous characters).

The polyphyletic relationship between *Bangia* and *Porphyra* is well established in the literature; however, this was the first phylogenetic treatment of all currently publicly available nSSU rRNA as well as directly corresponding *rbc*L sequences. The taxonomic breadth of this study is important as the identification of as many independent clades as possible is required for the construction of an updated and robust taxonomy as discussed by Nelson *et al.,* (Nelson et al., 2006). The revised generic delineation identified here as a series of *"Bangia"* and *"Porphyra"* clades should reflect, at minimum, a monophyly of each filamentous and sheet gametophyte. For example, each lineage of the filamentous *"Bangia"* species intercalated with sheet-like *"Porphyra"* isolates represents a genus. This process was observed in the delineation of each independent lineage of Bangiales (Figure 5-1). There were two exceptions to this pattern. First, the separation of *"Porphyra"* Clade 3, *Porphyra* (Type) and *"Porphyra"* Clade 2, which here were treated as independent entities due to the geographic isolation of the sequences in *"Porphyra"* Clade 3. Second, "*Bangia*" Clade 1, where isolates from two clades were maintained as a single evolutionary entity since one clade demonstrated no phylogenetic support and there was some geographic overlap among the taxa included. With a higher sampling intensity of isolates in *"Bangia"* Clade 1, taxonomic status will likely be resolved; however, changing taxonomy without resolution may require a retraction and is therefore not suggested in this case. The species-rich "*Porphyra*" Clade 8, which was the most morphologically diverse clade, was not further subdivided, as the phylogenetic support of constituent clades was not uniformly strong. A more detailed morphological and molecular phylogenetic analysis of this clade is required, and will likely result in greater phylogenetic resolution and subsequent taxonomic revisions.

The presence of multiple well-supported clades of *"Bangia"* and *"Porphyra"*, each with consistent biogeography agreed with other gene phylogenies in this thesis (Chapters 2 and 3). Any revision to current taxonomy would therefore likely require the erection of at least three and possibly four new genera for filamentous Bangiophyceae, depending on the resolution of isolates in "*Bangia*" Clade 1. The genus *Bangia* would be maintained for the freshwater *Bangia atropurpurea* due to taxonomic precedence as Pfeiffer (1871-1873: 361) proposed *Bangia atropurpurea* (Mert. ex Roth) C. Agardh (*Conferva atropurpurea* Mert. ex Roth, 1806: 208, pl. VI) as the lectotype. The presence of this lectotype factored into the taxonomic correction of the proposal to rename *Bangia atropurpurea* as *Bangiadulcis atropurpurea* (Nelson, 2007; Silva and Nelson, 2008). As a result, each of the proposed filamentous genera would be for marine specimens. Only one of these filamentous marine Bangiophyceae *s.s.* genera (*"Bangia"* Clade 2) contained multiple species that were clearly morphologically distinct, *"Bangia" fuscopurpurea*, *"Bangia" vermicularis* and *"Bangia" maxima*.

A more complete examination of the ecological, biogeographic and molecular sequence characteristics defining each of these 16 taxonomic lineages is not presented here as this is the focus of an upcoming publication that I am contributing to, but not exclusively responsible for (Broom et al., *in press*).

### 5.5.2 Florideophyceae

The nSSU rRNA gene sequence alignment for the Florideophyceae was less variable than the corresponding Bangiophyceae *s.s.* alignment. As a result, fewer character sites required

removal from the alignment and removal of sites did not have an effect on phylogenetic topology at the genus level or higher. Additionally, due to increased morphological complexity relative to the Bangiophyceae *s.s.* the presence of cryptic species was less prevalent in the Florideophyceae. The general topology of the Florideophyceae phylogeny was, for the most part, consistent with the taxonomy of recent treatments (Saunders and Hommersand, 2004; Verbruggen et al*.,* 2010). In Saunders and Hommersand (2004) four subclass lineages of Florideophyceae were recognized based on ultrastructure and literature-based consensus taxonomy. Three of those proposed lineages were fully monophyletic and well supported in these analyses (Figures 5-2, 5-5), Hildenbrandiophycidae (Lineage 1), Ahnfeltiophycidae (Lineage 3) and Rhodymeniophycidae (Lineage 4). Alternatively, the Nemaliophycidae (Lineage 2) was polyphyletic, with the Corallinales and Rhodogorgonales well supported and distinct from the remaining orders in the Nemaliophycidae.

The bifurcation of the Nemaliophycidae does not change the parsimony state (i.e. is equally parsimonious) inferred from pit-plug morphologies as presented in Saunders and Hommersand (2004). This separation is also consistent with more recent taxonomic treatments within the Florideophyceae (Le Gall and Saunders, 2007; Verbruggen *et al.,* 2010). Notably, in the analyses presented here the resolution of the Corallinales was achieved using a single gene (nSSU rRNA), whereas the similar resolution and support of the analogous Corallinophycidae by Le Gall and Saunders (2007) required three separate genes (EFP2, nSSU rRNA, nLSU rRNA). Since there is a broader taxonomic sampling for the nSSU rRNA relative to the genes used in mutigene phylogenies, a more inclusive resolved taxonomy can be proposed when this single gene is used effectively. Furthermore, the

phylogenetic resolution achieved in a 14-loci multigene analysis of the Rhodophyta (Verbruggen et al., 2010) showed only a moderate improvement over the single gene nSSU rRNA analyses here, and none of these differences were supported sufficiently to propose taxonomic changes. It is likely that multigene analyses taking advantage of secondary structure models of evolution for suitable gene regions (i.e. RNA) would result in a higher degree of support for phylogenetic metrics. Such analyses should be undertaken when the multigene data sets are suitably mature.

The subclasses Hildenbrandiophycidae, Nemaliophycidae, Corallinophycidae and Ahnfeltiophycidae are relatively species poor, which has resulted in a predominantly stable taxonomy. One notable exception is the Batrachospermales (Nemaliophycidae). While the Batrachospermales were monophyletic in these analyses, the Batrachospermaceae was strongly paraphyletic and would benefit from a formal taxonomic revision. Furthermore, based on the phylogenetic analyses here, *Audouinella macrospora* (Wood) Sheath & Burkholder should be recognized as a lineage within the Batrachospermales and not the Acrochaetiales, consistent with its homotypic synonym *Batrachospermum macrospora* (Wood) Collins (Guiry and Dhonncha 2002) and previous phylogenetic results (Necchi and Zucchi, 1997; Pueschel et al., 2000; Müller et al., 2002). In contrast to the relative taxonomic stability of the species poor subclasses, the Rhodymeniophycidae presented several notable discrepancies. These are discussed in the order encountered in the phylogeny (Figure 5-5).

The larger of the two families within the strongly supported Bonnemaisoniales, the Bonnemaisoniaceae was strongly polyphyletic in these analyses. While the majority of taxa belonging to this family formed a clade, one isolate, *Delisea hypneoides* Harvey, was

strongly supported as monophyletic within the other family in the order, the Naccariaceae. In the only other phylogeny with broad taxonomic sampling across the Florideophyceae (Le Gall and Saunders, 2007), *Delisea hypneoides* was resolved within the Bonnemaisoniaceae; however, there was no phylogenetic support noted in that study. It is possible that the broader taxonomic sampling and choice of nucleotide model of evolution uncovered a homoplasious morphology in *Delisea hypneoides* as all other members of genus *Delisea* resolved correctly within the Bonnemaisoniaceae (Figure 5-2). This scenario would require a re-evaluation of the taxonomic status of *Delisea hypneoides*. The more likely scenario is either the sequence was incorrectly identified or the phylogenetic placement is an artifact of the nSSU rRNA gene itself. Regardless, the sequence of this organism (Accession # EF033585) needs to be verified before it is used in further taxonomic research. Notably, a recent family-level phylogenetic analysis of 14 genes (Verbruggen *et al.,* 2010) utilized the *Delisea hypneoides* nSSU rRNA gene sequence. Of the 14 genes in that study, only six were present from the Bonnemaisoniaceae and from five different species. The use of the potentially problematic *Delisea hypneoides* sequence did not influence the phylogenetic topology in that case as the Bonnemaisoniaceae and Naccariaceae were each represented by a single concatenated sequence set. Future studies, however, need to address the provenance of this sequence.

In these analyses the Calosiphoniaceae (*Smitzia*) and Acrosymphytaceae (*Acrosymphyton*) were strongly excluded from the Gigartinales (Figures 5-2, 5-4, 5-5). The Acrosymphytales was recently erected (Withall and Saunders 2006), containing the Acrosymphytaceae and since the type genus *Acrosymphyton* has gene sequence data available that taxonomy was adopted here. In contrast, the Calosiphoniaceae type genus,

*Calosiphonia*, was not included in these analyses therefore the family was designated as *Incertae sedis* (*I.s.*; Figures 5-2, 5-5).

While phylogenetic resolution of the nSSU rRNA gene region has previously not been harnessed, the correct use of multigene data sets can also be very useful for resolving phylogenetic relationships. The order Plocamiales in particular appears to benefit from the use of several genes in phylogenetic analyses. The monophyletic relationship of two families within the Plocamiales (Sarcodiaceae and Plocamiaceae) was not supported here. This monophyly was strongly resolved in multigene analyses (Verbruggen *et al.,* 2010) and therefore maintained in the taxonomy presented here despite lack of phylogenetic resolution with the nSSU rRNA gene.

In these analyses there was a strongly supported monophyletic lineage containing the Halymeniales, Sebdeniales and Rhodymeniales; however, the Halymeniales were a polyphyletic taxon. Within the Halymeniales these analyses further supported the separation of the genus *Tsengia* from its traditional class the Nemastomataceae *sensu* Masuda and Guiry (1995) and elevation to the Tsengiaceae subordinate to the Halymeniales *sensu* Saunders et al (2004). However, while this placement within the Halymeniales was strongly supported in multigene analyses (Verbruggen *et al.,* 2010), it was not supported here. As the Halymeniales and Sebdeniales are not fully resolved here, the taxonomy suggested by the results of Verbruggen *et al.,* (2010) was maintained. In this taxonomy, the Sebdeniaceae constitute their own order, the Sebdeniales, consistent with the taxonomy of Withall and Saunders (2006). While the Rhodymeniales was strongly supported as monophyletic in these analyses, there appeared to be a phylogenetic affinity between *Gelidiopsis variabilis* (Lomentariaceae)

and *Gloioderma fruticulosa* (Faucheaceae), resolved in both full-taxon and reduced-taxon analyses (Figures 5-2, 5-5), but only supported in reduced taxon-analyses (0.91 BPP/86 MLBS). Large-scale phylogenies of the Rhodymeniales do resolve the sister-relationship between Faucheaceae and Lomentariaceae (Le Gall and Saunders, 2007; Verbruggen *et al.,* 2010); however, these studies do not include multiple members of the Faucheaceae, which is the case here, and therefore the paraphyly of Faucheaceae was not observed. A phylogenetic analysis of this relationship with higher taxon sampling is required to characterize this relationship. Similarly, the Rhodymeniaceae were paraphyletic in full taxon analysis (Figure 5-2); however, it was weakly supported as monophyletic in reduced taxon analysis (Figure 5-5). It is likely that this family is monophyletic and would require more thorough taxon sampling in future analyses.

The Gracilariaceae was a fully supported lineage but was paraphyletic due to the inclusion of the Pterocladiophilaceae. As the higher order branching within the Gracilariales was not fully resolved, it is likely that this placement of the Pterocladiophilaceae was an artifact. Interestingly, the Pterocladiophilaceae was only represented by multiple *Holmsella pachyderma* (Reinsch) Sturch, a species parasitic on *Gracilaria* and *Gracilariopsis*. Cross-contamination of these samples may have contributed to the ambiguous resolution observed. Unfortunately, there were no other sequences from the Pterocladiophilaceae with which to test this hypothesis.

The Ceramiales is a well-studied species rich lineage within the Florideophyceae and the taxonomy resolved here significantly varied from the most recent large-scale taxonomic review by Schneider and Wynne (2007). For example, the genus *Sciurothamnion*,

canonically within the Ceramiaceae, was fully supported within the Callithamniaceae, consistent with Hommersand *et al.,* (2005). These analyses resolved three well supported but independent lineages within the Ceramiaceae *sensu* Schneider and Wynne (2007). As these lineages, the Ceramiaceae, Callithamniaceae and Wrangeliaceae, were each well resolved in all phylogenetic analyses and covered a broad evolutionary distance they should be maintained as independent families *sensu* Choi *et al.,* (2008). Interestingly, Inkyuleeaceae is moderately well supported as part of the Ceramiales in these analyses reflecting its current taxonomic placement (Choi *et al.,* 2008). This is directly contradictory to multigene analyses (Verbruggen *et al.,* 2010), which resolved it as sister to the Calosiphoniaceae (Gigartinales), although it was not well supported by all metrics in that analysis. The family Calosiphoniaceae was not included in these analyses, so this relationship was not tested; however, it is unlikely the Inkyuleeaceae would resolve outside the Ceramiales.

More derived families within the Ceramiales were also considerably polyphyletic consistent with previous work (Verbruggen *et al.,* 2010; Lin *et al.,* 2001; Choi *et al.,* 2008). The families Dasyaceae and Delesseriaceae contained several independent lineages (Figures 5-2, 5-6). While there was no strong phylogenetic support to suggest taxonomy for these taxa, there are clearly a number of distinct lineages. If the Rhodomelaceae and Sarcomeniaceae were to remain separate families, each distinct lineage would require the same rank and at least six classes would need to be erected to replace the Dasyaceae and Delesseriaceae. A full phylogenetic treatment of these taxa is therefore required.

The Gigartinales is the most family-rich order in the Florideophyceae and its infraordinal phylogenetic structure has been difficult to resolve (Saunders et al., 2004;

Verbruggen *et al.,* 2010). This study is the largest taxonomic treatment of these taxa to date. Similar to a recent multigene analysis (Verbruggen *et al.,* 2010) most of the higher-order branching relationships within the Gigartinales were not well resolved in these analyses and consequently characterized as a polytomy (Figure 5-5). In full taxon analysis many higher order branching relationships were not supported by phylogenetic metrics of support (MLBS), although many of the familial clades were. While this is consistent with published multigene phylogenies, some suprafamilial relationships were either very well supported or moderately so. Novel phylogenetic topologies observed within the Gigartinales are discussed in the order in which they are encountered in the reduced taxon set analyses (Figure 5-5).

The intercalation of the Gigartinaceae and Phyllophoraceae was well supported within only the reduced taxon set analyses (Figure 5-5). In this case the genus *Mastocarpus* (Phyllophoraceae) resolved as sister to *Chondrus* (Gigartinaceae). The taxonomic placement of *Mastocarpus* is not stable, previously being a member of the Gigartinaceae. In a previous molecular study using the *rbc*L gene region (Fredericq and Ramirez, 1996) *Mastocarpus* allied strongly with the Phyllophoraceae. Conversely, in more recent studies phylogenetic resolution with the nSSU rRNA gene was weak and inconclusive (Saunders et al., 2004). Based on strong *rbc*L taxonomic resolution the genus was placed within the Phyllophoraceae, where it remains (Saunders et al., 2004; Schneider and Wynne, 2007). These results suggest strong support for the genus to be reverted back to the Gigartinaceae and demonstrate a direct specific example for the utility of structural models of sequence evolution in phylogenetic analyses. A revision of the taxonomy of *Mastocarpus* should not

be formally suggested until the disagreement between the nSSU rRNA and *rbc*L genes is resolved or confirmed.

The Peyssonneliaceae, also generally considered to be part of the Gigartinales, has been the subject of a recent taxonomic revision (Krayesky et al., 2009). There the elevation of the family to the ordinal level, the Peyssonneliales, was suggested based on morphological and molecular sequence evidence. Notably, phylogenetic position of the Peyssonneliaceae was not resolved in recent multigene sequence analyses (Verbruggen *et al.,* 2010). While the Peyssonneliaceae was resolved as monophyletic with the Gigartinales in all analyses presented here, the branch length leading to the clade as well as the stronger support for the Gigartinales excluding the Peyssonneliaceae (Figure 5-5) suggests support for the Peyssonneliales and Gigartinales *sensu* Krayesky *et al.,* (2009) and such taxonomy is adopted here.

The Schmitziellaceae *sensu* Saunders et al. (2004) are similarly supported in these analyses, clearly locating the constituent genus *Schmitziella* within the Gigartinales. The taxonomy of *Schmitziella* has been particularly problematic, being assigned to the Corallinaceae (Batters, 1892; Kylin, 1956) at various levels of tribe (Svedelius, 1911) and subfamily (Johansen, 1969). Investigations by Woelkerling and Irvine (1982) recognized *Schmitziella* as not associated with the Corallinaceae, showing some similarities to the Acrochaetiaceae. This taxonomy was further corroborated (Pueschel, 1989), eliminating *Schmitziella* from both the Corallinaceae and Acrochaetiaceae based on pit plug morphology, proposing its placement within the Gigartinales, a proposal supported by the results here and of Verbruggen *et al.,* (2010).

The Acrotylaceae consisted of an almost fully supported clade, which contained multiple taxa including *Amphiplexia* and *Antrocentrum* (Figure 5-4) with the other representative taxa (*Hennedya, Acrotylus and Claviclonium*) as monotypic lineages with no resolved phylogenetic affiliation. This was not resolved with the reduced taxa analyses (Bayesian). Similarly, The Caulacanthaceae contained a single sequence interrupting monophyly of the remaining taxa in the family. Additionally, Cystocloniaceae was a polyphyletic taxon in these analyses; however, the genera *Cystoclonium* and *Stictosporum* were strongly monophyletic and the genus *Calliblepharis* was ambiguously placed sister to the Solieriaceae. The unresolved monotypic lineages for each of these three families likely have two significant causes disrupting monophyly, poor taxon sampling and reduced phylogenetic resolution or poor sequence quality, which cannot be evaluated without source material. The phylogenetic topology of these clades does not suggest a taxonomic restructuring at this time as the disparate taxa are not separately resolved, merely unresolved.

Some suprafamilial phylogenetic resolution was observed in the Dumontiaceae-containing clade within the Gigartinales (Figures 5-4, 5-5), which contained the families Dumontiaceae, Gainiaceae, Kallymeniaceae and Rhizophyllidaceae. These taxa do form a monophyletic lineage in multigene phylogenies (Verbruggen *et al.,* 2010), and consequently likely do represent a monophyletic taxon. Multiple strongly supported clades within this group strongly suggest the Dumontiaceae is a polyphyletic taxon. Reduced taxon analyses reduced the number of clades of Dumontiaceae to three (from four in full taxon analyses). The extra clades in full taxon analyses collapsed into the large clade of Dumontiaceae. Regardless, the resolution of the clades is not sufficient in these analyses to suggest a

taxonomic revision. A more specific and taxon rich analysis of the clade should be undertaken.

The remaining phylogenetic relationships were no more resolved than multigene phylogenies and require further analyses before taxonomic revisions can be suggested. Interestingly, the subsetting of taxa and introduction of Bayesian analyses (Figure 5-5) did not significantly change phylogenetic topology, reinforcing full-taxa analyses. Furthermore, as these analyses were, on average, at least as successful as multigene analyses with stochastic models of sequence evolution (Saunders et al, 2004; Verbruggen *et al.,* 2010) it appears as though conflicting phylogenetic signal from biologically naive models interfered with multigene analyses. As it is generally accepted that more sequence data will improve phylogenetic resolution, assuming orthology (Dunn *et al.,* 2008), the reanalysis of multigene alignments using structurally informed models of sequence evolution for non-translated RNA molecules would improve phylogenetic resolution.

## 5.6 Conclusions

Incorporating parametric structural models of RNA sequence evolution into the phylogenetic analysis of Eurhodophytina taxa resulted in a significant increase in phylogenetic resolution. As a goal, taxonomy should be congruent with evolutionary history; however, this is not currently the case within the Eurhodophytina. The Bangiophyceae *sensu stricto*, including only the Bangiales, were very strongly characterized as having a minimum of 16 generic lineages. In all previous work, however, resolution of some filamentous and sheet gametophyte clades was weak or unresolved. In order to accommodate these results, marine

filamentous isolates previously deemed *Bangia* require at least three new genera. Similarly, a total of eight new genera should be erected to accommodate species of *Porphyra*. Monotypic generic lineages *Bangia atropurpurea*, *Dione arcuata, Minerva aenigmata, Porphyra purpurea* and *Pseudobangia kaycoleia* should be maintained. These changes are fully proposed in Broom et al. (*in press*).

In contrast to the Bangiophyceae *sensu stricto*, the taxonomic changes within the Florideophyceae suggested by these results are subtle, which is likely a function of the increased morphological variation within the Class and therefore fewer cryptic species or lineages. This is the most taxon rich phylogenetic analysis of the Florideophyceae and resolved topologies typically already have precedence within the taxonomic literature; however, topologies here were resolved with a single gene and typically with a higher degree of confidence. For example, the separation of the Nemaliophycidae *sensu* Saunders and Hommersand (2004) into the Corallinophycidae and the Nemaliophycidae increases the number of Florideophyceae subclasses to five. Furthermore, pre-existing taxonomic suggestions at the ordinal level were also corroborated. The Acrosymphytales were supported here as an independent order as was the demarcation of the Peyssonneliales from the Gigartinales *sensu* Kreyesky et al. (2009). Family-level results indicated the elevation of *Tsengia* to the Tsengiaceae subordinate to the Halymeniales and the resolution of three independent lineages (Ceramiaceae, Callithamniaceae and Wrangeliaceae) within the Ceramiaceae *sensu* Schneider and Wynne (2007).

Some novel taxonomic observations were also resolved in these analyses. For example, there was strong support for the return of *Mastocarpus* (Phyllophoraceae) to the

Gigartinaceae, potentially resolving the ambiguous familial placement of this genus. Phylogenetic results also noted sequences with unclear derivation. One example is *Holmsella pachyderma* (Pterocladiophilaceae), which resolved within the Gracilariaceae potentially due to cross contamination, as the species is parasitic on members of the Gracilariaceae.

The most significant contribution that single-gene phylogenies offer taxonomy is a consistent, easily generated dataset. The largest such pool for Rhodophyta is the nSSU rRNA gene, and to date the phylogenetic signal has not been fully utilized in analyses of this gene region. As such, large-scale analysis of the Florideophyceae identified areas of the phylogenetic tree that require further taxonomic analyses. These include the strong phylogenetic association between Lomentariaceae and Faucheaceae within the Rhodymeniales. The polyphyly between Dasyaceae and Delesseriaceae, with several lineages of each, also requires significant taxonomic revision. Additionally, there was evidence of poor phylogenetic resolution in some lineages caused by either insufficient taxonomic sampling or poor sequence quality. For example, the families Acrotylaceae, Caulacanthaceae, and Cystocloniaceae had the majority of isolates predominantly monophyletic interrupted by ambiguous placement of monotypic lineages.

The phylogenetic resolution of molecular sequences is highly dependent on the use of appropriate models of sequence evolution. As parametric secondary structure models of RNA are much more biologically realistic than stochastic models currently widely used, reevaluating current mature data sets of RNA sequences, e.g. nuclear rRNA, would provide significant improvement over current phylogenetic treatments with minimal research

investment. As demonstrated, the utility of these data has not been fully realized and will help to direct future multigene and phylogenomic research.

# Chapter 6

# Taxonomic signatures in the nSSU of Rhodophtya and the potential for species identification

## 6.1 OVERVIEW

Phylogenetic relationships among taxa over broad evolutionary scales are often difficult to resolve due to mutational saturation of orthologous genes. Incorporating additional gene sequence information is one promising method for improving phylogenetic resolution; however, generation of taxonomically consistent multigene datasets has proven difficult and expensive. Alternatively, investigation of higher order secondary structure of orthologous RNA molecules has been proposed as a complimentary approach to phylogenetic analyses.

The nuclear small subunit ribosomal RNA (nSSU rRNA) gene sequence dataset is the most complete orthologous dataset for the early-diverging crown eukaryotic phylum Rhodophyta (red algae). By using secondary structure signatures or molecular morphometrics the evolutionary relationships within the Rhodophyta were evaluated in order to address ambiguous phylogenetic relationships. Furthermore, there is currently no easy method for screening large datasets for the presence of taxonomically useful or biologically active secondary structure characteristics of the nSSU rRNA. Computational tools developed for analyses here were made publicly available under the General Public License (GPL) v.3.0.

Morphometrics of the nSSU rRNA secondary structure were useful in supporting and improving phylogenetic and taxonomic resolution of non-Eurhodophytina red algae, including support for the recently erected Dixoniellales. More recently derived Eurhodophytina taxa, however, did not show appreciable improvement in phylogenetic resolution, due primarily to lack of variation among structures. This novel approach to phylogenetic analyses, although able to distinguish isolates at all taxonomic levels, requires the development of a distance metric tailored to the types of data generated. Euclidian distance among structures appeared to obscure relationships for more recently derived taxa and seemed to be highly susceptible to sequence and structural artifacts such as missing or poorly aligned characters. Addressing these concerns is an area of ongoing investigation.

An unforeseen utility of this approach to nSSU rRNA morphometrics was the easy and rapid identification of deviations from the consensus secondary structure. These deviations manifested as long branches to terminal nodes and topological incongruence between the species tree and the morphometric clustering.

## 6.2 INTRODUCTION

Species identification and the development of taxonomy congruent with evolutionary history have been difficult within the red algae (Rhodophyta), particularly due to homoplasious and simple morphologies within most Bangiophyceae *s.l.* lineages. Using molecular sequence data and phylogenetics to address these problems has been the focus of considerable research (Freshwater *et al.*, 1994; Ragan *et al.*, 1994; Müller *et al.*, 2005; Nelson *et al.*, 2005; Saunders, 2005; Robba *et al.*, 2006; Lynch *et al.*, 2008); however, the age of the phylum,

>1.3 billion years (Butterfield, 2000; Butterfield, 2001) to as many as 2 billion years (Tappan, 1976), makes phylogenetic resolution of some relationships difficult due to loss of phylogenetic signal in sequence data.

Woese (1987) rationalized that the phylogenetic examination of the higher-order structure of ribosomal RNA (rRNA) would clarify the evolutionary stages of its structure and aid in extrapolating the significance of changes and variation in rRNA. This concept helped differentiate the bacterial subdivisions (Woese, 1987) and lead to the delineation of the domains Eukarya, Eubacteria and Archaea (Winker and Woese, 1991) using structural characteristics, or signatures, from the nuclear small subunit (nSSU) rRNA. There are different types of phylogenetically constrained elements, ranging from single nucleotides and base pairs to hairpin loops, non-canonical pairings and insertions/deletions. Based on the findings of Woese (1987) and Winker and Woese (1991), such sequence signatures can be synapomorphic for different phylogenetic levels. Furthermore, there is evidence for using structural signatures from the nSSU rRNA as support for taxonomic revisions within the Rhodophyta (Müller *et al.,* 2004), an approach that has not been fully explored.

The objective of this study is two-fold. First, exploring nucleotide and secondary structure signatures in a taxonomically diverse set of Rhodophyta nSSU rRNA genes using structural element encoding and molecular morphometrics. Second, using multivariate analysis of encoded secondary structure information for the nSSU rRNA gene to resolve ambiguous phylogenetic relationships within the Rhodophyta. There is currently no easy method for screening large data sets for the presence of taxonomically useful secondary structure characteristics (e.g. synapomorphies). Until now such screening has primarily been

done manually using small datasets. The computational tools presented here provide a simple screening and analysis workflow to derive a set of molecular morphometric characters and display abstracted structural distance among taxa. This workflow accepts sequences of any RNA gene for which the secondary structure of at least one sequence is known.

## 6.3 METHODS

### 6.3.1 Sequence alignment and structure prediction

Rhodophyta nSSU rRNA gene sequence alignments, provided by the Comparative RNA Website (Cannone et al., 2002) using sequences available from GenBank and appended with novel sequences from the Bangiales Working Group (Broom et al. *in press*), were constructed based on evaluations of compensatory substitutions using comparative sequence analysis of eukaryotic nSSU rRNA gene. In order to reduce null comparisons (comparing lengths of structural elements when one sequence is truncated and is therefore of length zero), only sequences of full or nearly full length were included. Sequences truncated to less than 95% of the gene region were removed and remaining sequences were screened against NCBI's GenBank database to ensure current user-submitted taxonomic status. Additionally, preliminary phylogenetic analysis using the BioNJ algorithm (Gascuel, 1997) was performed using SeaView v.4.0 (Gouy et al., 2010) in order to evaluate the alignment for any obvious identification errors not reflected in GenBank annotations. To facilitate screening of sequences, the data were analyzed in three subsets, non-Eurhodophytina, Bangiophyceae *sensu stricto* (*s.s.*) and Florideophyceae fractions using taxon membership similar to phylogenies in previous chapters, including outgroup taxa (Figures 4-2,5-1,5-5). Sequence

alignments were subsequently evaluated for secondary structure signatures using a series of text processing and analysis algorithms outlined in the following sections. Programs were implemented in the Python programming language and are publicly available and released under the GNU General Public License v.3.0.

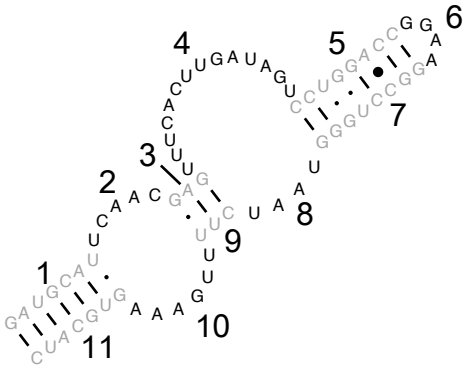
### 6.3.2 Structural variation and abstraction of the nSSU rRNA

A limited number of Rhodophyta lineages have fully derived secondary structure diagrams for the nSSU rRNA gene making direct comparisons of the phylogenetic distribution of structural elements challenging. A total of eight non-Florideophyceae and 26 Florideophyceae structures are currently available (Cannone *et al*., 2002) and are not evenly distributed among different lineages. Regardless, comparisons of derived structures through digital overlays were used to establish a baseline presence of structural variations during Rhodophyta evolution.

While there is a lack of derived secondary structure diagrams for organisms within the Rhodophyta, structurally informed alignments contain most of the information on secondary structure and can be used as a surrogate for structural comparisons. The basic secondary structure of the nSSU rRNA gene is highly conserved across the Rhodophyta, with almost all of the known variation occurring as length polymorphisms of individual structural elements (e.g. helices and loops). To examine the evolutionary distribution of these length polymorphisms, the secondary structure of the nSSU rRNA gene was codified as a series of length measurements of structural elements starting from the 5' end of the molecule. The sequence alignment was partitioned based on pairing and non-pairing sites derived from

secondary structure. Transitions in the alignment from paired to non-paired sites accounted for helical, loop and bulge structural elements (Figure 6-1). As bulges can occur on a single side of a helical structure, each side of such structures was treated independently. Structural elements that comprise of combinations of these secondary structure elements (e.g. helix junctions) were characterized by their constituent parts and not as a whole unit in order to more easily identify and weigh length polymorphisms. The nucleotide length of each element was then calculated independently for each sequence in the alignment.

**Figure 6-1.** Schematic indicating structural elements accounted for within the structural abstraction of RNA molecules. This segment is comprised of 11 individual structural elements, numbered sequentially. Grey and black text is alternated to delineate elements.


1
2
3
4
5
6
7
8
9
10
11

The partitioning of the data into three groups of taxa reflects the general conservation of structure across most lineages of Rhodophyta compared to the relative diversity of secondary structure observed within the Florideophyceae. This also enabled the use of different consensus structures for each partition and mirrored partitions used in previous chapters (Chapters 4 and 5). Consensus secondary structures used for each partition were derived from *Erythrotrichia carnea* (GenBank Accession #: L26189; non-Eurhodophytina), *Bangia fuscopurpurea* NWT (GenBank Accession #: AF043355; Bangiophyceae *s.s.*) and *Palmaria palmata* (GenBank Accession #: Z14142; Florideophyceae). Each of the three alignments was trimmed to the smallest included sequence to ensure that no artificial null comparisons were performed, which can strongly influence clustering algorithms.

Encoded length polymorphisms were transformed into a distance matrix using the Euclidian distance metric and subsequently clustered using the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering and Neighbor Joining algorithms. Distance, UPGMA and Neighbor Joining calculations were performed using the r-project for statistical computing environment (R Development Core Team, 2006) and the analysis of phylogenetics and evolution (APE) package (Paradis et al., 2004).

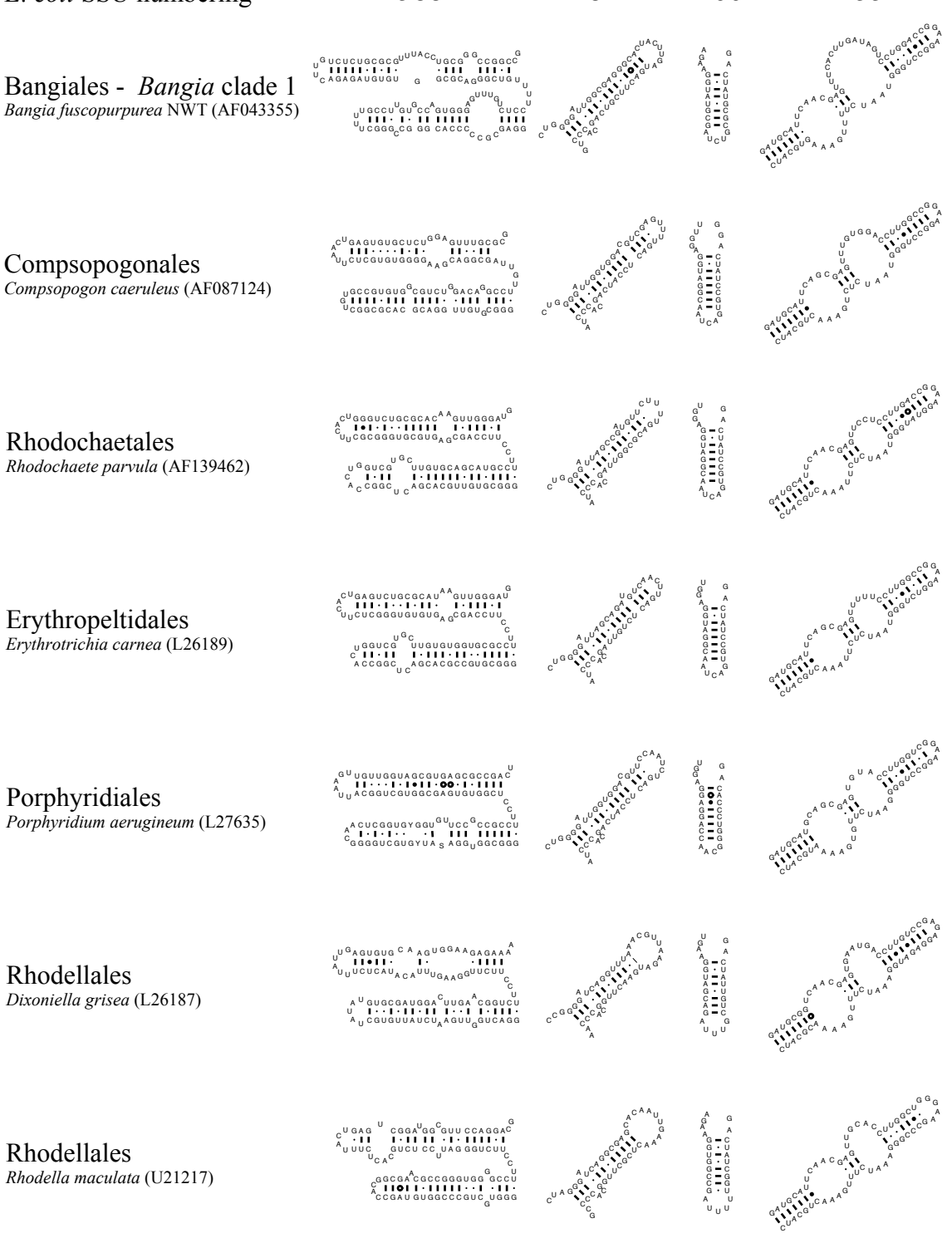
Secondary structure diagrams, based on the comparative structure models, are available from the Comparative RNA Website (Cannone et al., 2002).

## 6.4 RESULTS

When existing secondary structure diagrams were compared using digital overlay some small structural variations were almost exclusively observed as length polymorphisms of existing

structural elements (e.g. loops, helices). Furthermore, patterns of structural variation observed within existing diagrams were highly conserved within lineages across sequences in the structural alignment. These variations appeared to be phylogenetically distributed, where structures within an order formed morphometric islands (i.e. there were few intermediate states between structures among different ordinal lineages and there was very little flexibility within those lineages). A limited subset of variation for the non-Florideophyceae taxa is presented in Figure 6-2. Such conservation patterns suggested further investigation of the phylogenetic distribution of structural length polymorphisms was warranted.

**Figure 6-2.** Secondary structure elements from four regions of the nuclear small subunit ribosomal RNA for currently derived secondary structure diagrams of non-Florideophyceae taxa. The starting number of the corresponding structural element from the *Escherichia coli* secondary structure identifies each of the four regions. Structures were obtained from the Comparative RNA Website (Cannone et al., 2002).

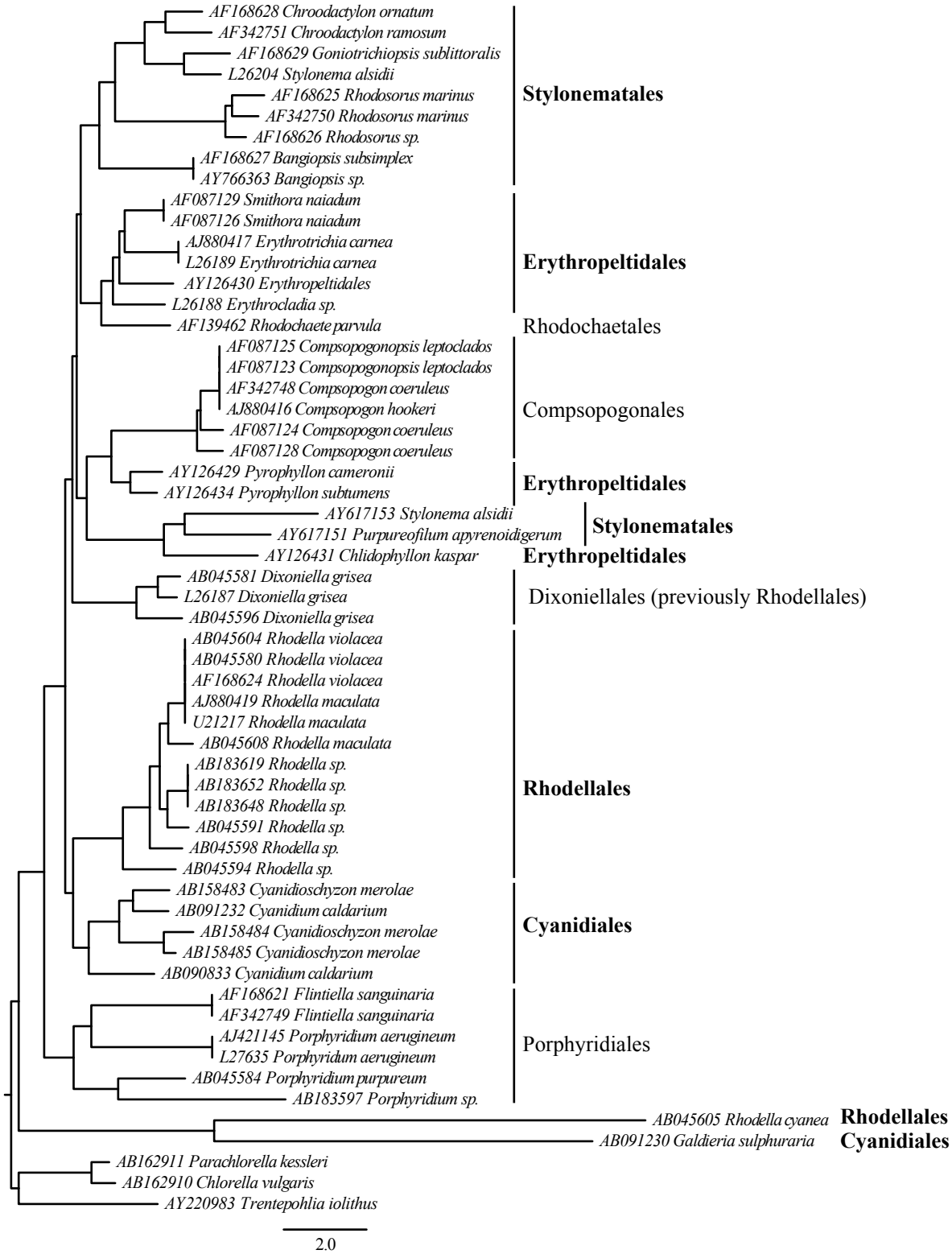

*E. coli* SSU numbering
588
841
1166
1255
Bangiales - *Bangia* clade 1
*Bangia fuscopurpurea* NWT (AF043355)
Compsopogonales
*Compsopogon caeruleus* (AF087124)
Rhodochaetales
*Rhodochaete parvula* (AF139462)
Erythropeltidales
*Erythrotrichia carnea* (L26189)
Porphyridiales
*Porphyridium aerugineum* (L27635)
Rhodellales
*Dixoniella grisea* (L26187)
Rhodellales
*Rhodella maculata* (U21217)

Cluster topologies resulting from the UPGMA algorithm tended to have compressed branch lengths at higher order branching events (i.e. above the genus level). This made it difficult to resolve some clades and made taxonomic inferences at the scope of this study, i.e., ordinal and supraordinal, problematic. Cluster results from UPGMA are therefore not presented. In contrast to UPGMA clustering, Neighbor Joining resulted in tree topologies that were, for the most part, unambiguous. The tree topologies were generally consistent with nucleotide-based phylogenies (Chapters 2-5), but were most similar for non-Eurhodophytina taxa.

### 6.4.1 Non-Eurhodophytina nSSU rRNA morphometrics

The tree topology from nSSU rRNA morphometrics of non-Eurhodophytina taxa closely mirrored phylogenetic inference from sequence data (Figure 6-3). In general, each ordinal clade from phylogenetic analysis was monophyletic in this analysis. Exceptions included a mixed clade of three taxa, *Stylonema alsidii* (AY617153), *Purpureofilum apyrenoidigerum* (AY617151) *and Chlidophyllon kaspar* (AY126431) as well as two taxa in a long-branch association, *Rhodella cyanea* (AB045605) and *Galdieria sulphuraria* (AB091230). All remaining isolates from these orders (Stylonematales, Rhodellales, Erythropeltidales and Cyanidiales) were monophyletic, which accounted for the large majority of isolates.

**Figure 6-3.** Neighbor Joining tree of non-Eurhodophytina taxa derived from a Euclidean pair-wise distance matrix constructed from helix and loop length measurements of the consensus nSSU rRNA secondary structure. Consensus structure was derived from the model for *Erythrotrichia carnea* (GenBank Accession #: L26189). Polyphyletic orders are bolded.


AF168628 Chroodactylon ornatum
AF342751 Chroodactylon ramosum
AF168629 Goniotrichiopsis sublittoralis
L26204 Stylonema alsidii
AF168625 Rhodosorus marinus
AF342750 Rhodosorus marinus
AF168626 Rhodosorus sp.
AF168627 Bangiopsis subsimplex
AY766363 Bangiopsis sp.
Stylonematales
AF087129 Smithora naiadum
AF087126 Smithora naiadum
AJ880417 Erythrotrichia carnea
L26189 Erythrotrichia carnea
AY126430 Erythropeltidales
L26188 Erythrocladia sp.
Erythropeltidales
AF139462 Rhodochaete parvula
Rhodochaetales
AF087125 Compsopogonopsis leptoclados
AF087123 Compsopogonopsis leptoclados
AF342748 Compsopogon coeruleus
AJ880416 Compsopogon hookeri
AF087124 Compsopogon coeruleus
AF087128 Compsopogon coeruleus
Compsopogonales
AY126429 Pyrophyllon cameronii
AY126434 Pyrophyllon subtumens
Erythropeltidales
AY617153 Stylonema alsidii
AY617151 Purpureofilum apyrenoidigerum
Stylonematales
AY126431 Chlidophyllon kaspar
Erythropeltidales
AB045581 Dixoniella grisea
L26187 Dixoniella grisea
AB045596 Dixoniella grisea
Dixoniellales (previously Rhodellales)
AB045604 Rhodella violacea
AB045580 Rhodella violacea
AF168624 Rhodella violacea
AJ880419 Rhodella maculata
U21217 Rhodella maculata
AB045608 Rhodella maculata
AB183619 Rhodella sp.
AB183652 Rhodella sp.
AB183648 Rhodella sp.
AB045591 Rhodella sp.
AB045598 Rhodella sp.
AB045594 Rhodella sp.
Rhodellales
AB158483 Cyanidioschyzon merolae
AB091232 Cyanidium caldarium
AB158484 Cyanidioschyzon merolae
AB158485 Cyanidioschyzon merolae
AB090833 Cyanidium caldarium
Cyanidiales
AF168621 Flintiella sanguinaria
AF342749 Flintiella sanguinaria
AJ421145 Porphyridium aerugineum
L27635 Porphyridium aerugineum
AB045584 Porphyridium purpureum
AB183597 Porphyridium sp.
Porphyridiales
AB045605 Rhodella cyanea
Rhodellales
AB091230 Galdieria sulphuraria
Cyanidiales
AB162911 Parachlorella kessleri
AB162910 Chlorella vulgaris
AY220983 Trentepohlia iolithus
2.0

Most taxa with incorrect placement relative to molecular phylogenies likely had sequence artifacts that influenced the morphometric analysis. The sequence for *Rhodella cyanea* (GenBank Accession #: AB045605), for example, was partially offset in the sequence alignment, which had the effect of displacing one of the loop regions thereby making it considerably larger than other Rhodellales taxa and similar in size to the *Galdieria sulphuraria* sequence (GenBank Accession #: AB091230). Furthermore, the three taxa in the mixed clade, *Stylonema alsidii*, *Purpureofilum apyrenoidigerum* and *Chlidophyllon kaspar*, all had the same gap at the beginning of the alignment, which influenced the size of the first helix element. This is likely an alignment error as all three sequences had nucleotides offset 5' of this gap that closely matched the anticipated sequence data that should have been present in this gap based on other, related taxa. Correction of the two above points resolved the mixed clade and the position of *Rhodella cyanea*, leaving only *Galdieria sulphuraria* distinct from its ordinal clade, Cyanidiales (results not shown as they were derived from a modified structural alignment).

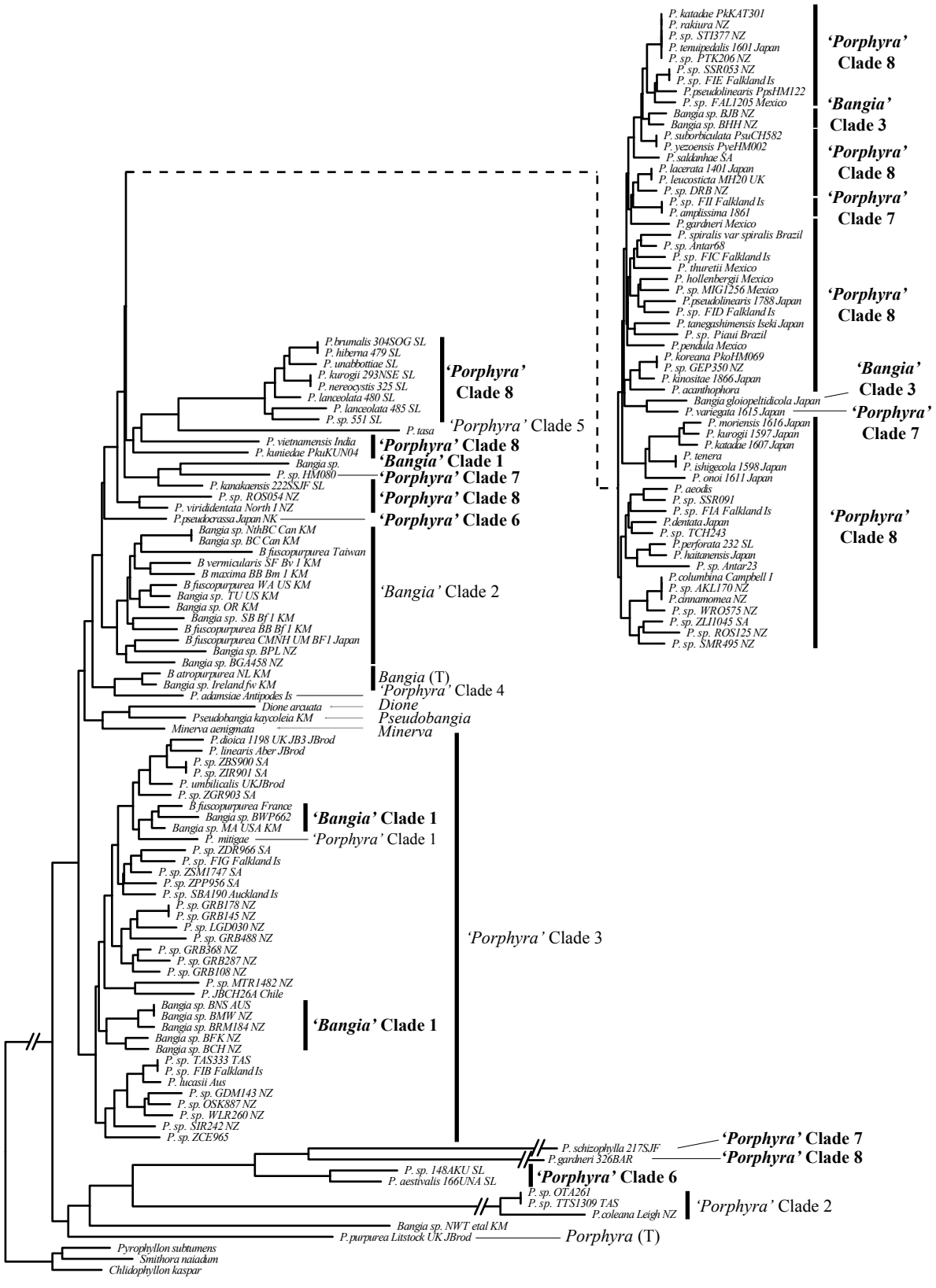
Although the topology of Neighbor Joining clustering contained monophyletic ordinal lineages consistent with previous phylogenetic work (Chapter 4), some supraordinal taxonomy was incongruent with previous results. Here the Porphyridiales were sister to the majority of taxa instead of their placement within the Porphyridiophyceae *sensu* Chapter 4 (i.e. Porphyridiales, Compsopogonales, Rhodochaetales and Erythropeltidales). Notably, all three sequences from *Dixoniella grisea* formed a distinct clade consistent with an ordinal delineation, the Dixoniellales *sensu* Yokoyama et al. (2009).

### 6.4.2 Bangiophyceae *sensu stricto* nSSU rRNA morphometrics

Molecular morphometric analysis of the nSSU rRNA gave mixed results for the Bangiophyceae *s.s.* (Figure 6-4) relative to phylogenies inferred from sequence data. Notably, *Bangia* clade 1 was distinctly more polyphyletic in these analyses relative to its ambiguous resolution in molecular phylogenies (Chapters 2 and 5). Four previously monophyletic groups were not in this analysis. Isolates from *Porphyra* clade 7 and *Porphyra* clade 8 were distributed throughout the tree, while *Porphyra* clade 6 and *Bangia* clade 3 were not monophyletic due to a single incongruous sequence. *Porphyra* clade 3 interestingly contained within it both lineages of *Bangia* clade 1 in contrast to their sister relationship in molecular phylogenies. In these analyses the majority of the nine multi-isolate clades were monophyletic (two), or nearly so (five).

Some isolates in these analyses occupied long branches, including *P. schizophylla*, *P. gardneri*, *P. purpurea*, *Bangia sp.* NWT and *Porphyra* clade 2 (Figure 6-4). The long branches in these analyses corresponded to unique structural signatures resulting in exaggerated Euclidian distances. For example, in *P. purpurea* the tenth loop element from the 5' end of the sequence was 33 nucleotides long whereas all other Bangiales taxa had fewer than 25 nucleotides at this site, and all but three had 22 or fewer.

**Figure 6-4.** Neighbor Joining tree of Bangiophyceae *sensu stricto* taxa derived from a Euclidean pair-wise distance matrix constructed from helix and loop length measurements of the consensus nSSU rRNA secondary structure. Consensus structure was derived from the model for *Bangia fuscopurpurea* NWT (GenBank Accession #: AF043355). Paraphyletic and polyphyletic groups are bolded.


P. katadae PkKAT301
P. rakiura NZ
P. sp. STI377 NZ
P. tenuipedalis 1601 Japan
P. sp. PTK206 NZ
P. sp. SSR053 NZ
P. sp. FIE Falkland Is
P.pseudolinearis PpsHM122
P. sp. FAL1205 Mexico
Bangia sp. BJB NZ
Bangia sp. BHH NZ
P. suborbiculata PsuCH582
P. yezoensis PyeHM002
P. saldanhae SA
P. lacerata 1401 Japan
P. leucosticta MH20 UK
P. sp. DRB NZ
P. sp. FII Falkland Is
P. amplissima 1861
P.gardneri Mexico
P. spiralis var spiralis Brazil
P. sp. Antar68
P. sp. FIC Falkland Is
P. thuretii Mexico
P. hollenbergii Mexico
P. sp. MIG1256 Mexico
P.pseudolinearis 1788 Japan
P. sp. FID Falkland Is
P. tanegashimensis Iseki Japan
P. sp. Piaui Brazil
P.pendula Mexico
P. koreana PkoHM069
P. sp. GEP350 NZ
P. kinositae 1866 Japan
P. acanthophora
Bangia gloiopeltidicola Japan
P. variegata 1615 Japan
P. moriensis 1616 Japan
P. kurogii 1597 Japan
P. katadae 1607 Japan
P. tenera
P. ishigecola 1598 Japan
P. onoi 1611 Japan
P. aeodis
P. sp. SSR091
P. sp. F1A Falkland Is
P.dentata Japan
P. sp. TCH243
P.perforata 232 SL
P. haitanensis Japan
P. sp. Antar23
P.columbina Campbell I
P. sp. AKL170 NZ
P.cinnamomea NZ
P. sp. WRO575 NZ
P. sp. ZLI1045 SA
P. sp. ROS125 NZ
P. sp. SMR495 NZ
'Porphyra' Clade 8
'Bangia' Clade 3
'Porphyra' Clade 8
'Porphyra' Clade 7
'Porphyra' Clade 8
'Bangia' Clade 3
'Porphyra' Clade 7
'Porphyra' Clade 8
P.brumalis 304SOG SL
P. hiberna 479 SL
P. unabbottiae SL
P. kurogii 293NSE SL
P. nereocystis 325 SL
P. lanceolata 480 SL
P. lanceolata 485 SL
P. sp. 551 SL
'Porphyra' Clade 8
P. tasa
'Porphyra' Clade 5
P. vietnamensis India
P. kuniedae PkuKUN04
'Porphyra' Clade 8
'Bangia' Clade 1
Bangia sp.
P. sp. HM080
'Porphyra' Clade 7
P. kanakaensis 222SSJF SL
P. sp. ROS054 NZ
'Porphyra' Clade 8
P. virididentata North I NZ
P.pseudocrassa Japan NK
'Porphyra' Clade 6
Bangia sp. NthBC Can KM
Bangia sp. BC Can KM
B fuscopurpurea Taiwan
B vermicularis SF Bv 1 KM
B maxima BB Bm 1 KM
B fuscopurpurea WA US KM
Bangia sp. TU US KM
Bangia sp. OR KM
Bangia sp. SB Bf 1 KM
B fuscopurpurea BB Bf 1 KM
B fuscopurpurea CMNH UM BF1 Japan
Bangia sp. BPL NZ
Bangia sp. BGA458 NZ
'Bangia' Clade 2
B atropurpurea NL KM
Bangia sp. Ireland fw KM
Bangia (T)
P. adamsiae Antipodes Is
'Porphyra' Clade 4
Dione arcuata
Pseudobangia kaycoleia KM
Minerva aenigmata
Dione
Pseudobangia
Minerva
P.dioica 1198 UK JB3 JBrod
P. linearis Aber JBrod
P. sp. ZBS900 SA
P. sp. ZIR901 SA
P. umbilicalis UKJBrod
P. sp. ZGR903 SA
B fuscopurpurea France
Bangia sp. BWP662
Bangia sp. MA USA KM
'Bangia' Clade 1
P. mitigae
'Porphyra' Clade 1
P. sp. ZDR966 SA
P. sp. FIG Falkland Is
P. sp. ZSM1747 SA
P. sp. ZPP956 SA
P. sp. SBA190 Auckland Is
P. sp. GRB178 NZ
P. sp. GRB145 NZ
P. sp. LGD030 NZ
P. sp. GRB488 NZ
P. sp. GRB368 NZ
P. sp. GRB287 NZ
P. sp. GRB108 NZ
P. sp. MTR1482 NZ
P. JBCH26A Chile
Bangia sp. BNS AUS
Bangia sp. BMW NZ
Bangia sp. BRM184 NZ
'Bangia' Clade 1
Bangia sp. BFK NZ
Bangia sp. BCH NZ
P. sp. TAS333 TAS
P. sp. FIB Falkland Is
P. lucasii Aus
P. sp. GDM143 NZ
P. sp. OSK887 NZ
P. sp. WLR260 NZ
P. sp. SIR242 NZ
P. sp. ZCE965
'Porphyra' Clade 3
P. schizophylla 217SJF
P.gardneri 326BAR
'Porphyra' Clade 7
'Porphyra' Clade 8
P. sp. 148AKU SL
P. aestivalis 166UNA SL
'Porphyra' Clade 6
P. sp. OTA261
P. sp. TTS1309 TAS
P.coleana Leigh NZ
'Porphyra' Clade 2
Bangia sp. NWT etal KM
P.purpurea Litstock UK JBrod
Porphyra (T)
Pyrophyllon subtumens
Smithora naiadum
Chlidophyllon kaspar
3.0

As structures are highly conserved within the Bangiales, including the lengths of most structural signatures evaluated here, the presence of artifactual null comparisons can strongly influence distance measurements (e.g. comparing lengths of an element for which one sequence in a pair-wise comparison has been misaligned or is missing sequence data). To ensure such comparisons were not influencing tree topology an aggressive exclusion of sites with missing or unaligned sequence data was performed, but did not resolve taxonomic inconsistencies.

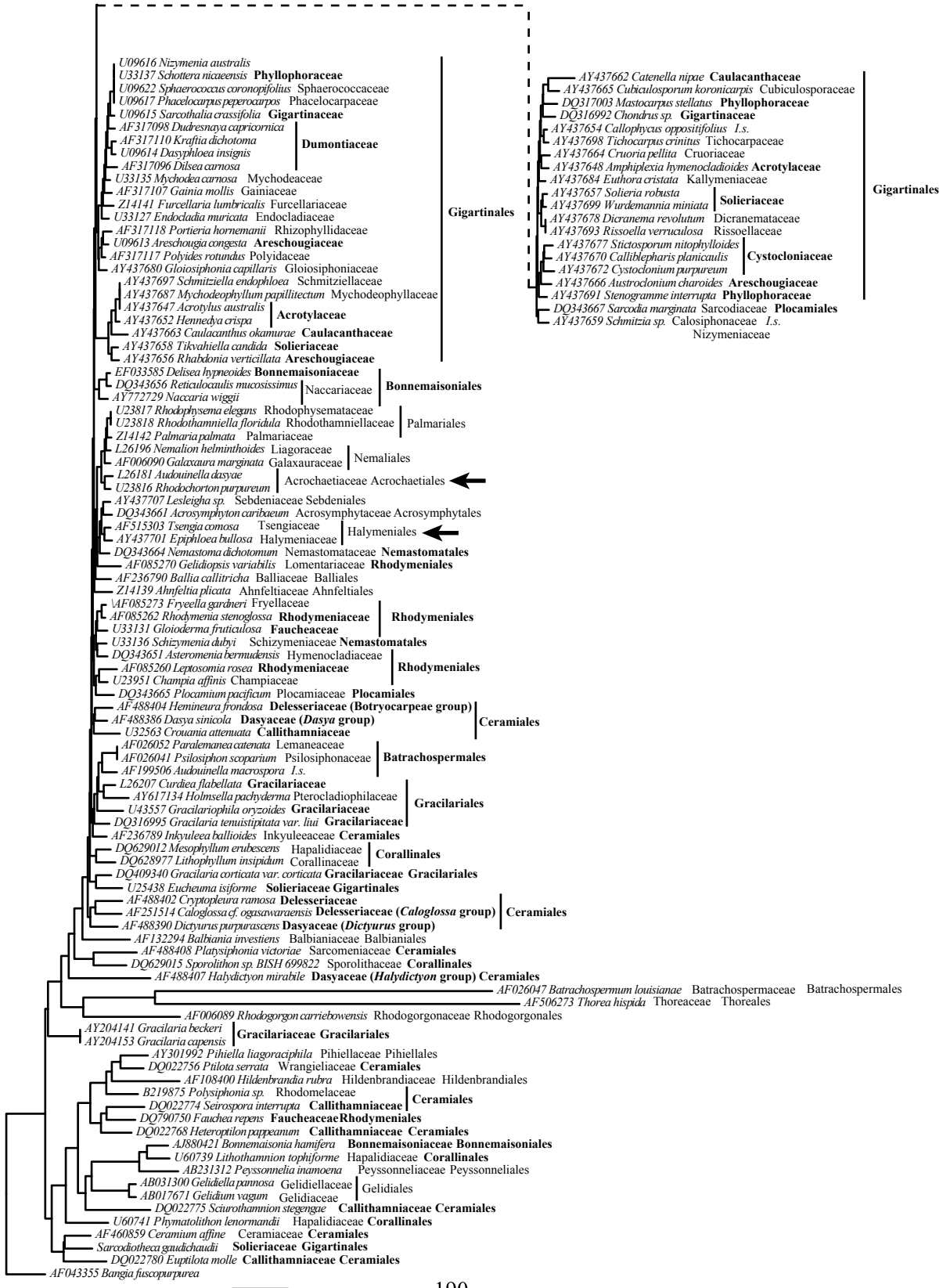
### 6.4.3 Florideophyceae nSSU rRNA morphometrics

Morphometric analysis of the Florideophyceae nSSU rRNA secondary structure was largely inconsistent with current phylogenetic and taxonomic schemes (Figure 6-5) and the majority of order and family level clades were paraphyletic or polyphyletic. Furthermore, only one of five Florideophyceae subclasses (see Figure 5-5), the Hildenbrandiophycidae (Lineage 1 *sensu* Saunders and Hommersand (2004)), was monophyletic in these analyses; however, its position was derived within one of the two major groups of taxa here, a significant deviation from its phylogenetic position (Figures 5-2, 5-5). Notably, while few distinct taxonomic patterns consistent with phylogenetic consensus were observed in the molecular morphometric Neighbor Joining topology, two orders that were previously resolved as paraphyletic were monophyletic (Chapter 5), the Acrochaetiales and Halymeniales.

Morphometric structural analysis was able to clearly identify sequences that deviated from the consensus secondary structure (*Palmaria palmata*, GenBank Accession #: Z14142). The majority of the isolates occupied relatively short branches indicating that structure

abstractions were largely consistent across most taxa. One major exception was a lineage of three taxa, *Rhodogorgon carriebowensis* (GenBank Accession #: AF006089), *Batrachospermum louisianae* (GenBank Accession #: AF026047) and *Thorea hispida* (GenBank Accession #: AF506273), which each occupied long branches within a single clade. Each of these three sequences had a single large loop expansion relative to other Florideophyceae isolates. *Batrachospermum louisianae* and *Thorea hispida* contained very large expansions (65 nucleotides vs. five and 70 nucleotides vs. eight respectively), while *Rhodogorgon carriebowensis* contained a smaller expansion (22 nucleotides vs. seven). Although these sequences grouped together in this analysis, the loop expansion for each of these sequences occurred in different regions of the nSSU rRNA.

**Figure 6-5.** Neighbor Joining tree of Florideophyceae taxa derived from a Euclidean pair-wise distance matrix constructed from helix and loop length measurements of the consensus nSSU rRNA secondary structure using the structural model for *Palmaria palmata* (GenBank Accession #: Z14142). Paraphyletic and polyphyletic groups (order and family) are bolded. Orders identified by block arrows are paraphyletic or polyphyletic in phylogenetic analyses but monophyletic here.


U09616 Nizymenia australis
U33137 Schottera nicaeensis Phyllophoraceae
U09622 Sphaerococcus coronopifolius Sphaerococcaceae
U09617 Phacelocarpus peperocarpos Phacelocarpaceae
U09615 Sarcothalia crassifolia Gigartinaceae
AF317098 Dudresnaya capricornica
AF317110 Kraftia dichotoma
U09614 Dasysiphloea insignis
Dumontiaceae
AF317096 Dilsea carnosa
U33135 Mychodea carnosa Mychodeaceae
AF317107 Gainia mollis Gainiaceae
Z14141 Furcellaria lumbricalis Furcellariaceae
U33127 Endocladia muricata Endocladiaceae
AF317118 Portieria hornemanii Rhizophyllidaceae
U09613 Areschougia congesta Areschougiaceae
AF317117 Polyides rotundus Polyidaceae
AY437680 Gloiosiphonia capillaris Gloiosiphoniaceae
AY437697 Schmitziella endophloea Schmitziellaceae
AY437687 Mychodeophyllum papillitectum Mychodeophyllaceae
AY437647 Acrotylus australis
AY437652 Hennedya crispa
Acrotylaceae
AY437663 Caulacanthus okamurae Caulacanthaceae
AY437658 Tikvahiella candida Solieriaceae
AY437656 Rhabdonia verticillata Areschougiaceae
Gigartinales
AY437662 Catenella nipae Caulacanthaceae
AY437665 Cubiculosporum koronicarpis Cubiculosporaceae
DQ317003 Mastocarpus stellatus Phyllophoraceae
DQ316992 Chondrus sp. Gigartinaceae
AY437654 Callophycus oppositifolius I.s.
AY437698 Tichocarpus crinitus Tichocarpaceae
AY437664 Cruoria pellita Cruoriaceae
AY437648 Amphiplexia hymenocladioides Acrotylaceae
AY437684 Euthora cristata Kallymeniaceae
AY437657 Solieria robusta
AY437699 Wurdemannia miniata
Solieriaceae
AY437678 Dicranema revolutum Dicranemataceae
AY437693 Rissoella verruculosa Rissoellaceae
AY437677 Stictosporum nitophylloides
AY437670 Calliblepharis planicaulis
AY437672 Cystoclonium purpureum
Cystocloniaceae
AY437666 Austroclonium charoides Areschougiaceae
AY437691 Stenogramme interrupta Phyllophoraceae
DQ343667 Sarcodia marginata Sarcodiaceae Plocamiales
AY437659 Schmitzia sp. Calosiphonaceae I.s. Nizymeniaceae
Gigartinales
EF033585 Delisea hypneoides Bonnemaisoniaceae
DQ343656 Reticulocaulis mucosissimus
AY772729 Naccaria wiggii
Naccariaceae
Bonnemaisoniales
U23817 Rhodophysema elegans Rhodophysemataceae
U23818 Rhodothamniella floridula Rhodothamniellaceae
Palmariales
Z14142 Palmaria palmata Palmariaceae
L26196 Nemalion helminthoides Liagoraceae
AF006090 Galaxaura marginata Galaxauraceae
Nemaliales
L26181 Audouinella dasyae
U23816 Rhodochorton purpureum
Acrochaetiaceae Acrochaetiales
AY437707 Lesleigha sp. Sebdeniaceae Sebdeniales
DQ343661 Acrosymphyton caribaeum Acrosymphytaceae Acrosymphytales
AF515303 Tsengia comosa Tsengiaceae
AY437701 Epiphloea bullosa Halymeniaceae
Halymeniales
DQ343664 Nemastoma dichotomum Nemastomataceae Nemastomatales
AF085270 Gelidiopsis variabilis Lomentariaceae Rhodymeniales
AF236790 Ballia callitricha Balliaceae Balliales
Z14139 Ahnfeltia plicata Ahnfeltiaceae Ahnfeltiales
\AF085273 Fryeella gardneri Fryellaceae
AF085262 Rhodymenia stenoglossa Rhodymeniaceae
Rhodymeniales
U33131 Glioiderma fruticulosa Faucheaceae
U33136 Schizymenia dubyi Schizymeniaceae Nemastomatales
DQ343651 Asteromenia bermudensis Hymenocladiaceae
AF085260 Leptosomia rosea Rhodymeniaceae
Rhodymeniales
U23951 Champia affinis Champiaceae
DQ343665 Plocamium pacificum Plocamiaceae Plocamiales
AF488404 Hemineura frondosa Delesseriaceae (Botryocarpeae group)
AF488386 Dasya sinicola Dasyaceae (Dasya group)
U32563 Crouania attenuata Callithamniaceae
Ceramiales
AF026052 Paralemanea catenata Lemaneaceae
AF026041 Psilosiphon scoparium Psilosiphonaceae
AF199506 Audouinella macrospora I.s.
Batrachospermales
L26207 Curdiea flabellata Gracilariaceae
AY617134 Holmsella pachyderma Pterocladiophilaceae
U43557 Gracilariophila oryzoides Gracilariaceae
DQ316995 Gracilaria tenuistipitata var. liui Gracilariaceae
Gracilariales
AF236789 Inkyuleea ballioides Inkyuleeaceae Ceramiales
DQ629012 Mesophyllum erubescens Hapalidiaceae
DQ628977 Lithophyllum insipidum Corallinaceae
Corallinales
DQ409340 Gracilaria corticata var. corticata Gracilariaceae Gracilariales
U25438 Eucheuma isiforme Solieriaceae Gigartinales
AF488402 Cryptopleura ramosa Delesseriaceae
AF251514 Caloglossa cf. ogasawaraensis Delesseriaceae (Caloglossa group)
AF488390 Dictyurus purpurascens Dasyaceae (Dictyurus group)
Ceramiales
AF132294 Balbiania investiens Balbianiaceae Balbianiales
AF488408 Platysiphonia victoriae Sarcomeniaceae Ceramiales
DQ629015 Sporolithon sp. BISH 699822 Sporolithaceae Corallinales
AF488407 Halydictyon mirabile Dasyaceae (Halydictyon group) Ceramiales
AF026047 Batrachospermum louisianae Batrachospermaceae Batrachospermales
AF506273 Thorea hispida Thoreaceae Thoreales
AF006089 Rhodogorgon carriebowensis Rhodogorgonaceae Rhodogorgonales
AY204141 Gracilaria beckeri
AY204153 Gracilaria capensis
Gracilariaceae Gracilariales
AY301992 Pihiella liagoraciphila Pihiellaceae Pihiellales
DQ022756 Ptilota serrata Wrangieliaceae Ceramiales
AF108400 Hildenbrandia rubra Hildenbrandiaceae Hildenbrandiales
B219875 Polysiphonia sp. Rhodomelaceae
DQ022774 Seirospora interrupta Callithamniaceae
Ceramiales
DQ790750 Fauchea repens FaucheaceaeRhodymeniales
DQ022768 Heteroptilon pappeanum Callithamniaceae Ceramiales
AJ880421 Bonnemaisonia hamifera Bonnemaisoniaceae Bonnemaisoniales
U60739 Lithothamnion tophiforme Hapalidiaceae Corallinales
AB231312 Peyssonnelia inamoena Peyssonneliaceae Peyssonneliales
AB031300 Gelidiella pannosa Gelidiellaceae
AB017671 Gelidium vagum Gelidiaceae
Gelidiales
DQ022775 Sciurothamnion stegengae Callithamniaceae Ceramiales
U60741 Phymatolithon lenormandii Hapalidiaceae Corallinales
AF460859 Ceramium affine Ceramiaceae Ceramiales
Sarcodiotheca gaudichaudii Solieriaceae Gigartinales
DQ022780 Euptilota molle Callithamniaceae Ceramiales
AF043355 Bangia fuscopurpurea
7.0

## 6.5 DISCUSSION

In almost all cases variation in length elements of the nSSU rRNA secondary structure was sufficient to distinguish taxa, including among Florideophyceae, as only conspecific isolates occupied the same terminal nodes in the trees (Figures 6-3 to 6-5); however, only non-Eurhodophytina taxa demonstrated enough accumulated variation for accurate taxonomic resolution. Although few taxonomic insights were clearly observed in these analyses, the monophyly of the Dixoniellales (Figure 6-3) does support the recent erection of the order (Yokoyama et al., 2009); however, other genera belonging to the Dixoniellales, *Glaucosphaera* and *Neorhodella*, were not present.

Structural changes in biomolecules such as proteins and RNA tend to accumulate slowly relative to its sequence and it is likely that Florideophyceae taxa have evolved too recently for enough structural changes to accumulate. The Rhodophyta are an early diverging lineage of eukaryotes, with putative fossil evidence of unicellular species superficially similar to the extant Porphyridiales ca. 2 billion years before present (Tappan, 1976). Even morphologically modern Rhodophyta fossils similar to modern Bangiales taxa have a considerable fossil history, dated from ca. 1.2 billion years before present (Butterfield *et al.*, 1990; Butterfield, 2000). In contrast, the Florideophyceae is the most recently evolved class of Rhodophyta. Although the radiation of the major subclass lineages of Florideophyceae was likely to have occurred as much as 600 million years ago and there is evidence for early divergence of orders (see Saunders and Hommersand (2004) for review), it appears as though

nSSU rRNA structural changes have been too slow to be phylogenetically useful within the class.

The Neighbor Joining algorithm was an improvement over UPGMA clustering in analysis of these nSSU rRNA morphometric data, particularly due of the lack of gradual transition between nSSU rRNA structures of different taxa. In general structures were identical or nearly so for closely related taxa and there were few to no intermediate steps existing in current sequences among taxonomic groups. For UPGMA clustering this resulted in long branches to terminal nodes relative to the compressed branch lengths present for higher order relationships. Consequently, broader taxonomic relationships were obscured. Corresponding tree topology was not as ambiguous in Neighbor Joining analyses, most likely due to algorithm differences (e.g. optimality criterion). The lack of gradual transitions among taxa also made these analyses very susceptible to data artifacts such as unknown characters and truncations, which resulted in artificial exaggeration of distances. For example, nSSU rRNA gene sequences from *Stylonema alsidii*, *Purpureofilum apyrenoidigerum* and *Chlidophyllon kaspar*, all had the same missing data for the 5' region of the alignment, which influenced the size of the first helix element and forced the sequences into a polyphyletic group (Figure 6-3).

The Euclidian distance metric is likely not the most appropriate metric for these data. Structural abstractions of the SSU have been attempted in previous work (Caetano-Anolles 2002a); however, previously existing model derivations do not scale well to the type of structural abstraction performed here. Similarly it is likely that a tree topology is not the optimal display method for these data and future analysis should contrast the use of

ordination in addition to different, more biologically realistic distance metrics. Furthermore, support metrics such as bootstrapping that are suitable for these analyses should be proposed. Since different regions are constrained by different evolutionary pressures, a straight forward resampling procedure would be inappropriate. Improving resolution with customized non-Euclidian distance metrics may also broaden the timescale at which this method is useful with nSSU rRNA molecules. Notably, since the method is dependent on accumulated structural changes, other orthologous RNA molecules could be used for resolution at different evolutionary timescales.

A secondary utility for this method, independent of lineage age, is the rapid screening of sequences for unique (large or small) structural elements that deviate from the consensus nSSU rRNA secondary structure. For example, sequences within the genus *Thorea* have previously observed unique secondary structure elements in the nSSU rRNA (Müller et al., 2002) that was clearly manifested here as a long terminal branch to the genus (Figure 6-5). Such information could be useful for similar branching patterns observed here, including Bangiophyceae *s.s.* sequences in *'Porphyra'* clade 2 and *Porphyra purpurea* (T) (Figure 6-4) and Florideophyceae isolates *Batrachospermum louisianae* and *Rhodogorgon carriebowensis* (Figure 6-5). Each of the large loop expansions present in these sequences likely has a specific non-loop secondary structure. Furthermore, as these analyses were performed on a dataset filtered by sequence identity and phylogeny, each of these sequences with potential unique structural elements represents complete clades of taxa, increasing the potential taxonomic utility of these findings.

The outlined methodology for evaluating nSSU rRNA secondary structure is useful for several reasons. It is much faster than phylogenetic analyses, with results obtained instantly for small data sets and within minutes for thousands of taxa. As such, this approach, as it matures, can be used to complement traditional phylogenetic analyses. Additionally, RNA morphometrics can be used to determine significant sites (active sites, structural synapomorphies in RNA, etc.) based on differences, primarily branch lengths, between the morphometric and species tree topologies. These characteristics were not addressed here because a quantification and qualification of topological incongruence, the reliability of the Euclidian distance metric for these types of data and the clarification of the influence of single topology differences requires a more thorough and directed investigation.

## 6.6 CONCLUSIONS

Morphometrics of the nSSU rRNA secondary structure, specifically the multivariate analysis of length polymorphisms in constituent helix and loop elements, was useful in supporting and improving phylogenetic and taxonomic resolution of non-Eurhodophytina red algae. This novel approach to phylogenetic analyses, although able to distinguish isolates at all taxonomic levels, requires the development of a distance metric tailored to the types of data generated. Euclidian distance did not adequately represent the morphometric islands within these data (i.e. the general conservation of structure among closely related species) as well as factors such as the differing potential of expansion/contraction of helices and loops enforced by structural constraints on the molecule. As a result, Euclidian distance among structures appeared to obscure relationships within the data for recently derived taxa and seemed to be

highly susceptible to sequence and structural artifacts such as missing or poorly aligned data. Addressing these concerns is an area of ongoing investigation.

An unforeseen utility of this approach to nSSU rRNA morphometrics was the easy and rapid identification of deviations from the consensus secondary structure. These deviations manifested as long branches to terminal nodes and topological incongruence between the species tree and the morphometric clustering. This approach to RNA morphometrics was sensitive enough for a deviation within one of hundreds of structural elements to be manifested as a divergent topology. Specifically tailoring these analyses towards filtering for these deviations, including modeling approximate secondary structure of large deviations, will be useful in the characterization of synapomorphic structural elements within rRNA specifically and active or functional sites of RNA molecules in general.

# Chapter 7
# General Conclusions

Despite active research into Rhodophyta taxonomy there still exists considerable ambiguity in the definition and identification of species, resulting in cryptic species and widespread errors in taxonomy. Furthermore, there are limited data for characteristics such as biogeography and inter and intra-specific genetic diversity for Rhodophyta taxa, especially for the Bangiophyceae *sensu lato* (*s.l.*) defined as all non-Florideophyceae taxa. The definition, clear identification and resolved taxonomy of species are crucial for understanding and framing questions regarding the ecology and evolutionary history of an organism. Research into this aspect of Rhodophyta biology is especially important, as this crown eukaryotic group provides insights into the development of multicelluarity, sexuality and chloroplast diversification. Furthermore, there is urgency for a phylogenetic and taxonomic framework of these organisms due to the ongoing North American commercialization of the aquaculture crop *Porphyra* (Bangiales) and the anticipated release of the *Porphyra umbilicalis* Kützing genome, which will stimulate a considerable volume of research due to its novelty (the closest neighbouring genome, the biologically unique *Cyanidioschyzon merolae*, shared a common ancestor as much as 2 billion years ago).

Due to the significant amount of cryptic diversity within the Rhodophyta, specifically most non-Florideophyceae taxa, a baseline of intraspecific genetic diversity for different lineages and species is required. The work presented in Chapter 2 was the first attempt at such quantification for non-Florideophyceae Rhodophyta. The intraspecific genetic variation

and population dynamics over a series of localized boulders for the highly endemic species *Bangia maxima* N.L. Gardner were evaluated, providing insights in the sexuality, genetic variation and population structure of species within the Bangiales. The phylogenetic position of the morphologically unique *Bangia maxima* within one clade of the paraphyletic filamentous *Bangia fuscopurpurea* further supported the proposal of multiple cryptic species within the currently recognized genus *Bangia*. Moreover, the morphology of *Bangia maxima* may further indicate the homoplasious transition to the bladed thallus of *Porphyra* species within the Bangiales. While genetic variation in species of *Bangia* as a whole, specifically over gene regions such as nSSU rRNA, is well established, more work is necessary to understand the dynamics of genetic variation over small spatial scales. Since *Bangia maxima* is only known from one restricted location, it is possible that it is a true endemic species and its population dynamics may differ from other *Bangia* species. Consequently, the finding of highly consistent localized within-boulder and divergent among-boulder ISSR banding patterns may not be typical of the genus as a whole.

Sequence-based molecular identification of taxa is a useful construct for studying species, especially non-Florideophyceae Rhodophyta due to simple unicellular, filament or sheet gametophyte morphologies. Identification of Bangiophyceae *s.l.* through so-called DNA barcodes demonstrated promise in Chapter 3, although some taxonomic and phylogenetic inconsistencies were observed using protocols proposed for the Rhodophyta. The 5' 500-600 nucleotide cytochrome oxidase c subunit I fragment (COI) was proposed for the Rhodophyta, but not thoroughly tested for non-Florideophyceae taxa (Saunders, 2005), which, while currently not as species rich as the Florideophyceae, does account for the

majority of the evolutionary distance within the phylum. Results in Chapter 3 indicated that although the COI gene is suitable for many cases of molecular identification of Bangiales taxa, amplification of non-Eurhodophytina taxa with current primers is very limited. Furthermore, even when amplification was successful, there was unequal efficiency among lineages. Additionally, while discrimination was often strong (sequence identity among conspecifics was typically > 99% and different groups formed sequence 'islands' in the phylogeny), some groupings of isolates directly contradicted phylogenies using orthologous genes such as nSSU rRNA and *rbc*L. Furthermore, discrimination of endemic species (e.g. *Bangia vermicularis*) was unclear. Due to these limitations, COI, while promising, should not exclusively be used as the barcoding region for Rhodophyta. Ongoing research has determined that highly degenerate primers have been able to solve amplification concerns. Despite stated limitations, the work presented here is, to date, the most complete study of the biogeography of northern Atlantic and Pacific Bangiales isolates.

Certainly the most useful methodology for the clarification of cryptic and unresolved phylogenetic relationships presented in this thesis was the incorporation of secondary structure information into sequence alignment and models of sequence evolution used in phylogenetic analysis. It is clear that the evolutionary information in available nSSU rRNA gene sequence data has not being fully utilized in typical phylogenetic work, which is almost exclusively performed under the assumption that each nucleotide is an independent character. Incorporating secondary structure information by using more biologically realistic parametric secondary structure models of RNA successfully resolved many previously ambiguous phylogenetic relationships within the Rhodophyta. In fact, it was often more successful in

resolving these relationships than the incorporation of multiple genes. Analyses presented. This work, using the most diverse collection of samples currently assembled, demonstrated the utility of secondary structure models in resolving ambiguous phylogenetic relationships within the Rhodophyta and demonstrating how pervasive cryptic lineages are.

The Bangiophyceae *s.l.*, specifically non-Eurhodophytina taxa, demonstrated limited cryptic species in these analyses; however, increased taxon sampling would likely increase cryptic diversity based on the observed genetic diversity of isolates with the same species designation and the paucity of morphological and ecological characters for these taxa. The analyses presented in Chapter 4 represents a significant improvement over current taxonomic treatments, providing phylogenetic structure to what has previously been considered an unresolved polytomy (Yoon et al., 2006a). Both phylogenetic metrics and nucleotide signatures supported this resolution, specifically for the Compsopogonales, Erythropeltidales, Rhodochaetales and Porphyridiales group. The addition of sequences from more Bangiophyceae *s.l.* isolates will likely further increase taxonomic resolution.

Nuclear SSU rRNA analysis of the Bangiales (Chapter 5) was the most taxonomically complete phylogenetic analysis so far undertaken and demonstrated a large degree of homoplasious evolution of filamentous and foliose gametophyte morphologies. The Bangiophyceae *sensu stricto* (*s.s.*), including only the Bangiales, were very strongly characterized as having a minimum of 16 independent generic lineages, requiring three new genera of filamentous *Bangia*-like species and eight new genera of foliose *Porphyra*-like species. Monotypic generic lineages *Bangia atropurpurea*, *Dione arcuata, Minerva*

*aenigmata, Porphyra purpurea* and *Pseudobangia kaycoleia* should be maintained. The work presented here clarified the cryptic diversity present in the Bangiales and was the basis for the taxonomic revision of the order (Broom et al., in press).

Despite being less prone to cryptic species due a higher degree of morphological complexity relative to other Rhodophyta, there is still considerable ambiguity in many evolutionary relationships within the Florideophyceae. Chapter 5 presented the most species-rich, comprehensive phylogenetic analysis of the class. In contrast to the Bangiophyceae *s.s.* the taxonomic changes within the Florideophyceae suggested by these results were subtle. Most observed phylogenetic topologies typically had precedence in the taxonomic literature; however, topologies here were resolved with a single gene and typically with a higher degree of confidence. For example, the separation of the Nemaliophycidae *sensu* Saunders and Hommersand (2004) into the Corallinophycidae and the Nemaliophycidae, which increases the number of Florideophyceae subclasses to five was previously only observed in phylogenies with several gene loci (e.g. Verbrugget et al. 2010). Furthermore, pre-existing yet not fully accepted taxonomic suggestions were also corroborated, such as the Peyssoneliales *sensu* Kreyesky et al. (2009). In contrast, some novel taxonomic observations were resolved in this analysis of the Florideophyceae. For example, there was strong support for the return of *Mastocarpus* (Phyllophoraceae) to the Gigartinaceae, potentially resolving the taxonomic ambiguity of this genus.

The analysis of the Florideophyceae presented in Chapter 5 also identified areas of the phylogenetic tree that require further taxonomic attention. These include the strong phylogenetic association between Lomentariaceae and Faucheaceae within the

Rhodymeniales. The widespread polyphyly between Dasyaceae and Delesseriaceae, with several independent lineages of each, also requires significant taxonomic revision. Additionally, there was evidence of poor phylogenetic resolution in some lineages caused by either insufficient taxonomic sampling or poor sequence quality. For example, the families Acrotylaceae, Caulacanthaceae, and Cystocloniaceae each had the majority of isolates resolved as monophyletic groups interrupted by ambiguous placement of monotypic lineages.

Structural characteristics of the nSSU rRNA have been used to infer ancient evolutionary relationships (Woese, 1987; Winkler and Woese, 1991; Caetano-Anolles, 2002). By examining secondary structure characteristics of all known nSSU rRNA Rhodophyta sequences, the work presented in Chapter 6, although preliminary, demonstrated that secondary structure characteristics could provide additional support for phylogenies inferred from sequence analysis. Furthermore, this technique of molecular morphometrics could potentially be used to examine more than just evolutionary relationships, as it was adept at finding unique molecular characteristics of RNA molecules in general, which could represent either conserved or functional motifs.

Erection of taxonomic ranks based on molecular sequence analysis can be difficult, especially in the Rhodophyta where most lineages with cryptic species have no consistent discernable morphological, environmental or ecological support for their separation. Potentially, the genetic divergence and well-supported phylogenies presented here will help direct research into the novel suggested taxonomy that will identify characteristics further supporting these results. The analyses presented here significantly advanced understanding of the evolutionary distribution of cryptic species within the Rhodophyta. Furthermore, useful

methods for the characterization of such species were presented, as is a demonstration of the utility biologically realistic sequence models that parameterize nSSU rRNA structure in resolving ambiguous and problematic phylogenetic relationships. Most importantly, this work also represents a significant improvement toward taxonomy congruent with evolutionary history for the Rhodophyta.

# Appendix A

# NCBI GenBank Accession Numbers for nSSU rRNA Sequences Used in This Thesis

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| Bangiophyceae | Bangiales | Bangiaceae | *Bangia* | *atropurpurea* | AB053491 |
| | | | *Bangia* | *atropurpurea* | AB114638 |
| | | | *Bangia* | *atropurpurea* | AB114639 |
| | | | *Bangia* | *atropurpurea* | AF169340 |
| | | | *Bangia* | *atropurpurea* | AF169339 |
| | | | *Bangia* | *atropurpurea* | AF169341 |
| | | | *Bangia* | *atropurpurea* | AF043365 |
| | | | *Bangia* | *atropurpurea* | AF043365 |
| | | | *Bangia* | *atropurpurea* | AF043365 |
| | | | *Bangia* | *atropurpurea* | AF043365 |
| | | | *Bangia* | *atropurpurea* | L36066 |
| | | | *Bangia* | *atropurpurea* | D88387 |
| | | | *Bangia* | *fuscopurpurea* | AF175537 |
| | | | *Bangia* | *fuscopurpurea* | AB053488 |
| | | | *Bangia* | *fuscopurpurea* | AB053489 |
| | | | *Bangia* | *fuscopurpurea* | AF175529 |
| | | | *Bangia* | *fuscopurpurea* | AF175535 |
| | | | *Bangia* | *fuscopurpurea* | AF175531 |
| | | | *Bangia* | *fuscopurpurea* | AF175532 |
| | | | *Bangia* | *fuscopurpurea* | AF169334 |
| | | | *Bangia* | *fuscopurpurea* | AF043363 |
| | | | *Bangia* | *fuscopurpurea* | AF175533 |
| | | | *Bangia* | *fuscopurpurea* | AF043362 |
| | | | *Bangia* | *fuscopurpurea* | AF169338 |
| | | | *Bangia* | *fuscopurpurea* | AF043353 |
| | | | *Bangia* | *fuscopurpurea* | AF175536 |
| | | | *Bangia* | *fuscopurpurea* | AF175530 |
| | | | *Bangia* | *fuscopurpurea* | AF043354 |
| | | | *Bangia* | *fuscopurpurea* | AF043355 |
| | | | *Bangia* | *fuscopurpurea* | AF043355 |
| | | | *Bangia* | *fuscopurpurea* | AF043355 |
| | | | *Bangia* | *fuscopurpurea* | AF175534 |
| | | | *Bangia* | *fuscopurpurea* | AF169335 |
| | | | *Bangia* | *fuscopurpurea* | AF169337 |
| | | | *Bangia* | *fuscopurpurea* | AF043358 |
| | | | *Bangia* | *fuscopurpurea* | AF169336 |
| | | | *Bangia* | *fuscopurpurea* | AF175528 |
| | | | *Bangia* | *fuscopurpurea* | AF043359 |
| | | | *Bangia* | *fuscopurpurea* | AF043360 |
| | | | *Bangia* | *fuscopurpurea* | AF043356 |
| | | | *Bangia* | *fuscopurpurea* | AF043361 |
| | | | *Bangia* | *fuscopurpurea* | AF043357 |
| | | | *Bangia* | *fuscopurpurea* | AF342745 |
| | | | *Bangia* | *gloiopeltidicola* | AB053490 |
| | | | *Bangia* | sp. | AF043365 |
| | | | *Bangia* | sp. | UNP00639 |
| | | | *Bangia* | sp. | AY184335 |
| | | | *Bangia* | sp. | AY184336 |
| | | | *Bangia* | sp. | AY184337 |
| | | | *Bangia* | sp. | AY184338 |
| | | | *Bangia* | sp. | AY184339 |
| | | | *Bangia* | sp. | AY184341 |
| | | | *Bangia* | sp. | AY184342 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Bangia* | sp. | AY184344 |
| | | | *Bangia* | sp. | AY184345 |
| | | | *Bangia* | sp. | AY184346 |
| | | | *Bangia* | sp. | AY184348 |
| | | | *Bangia* | sp. | AY909600 |
| | | | *Bangia* | sp. | DQ084421 |
| | | | *Dione* | *arcuata* | AY184343 |
| | | | *Dione* | *arcuata* | AY465354 |
| | | | *Minerva* | *aenigmata* | AY184347 |
| | | | *Minerva* | *aenigmata* | AY465355 |
| | | | *Porphyra* | *abbottae* | AF175545 |
| | | | *Porphyra* | *acanthophora* | L26197 |
| | | | | *var acanthophora* | |
| | | | *Porphyra* | *acanthophora* | AY766359 |
| | | | | *var brasiliensis* | |
| | | | *Porphyra* | *amplissima* | AF358288 |
| | | | *Porphyra* | *amplissima* | AF358291 |
| | | | *Porphyra* | *amplissima* | AF358292 |
| | | | *Porphyra* | *amplissima* | AF358293 |
| | | | *Porphyra* | *amplissima* | AF358295 |
| | | | *Porphyra* | *amplissima* | AF358297 |
| | | | *Porphyra* | *amplissima* | AF358299 |
| | | | *Porphyra* | *amplissima* | AF358290 |
| | | | *Porphyra* | *amplissima* | AF358294 |
| | | | *Porphyra* | *amplissima* | AF358289 |
| | | | *Porphyra* | *amplissima* | AF358296 |
| | | | *Porphyra* | *amplissima* | AF358298 |
| | | | *Porphyra* | *amplissima* | AB015791 |
| | | | *Porphyra* | *amplissima* | L36048 |
| | | | *Porphyra* | *amplissima* | AH010573 |
| | | | *Porphyra* | *capensis* | AY766361 |
| | | | *Porphyra* | *carolinensis* | AF133792 |
| | | | *Porphyra* | *carolinensis* | AF378654 |
| | | | *Porphyra* | *carolinensis* | AF378653 |
| | | | *Porphyra* | *cf leucosticta* | AF175538 |
| | | | *Porphyra* | *cf plocamiestris* | AF175555 |
| | | | *Porphyra* | *cinnamomea* | AF136418 |
| | | | *Porphyra* | *dentata* | AB293462 |
| | | | *Porphyra* | *dentata* | AB293463 |
| | | | *Porphyra* | *dentata* | AB293464 |
| | | | *Porphyra* | *dentata* | AB013183 |
| | | | *Porphyra* | *drewiana* | AY766362 |
| | | | *Porphyra* | *fallax ssp fallax* | AF175541 |
| | | | *Porphyra* | *gardneri* | DQ084423 |
| | | | *Porphyra* | *haitanensis* | AB015795 |
| | | | *Porphyra* | *haitanensis* | AB013181 |
| | | | *Porphyra* | *kanakaensis* | AF175556 |
| | | | *Porphyra* | *katadae* | AB013184 |
| | | | *Porphyra* | *kuniedai* | AF123051 |
| | | | *Porphyra* | *lanceolata* | AY909594 |
| | | | *Porphyra* | *leucosticta* | AF358345 |
| | | | *Porphyra* | *leucosticta* | AF358347 |
| | | | *Porphyra* | *leucosticta* | AF358348 |
| | | | *Porphyra* | *leucosticta* | AF358350 |
| | | | *Porphyra* | *leucosticta* | AF358351 |
| | | | *Porphyra* | *leucosticta* | AF358346 |
| | | | *Porphyra* | *leucosticta* | AF358349 |
| | | | *Porphyra* | *leucosticta* | AH010575 |
| | | | *Porphyra* | *leucosticta* | AH010578 |
| | | | *Porphyra* | *leucosticta* | AF175557 |
| | | | *Porphyra* | *leucosticta* | AF342746 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Porphyra* | *leucosticta* | L26199 |
| | | | *Porphyra* | *lilliputiana* | AF136424 |
| | | | *Porphyra* | *lilliputiana* | AF378649 |
| | | | *Porphyra* | *lilliputiana* | AF378650 |
| | | | *Porphyra* | *lilliputiana* | AF378651 |
| | | | *Porphyra* | *lilliputiana* | AF378658 |
| | | | *Porphyra* | *lilliputiana* | AF378659 |
| | | | *Porphyra* | *lilliputiana* | AF378660 |
| | | | *Porphyra* | *lilliputiana* | AF378661 |
| | | | *Porphyra* | *lilliputiana* | AF378662 |
| | | | *Porphyra* | *lilliputiana* | AF378663 |
| | | | *Porphyra* | *lilliputiana* | AF378664 |
| | | | *Porphyra* | *lilliputiana* | AF378665 |
| | | | *Porphyra* | *linearis* | AF358325 |
| | | | *Porphyra* | *linearis* | AF358326 |
| | | | *Porphyra* | *linearis* | AF358328 |
| | | | *Porphyra* | *linearis* | AF358329 |
| | | | *Porphyra* | *linearis* | AF358330 |
| | | | *Porphyra* | *linearis* | AF358327 |
| | | | *Porphyra* | *linearis* | AF358324 |
| | | | *Porphyra* | *linearis* | AF175539 |
| | | | *Porphyra* | *miniata* | AF358365 |
| | | | *Porphyra* | *miniata* | AF358366 |
| | | | *Porphyra* | *miniata* | AF358359 |
| | | | *Porphyra* | *miniata* | AF358360 |
| | | | *Porphyra* | *miniata* | AF358361 |
| | | | *Porphyra* | *miniata* | AF358362 |
| | | | *Porphyra* | *miniata* | AF358364 |
| | | | *Porphyra* | *miniata* | AF358363 |
| | | | *Porphyra* | *miniata* | AH010602 |
| | | | *Porphyra* | *miniata* | AH010577 |
| | | | *Porphyra* | *miniata* | AF175547 |
| | | | *Porphyra* | *miniata* | AF175540 |
| | | | *Porphyra* | *miniata* | L26200 |
| | | | *Porphyra* | *nereocystis* | AF175542 |
| | | | *Porphyra* | *onoi* | AB015794 |
| | | | *Porphyra* | *perforata* | AY909592 |
| | | | *Porphyra* | *pseudolanceolata* | AF175543 |
| | | | *Porphyra* | *pseudolinearis* | AB293465 |
| | | | *Porphyra* | *pseudolinearis* | AB293466 |
| | | | *Porphyra* | *pseudolinearis* | AB293467 |
| | | | *Porphyra* | *pseudolinearis* | AB293468 |
| | | | *Porphyra* | *pseudolinearis* | AB293469 |
| | | | *Porphyra* | *pseudolinearis* | AB293470 |
| | | | *Porphyra* | *pseudolinearis* | AB293471 |
| | | | *Porphyra* | *pseudolinearis* | AB293472 |
| | | | *Porphyra* | *pseudolinearis* | AB293473 |
| | | | *Porphyra* | *pseudolinearis* | AB293474 |
| | | | *Porphyra* | *pseudolinearis* | AB293475 |
| | | | *Porphyra* | *pseudolinearis* | AB293476 |
| | | | *Porphyra* | *pseudolinearis* | AB293477 |
| | | | *Porphyra* | *pseudolinearis* | AB293478 |
| | | | *Porphyra* | *pseudolinearis* | AB293479 |
| | | | *Porphyra* | *pseudolinearis* | AB293480 |
| | | | *Porphyra* | *pseudolinearis* | AB293481 |
| | | | *Porphyra* | *pseudolinearis* | AB293482 |
| | | | *Porphyra* | *pseudolinearis* | AB293483 |
| | | | *Porphyra* | *pseudolinearis* | AB293484 |
| | | | *Porphyra* | *pseudolinearis* | AB293485 |
| | | | *Porphyra* | *pseudolinearis* | AB293486 |
| | | | *Porphyra* | *pseudolinearis* | AB293487 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Porphyra* | *pseudolinearis* | AB293488 |
| | | | *Porphyra* | *pseudolinearis* | AB293489 |
| | | | *Porphyra* | *pseudolinearis* | AB015793 |
| | | | *Porphyra* | *pseudolinearis* | AF116913 |
| | | | *Porphyra* | *pseudolinearis* | AB013185 |
| | | | *Porphyra* | *purpurea* | AF358368 |
| | | | *Porphyra* | *purpurea* | AF358370 |
| | | | *Porphyra* | *purpurea* | AF358373 |
| | | | *Porphyra* | *purpurea* | AF358372 |
| | | | *Porphyra* | *purpurea* | AF358374 |
| | | | *Porphyra* | *purpurea* | AF358375 |
| | | | *Porphyra* | *purpurea* | AF358371 |
| | | | *Porphyra* | *purpurea* | AF358369 |
| | | | *Porphyra* | *purpurea* | AF358367 |
| | | | *Porphyra* | *purpurea* | AH010597 |
| | | | *Porphyra* | *purpurea* | AH010574 |
| | | | *Porphyra* | *purpurea* | DQ535254 |
| | | | *Porphyra* | *purpurea* | DQ535255 |
| | | | *Porphyra* | *purpurea* | DQ535256 |
| | | | *Porphyra* | *purpurea* | DQ535260 |
| | | | *Porphyra* | *purpurea* | AF175550 |
| | | | *Porphyra* | *purpurea* | DQ535257 |
| | | | *Porphyra* | *purpurea* | DQ535259 |
| | | | *Porphyra* | *purpurea* | DQ535258 |
| | | | *Porphyra* | *purpurea* | AF175551 |
| | | | *Porphyra* | *purpurea* | L26201 |
| | | | *Porphyra* | *rakiura* | AY139682 |
| | | | *Porphyra* | *rakiura* | AY139684 |
| | | | *Porphyra* | *rakiura* | AF136425 |
| | | | *Porphyra* | *rediviva* | AF175544 |
| | | | *Porphyra* | *rosengurttii* | DQ834639 |
| | | | *Porphyra* | sp. | DQ084424 |
| | | | *Porphyra* | sp. | DQ084426 |
| | | | *Porphyra* | sp. | DQ084428 |
| | | | *Porphyra* | sp. | AB293500 |
| | | | *Porphyra* | sp. | AB293501 |
| | | | *Porphyra* | sp. | AB293502 |
| | | | *Porphyra* | sp. | AB293503 |
| | | | *Porphyra* | sp. | AB293504 |
| | | | *Porphyra* | sp. | AB293505 |
| | | | *Porphyra* | sp. | AB293506 |
| | | | *Porphyra* | sp. | AB293507 |
| | | | *Porphyra* | sp. | AB293508 |
| | | | *Porphyra* | sp. | AB293509 |
| | | | *Porphyra* | sp. | AB293510 |
| | | | *Porphyra* | sp. | AB293511 |
| | | | *Porphyra* | sp. | AB293512 |
| | | | *Porphyra* | sp. | AB293513 |
| | | | *Porphyra* | sp. | AB293514 |
| | | | *Porphyra* | sp. | AB293515 |
| | | | *Porphyra* | sp. | AB293516 |
| | | | *Porphyra* | sp. | AB293517 |
| | | | *Porphyra* | sp. | AB293518 |
| | | | *Porphyra* | sp. | AB293519 |
| | | | *Porphyra* | sp. | AB293520 |
| | | | *Porphyra* | sp. | AB293521 |
| | | | *Porphyra* | sp. | AB293522 |
| | | | *Porphyra* | sp. | AB293523 |
| | | | *Porphyra* | sp. | AB293524 |
| | | | *Porphyra* | sp. | AB293525 |
| | | | *Porphyra* | sp. | AB293526 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Porphyra* | sp. | AB293527 |
| | | | *Porphyra* | sp. | AB293528 |
| | | | *Porphyra* | sp. | AB293529 |
| | | | *Porphyra* | sp. | AB293530 |
| | | | *Porphyra* | sp. | AB293531 |
| | | | *Porphyra* | sp. | AB293532 |
| | | | *Porphyra* | sp. | AB293533 |
| | | | *Porphyra* | sp. | AB293534 |
| | | | *Porphyra* | sp. | AY139685 |
| | | | *Porphyra* | sp. | AF136427 |
| | | | *Porphyra* | sp. | AF136428 |
| | | | *Porphyra* | sp. | AF136426 |
| | | | *Porphyra* | sp. | AF136420 |
| | | | *Porphyra* | sp. | AF136422 |
| | | | *Porphyra* | sp. | AF117306 |
| | | | *Porphyra* | sp. | AB013182 |
| | | | *Porphyra* | sp. | AF136423 |
| | | | *Porphyra* | sp. | DQ084431 |
| | | | *Porphyra* | sp. | DQ084432 |
| | | | *Porphyra* | sp. | DQ084433 |
| | | | *Porphyra* | sp. | DQ084434 |
| | | | *Porphyra* | sp. | DQ084436 |
| | | | *Porphyra* | sp. | DQ084438 |
| | | | *Porphyra* | sp. | AF175546 |
| | | | *Porphyra* | sp. | AF175548 |
| | | | *Porphyra* | sp. | AF175554 |
| | | | *Porphyra* | sp. | AY100473 |
| | | | *Porphyra* | sp. | AY184349 |
| | | | *Porphyra* | sp. | AY184350 |
| | | | *Porphyra* | sp. | AY184351 |
| | | | *Porphyra* | sp. | AY184352 |
| | | | *Porphyra* | sp. | AY292624 |
| | | | *Porphyra* | sp. | AY292626 |
| | | | *Porphyra* | sp. | AY292627 |
| | | | *Porphyra* | sp. | AY292628 |
| | | | *Porphyra* | sp. | AY292629 |
| | | | *Porphyra* | sp. | AY292630 |
| | | | *Porphyra* | sp. | AY292631 |
| | | | *Porphyra* | sp. | AY292632 |
| | | | *Porphyra* | sp. | AY292633 |
| | | | *Porphyra* | sp. | AY292634 |
| | | | *Porphyra* | sp. | AY292636 |
| | | | *Porphyra* | sp. | AY292637 |
| | | | *Porphyra* | sp. | AY292638 |
| | | | *Porphyra* | sp. | AY292639 |
| | | | *Porphyra* | sp. | AY292640 |
| | | | *Porphyra* | sp. | AY292642 |
| | | | *Porphyra* | sp. | AY292644 |
| | | | *Porphyra* | sp. | AY299973 |
| | | | *Porphyra* | sp. | AY766357 |
| | | | *Porphyra* | sp. | AY766358 |
| | | | *Porphyra* | sp. | AY909601 |
| | | | *Porphyra* | sp. | AY909599 |
| | | | *Porphyra* | sp. | AY909598 |
| | | | *Porphyra* | sp. | AY909597 |
| | | | *Porphyra* | sp. | AY909596 |
| | | | *Porphyra* | sp. | AY909603 |
| | | | *Porphyra* | sp. | AY909595 |
| | | | *Porphyra* | sp. | AY909604 |
| | | | *Porphyra* | sp. | AY909593 |
| | | | *Porphyra* | sp. | AY909591 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Porphyra* | sp. | AY909590 |
| | | | *Porphyra* | sp. | AY909589 |
| | | | *Porphyra* | sp. | AY909588 |
| | | | *Porphyra* | sp. | AY909587 |
| | | | *Porphyra* | sp. | AY909584 |
| | | | *Porphyra* | sp. | AY909585 |
| | | | *Porphyra* | sp. | AY909583 |
| | | | *Porphyra* | sp. | AY909586 |
| | | | *Porphyra* | sp. | AY913952 |
| | | | *Porphyra* | sp. | EF033582 |
| | | | *Porphyra* | sp. | DQ834636 |
| | | | *Porphyra* | sp. | DQ834637 |
| | | | *Porphyra* | *spiralis var ampifolia* | L26177 |
| | | | *Porphyra* | *spiralis var spiralis* | AY766360 |
| | | | *Porphyra* | *suborbiculata* | AB293490 |
| | | | *Porphyra* | *suborbiculata* | AB293491 |
| | | | *Porphyra* | *suborbiculata* | AB293492 |
| | | | *Porphyra* | *suborbiculata* | AB293493 |
| | | | *Porphyra* | *suborbiculata* | AB293494 |
| | | | *Porphyra* | *suborbiculata* | AB293495 |
| | | | *Porphyra* | *suborbiculata* | AB293496 |
| | | | *Porphyra* | *suborbiculata* | AF378652 |
| | | | *Porphyra* | *suborbiculata* | AB015796 |
| | | | *Porphyra* | *suborbiculata* | AF378655 |
| | | | *Porphyra* | *suborbiculata* | AF378656 |
| | | | *Porphyra* | *suborbiculata* | AF378657 |
| | | | *Porphyra* | *suborbiculata* | AB013180 |
| | | | *Porphyra* | *suborbiculata* | AF117239 |
| | | | *Porphyra* | *tenera* | AB293497 |
| | | | *Porphyra* | *tenera* | AB000964 |
| | | | *Porphyra* | *tenera* | D86237 |
| | | | *Porphyra* | *tenera* | D86236 |
| | | | *Porphyra* | *tenera* | AB235850 |
| | | | *Porphyra* | *tenera* | AB235851 |
| | | | *Porphyra* | *tenera* | AB235852 |
| | | | *Porphyra* | *tenera* | AB029881 |
| | | | *Porphyra* | *tenera* | AB029882 |
| | | | *Porphyra* | *tenera* | AB013176 |
| | | | *Porphyra* | *tenera* | AB029883 |
| | | | *Porphyra* | *tenera* | AB029884 |
| | | | *Porphyra* | *tenera* | AB029879 |
| | | | *Porphyra* | *tenera* | AB029880 |
| | | | *Porphyra* | *tenera* | AB013175 |
| | | | *Porphyra* | *tenera* | AB100958 |
| | | | *Porphyra* | *tenera* | AB100959 |
| | | | *Porphyra* | *tenera* | AB101442 |
| | | | *Porphyra* | *tenuipedalis* | AB293498 |
| | | | *Porphyra* | *tenuipedalis* | AB015797 |
| | | | *Porphyra* | *torta* | AF175552 |
| | | | *Porphyra* | *umbilicalis* | AH010603 |
| | | | *Porphyra* | *umbilicalis* | AH010576 |
| | | | *Porphyra* | *umbilicalis* | AF358398 |
| | | | *Porphyra* | *umbilicalis* | AF358396 |
| | | | *Porphyra* | *umbilicalis* | AF358395 |
| | | | *Porphyra* | *umbilicalis* | AF358397 |
| | | | *Porphyra* | *umbilicalis* | AF358393 |
| | | | *Porphyra* | *umbilicalis* | AF358394 |
| | | | *Porphyra* | *umbilicalis* | X53500 |
| | | | *Porphyra* | *umbilicalis* | AF175553 |

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|---|---|---|---|---|---|
| | | | *Porphyra* | *umbilicalis* | AF175549 |
| | | | *Porphyra* | *umbilicalis* | AB013179 |
| | | | *Porphyra* | *umbilicalis* | L36049 |
| | | | *Porphyra* | *umbilicalis* | L26202 |
| | | | *Porphyra* | *variegata* | AB015792 |
| | | | *Porphyra* | *virididentata* | AF136421 |
| | | | *Porphyra* | *yezoensis* | AB293499 |
| | | | *Porphyra* | *yezoensis* | AY131005 |
| | | | *Porphyra* | *yezoensis* | AB235208 |
| | | | *Porphyra* | *yezoensis* | AB235209 |
| | | | *Porphyra* | *yezoensis* | AB235210 |
| | | | *Porphyra* | *yezoensis* | D79976 |
| | | | *Porphyra* | *yezoensis* | AB235849 |
| | | | *Porphyra* | *yezoensis* | AB235853 |
| | | | *Porphyra* | *yezoensis* | AB235854 |
| | | | *Porphyra* | *yezoensis* | AB013177 |
| | | | *Porphyra* | *yezoensis* | AB013178 |
| | | | *Pseudobangia* | *kaycoleia* | AF043364 |
| Cyanidiophyceae | Cyanidiales | Cyanidiaceae | *Cyanidioschyzon* | *merolae* | AB158483 |
| | | | *Cyanidioschyzon* | *merolae* | AB158484 |
| | | | *Cyanidioschyzon* | *merolae* | AB158485 |
| | | | *Cyanidium* | *caldarium* | AB090833 |
| | | | *Cyanidium* | *caldarium* | AB091231 |
| | | | *Cyanidium* | *caldarium* | AB091232 |
| | | Galdieriaceae | *Galdieria* | *partita* | AB090830 |
| | | | *Galdieria* | *daedala* | AF441362 |
| | | | *Galdieria* | *maxima* | AF441367 |
| | | | *Galdieria* | *sulphuraria* | AB090828 |
| | | | *Galdieria* | *sulphuraria* | AF441360 |
| | | | *Galdieria* | *sulphuraria* | AF441363 |
| | | | *Galdieria* | *sulphuraria* | AF441375 |
| | | | *Galdieria* | *sulphuraria* | AF342747 |
| Florideophyceae | Acrochaetiales | Acrochaetiaceae | *Rhodochorton* | *purpureum* | U23816 |
| | | | *Audouinella* | *hermannii* | AF026040 |
| | | | *Audouinella* | *dasyae* | L26181 |
| | | | *Audouinella* | *arcuata* | AF079786 |
| | | | *Audouinella* | *endophytica* | AF079789 |
| | | | *Audouinella* | *secundata* | AF079784 |
| | | | *Audouinella* | *tenue* | AF079796 |
| | | | *Audouinella* | *asparagopsis* | AF079795 |
| | | | *Audouinella* | *amphiroae* | AF079785 |
| | | | *Audouinella* | *caespitosa* | AF079787 |
| | | | *Audouinella* | *daviesii* | AF079788 |
| | | | *Audouinella* | *pectinata* | AF079790 |
| | | | *Audouinella* | *proskaueri* | AF079791 |
| | | | *Audouinella* | *rhizoidea* | AF079792 |
| | | | *Audouinella* | *tetraspora* | AF079793 |
| | | | *Audouinella* | *macrospora* | AF199505 |
| | | | *Audouinella* | *macrospora* | AF199506 |
| | Acrosymphytales | Acrosymphytaceae | *Acrosymphyton* | *purpureum* | AF317091 |
| | | | *Acrosymphyton* | *caribaeum* | DQ343661 |
| | | | *Schimmelmannia* | *schousboei* | AY437681 |
| | Ahnfeltiales | Ahnfeltiaceae | *Ahnfeltia* | *plicata* | Z14139 |
| | | | *Ahnfeltia* | *fastigiata* | DQ343668 |
| | Balbianiales | Balbianiaceae | *Rhododraparnaldia* | *oregonica* | AF026043 |
| | | | *Balbiania* | *investens* | AF132294 |
| | Balliales | Balliaceae | *Ballia* | *callitricha* | AF236790 |
| | | | *Ballia* | *callitricha* | AF236791 |
| | Batrachospermales | Batrachospermaceae | *Balliopsis* | *prieurii* | AF419245 |
| | | | *Nothocladus* | *nodosus* | U23815 |
| | | | *Sirodotia* | *huillensis* | AF026054 |

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|---|---|---|---|---|---|
| | | | *Sirodotia* | *suecica* | AF026053 |
| | | | *Tuomeya* | *americana* | AF026055 |
| | | | *Petrohua* | *bernabei* | EF033583 |
| | | | *Batrachospermum* | *boryanum* | AF026044 |
| | | | *Batrachospermum* | *helminthosum* | AF026046 |
| | | | *Batrachospermum* | *louisianae* | AF026047 |
| | | | *Batrachospermum* | *macrosporum* | AF026048 |
| | | | *Batrachospermum* | *turfosum* | AF026049 |
| | | | *Batrachospermum* | *virgatodecaisneanum* | AF026050 |
| | | | *Batrachospermum* | *gelatinosum* | AF026045 |
| | | Lemaneaceae | *Chantransia* | sp. | AF199507 |
| | | | *Lemanea* | *fluviatilis* | AY495972 |
| | | | *Lemanea* | *fluviatilis* | AY495973 |
| | | | *Lemanea* | *fluviatilis* | AY495974 |
| | | | *Lemanea* | *fluviatilis* | AY495975 |
| | | | *Lemanea* | *fluviatilis* | AY495976 |
| | | | *Lemanea* | *fluviatilis* | AF026051 |
| | | | *Lemanea* | *torulosa* | AY495971 |
| | | | *Paralemanea* | *annulata* | AY495979 |
| | | | *Paralemanea* | *catenata* | AY495977 |
| | | | *Paralemanea* | *catenata* | AY495978 |
| | | | *Paralemanea* | *catenata* | AY495980 |
| | | | *Paralemanea* | *catenata* | AF026052 |
| | | Psilosiphonaceae | *Psilosiphon* | *scoparium* | AF026041 |
| | Bonnemaisoniales | Bonnemaisoniaceae | *Bonnemaisonia* | *hamifera* | AJ880421 |
| | | | *Bonnemaisonia* | *hamifera* | L26182 |
| | | | *Ptilonia* | *australasica* | AY437646 |
| | | | *Delisea* | *pulchra* | AY437645 |
| | | | *Delisea* | *hypneoides* | EF033585 |
| | | | *Asparagopsis* | *armata* | AY772722 |
| | | | *Asparagopsis* | *taxiformis* | AY772723 |
| | | | *Asparagopsis* | *taxiformis* | AY772724 |
| | | | *Asparagopsis* | *taxiformis* | AY772725 |
| | | | *Asparagopsis* | *taxiformis* | AY772726 |
| | | | *Asparagopsis* | *taxiformis* | AY772727 |
| | | Naccariaceae | *Atractophora* | *hypnoides* | AY772728 |
| | | | *Naccaria* | *wiggii* | AY772729 |
| | | | *Reticulocaulis* | *mucosissimus* | DQ343656 |
| | Ceramiales | Callithamniaceae | *Aglaothamnion* | *halliae* | DQ022771 |
| | | | *Aglaothamnion* | *callophyllidicola* | AY643486 |
| | | | *Callithamnion* | *pikeanum* | AF488380 |
| | | | *Callithamnion* | *pikeanum* | DQ022770 |
| | | | *Callithamnion* | *collabens* | DQ022769 |
| | | | *Crouania* | *attenuata* | U32563 |
| | | | *Crouania* | *elisiae* | DQ022763 |
| | | | *Euptilota* | *formosissima* | DQ022778 |
| | | | *Euptilota* | *articulata* | DQ022777 |
| | | | *Euptilota* | *fergusonii* | DQ022779 |
| | | | *Euptilota* | *molle* | DQ022780 |
| | | | *Rhodocallis* | *elegans* | AF488383 |
| | | | *Seirospora* | *interrupta* | DQ022774 |
| | | | *Seirospora* | *viridis* | DQ022773 |
| | | | *Ptilocladia* | *pulchra* | DQ022762 |
| | | | *Falklandiella* | *harveyi* | DQ022764 |
| | | | *Georgiella* | *confluens* | DQ022765 |
| | | | *Diapse* | *ptilota* | DQ022766 |
| | | | *Aristoptilon* | *mooreanum* | DQ022767 |
| | | | *Heteroptilon* | *pappeanum* | DQ022768 |
| | | | *Carpothamnion* | *gunnianum* | DQ022772 |
| | | | *Sciurothamnion* | *stegengae* | DQ022775 |
| | | | *Sciurothamnion* | sp. | DQ022776 |

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|---|---|---|---|---|---|
| | | Ceramiaceae | *Antithamnionella* | *floccosa* | AF236788 |
| | | | *Antithamnionella* | sp. | AY168243 |
| | | | *Antithamnionella* | *spirographidis* | DQ022761 |
| | | | *Pterothamnion* | *villosum* | DQ343658 |
| | | | *Platythamnion* | *yezoense* | AY168242 |
| | | | *Antithamnion* | *sparsum* | AY168237 |
| | | | *Antithamnion* | *sparsum* | AY168238 |
| | | | *Antithamnion* | *sparsum* | AF236787 |
| | | | *Antithamnion* | *aglandum* | AY168234 |
| | | | *Antithamnion* | *aglandum* | AY643487 |
| | | | *Antithamnion* | *callocladum* | AY168236 |
| | | | *Antithamnion* | *callocladum* | AY643488 |
| | | | *Antithamnion* | *kylinii* | AY168240 |
| | | | *Antithamnion* | *nipponicum* | AY168235 |
| | | | *Antithamnion* | *nipponicum* | AY643489 |
| | | | *Antithamnion* | *defectum* | AY168239 |
| | | | *Antithamnion* | *densum* | AY168241 |
| | | | *Antithamnion* | *densum* | AY643485 |
| | | | *Centroceras* | *micracanthum* | DQ374385 |
| | | | *Centroceras* | *hyalacanthum* | DQ374387 |
| | | | *Centroceras* | *hyalacanthum* | DQ374388 |
| | | | *Centroceras* | *hyalacanthum* | DQ374389 |
| | | | *Centroceras* | *tetrachotomum* | DQ374379 |
| | | | *Centroceras* | *uncinatacanthum* | DQ374384 |
| | | | *Centroceras* | *clavulatum* | DQ374380 |
| | | | *Centroceras* | *clavulatum* | DQ374381 |
| | | | *Centroceras* | *clavulatum* | DQ374382 |
| | | | *Centroceras* | *clavulatum* | DQ374383 |
| | | | *Centroceras* | *clavulatum* | DQ022759 |
| | | | *Centroceras* | *clavulatum* | AY155521 |
| | | | *Centroceras* | *clavulatum* | DQ343657 |
| | | | *Centroceras* | *gasparrinii* | DQ374368 |
| | | | *Centroceras* | *gasparrinii* | DQ374369 |
| | | | *Centroceras* | *gasparrinii* | DQ374370 |
| | | | *Centroceras* | *gasparrinii* | DQ374371 |
| | | | *Centroceras* | *gasparrinii* | DQ374372 |
| | | | *Centroceras* | *gasparrinii* | DQ374373 |
| | | | *Centroceras* | *gasparrinii* | DQ374374 |
| | | | *Centroceras* | *gasparrinii* | DQ374375 |
| | | | *Centroceras* | *gasparrinii* | DQ374377 |
| | | | *Centroceras* | *gasparrinii* | DQ374378 |
| | | | *Centroceras* | *gasparrinii* | DQ374376 |
| | | | *Ceramium* | *diaphanum* | DQ022760 |
| | | | *Ceramium* | *rubrum* | AF236793 |
| | | | *Ceramium* | *rubrum* | L26183 |
| | | | *Ceramium* | *macilentum* | U32562 |
| | | | *Ceramium* | *paniculatum* | AF460864 |
| | | | *Ceramium* | *paniculatum* | AF460865 |
| | | | *Ceramium* | *affine* | AF460859 |
| | | | *Ceramium* | *horridum* | AF460858 |
| | | | *Ceramium* | *inkyuii* | AF460860 |
| | | | *Ceramium* | *inkyuii* | AF460861 |
| | | | *Ceramium* | *inkyuii* | AF460862 |
| | | | *Ceramium* | *inkyuii* | AF460863 |
| | | | *Ceramium* | *tenerrimum* | AF460866 |
| | | | *Ceramium* | *tenerrimum* | AF460867 |
| | | | *Ceramium* | *codicola* | AY155510 |
| | | | *Ceramium* | *codicola* | AY155511 |
| | | | *Ceramium* | *interruptum* | AY155512 |
| | | | *Ceramium* | *interruptum* | AY155513 |
| | | | *Ceramium* | *interruptum* | AY155514 |

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|---|---|---|---|---|---|
| | | | *Ceramium* | *interruptum* | AY155515 |
| | | | *Ceramium* | *interruptum* | AY155516 |
| | | | *Ceramium* | *sinicola* | AY155519 |
| | | | *Ceramium* | *sinicola* | AY155520 |
| | | | *Ceramium* | *sinicola* | AY155517 |
| | | | *Ceramium* | *sinicola* | AY155518 |
| | | Dasyaceae | *Dasya* | *baillouviana* | L26185 |
| | | | *Dasya* | *hutchinsiae* | AF488385 |
| | | | *Dasya* | *collabens* | AF488384 |
| | | | *Dasya* | *sinicola* | AF488386 |
| | | | *Dasya* | *villosa* | AF488387 |
| | | | *Eupogodon* | *planus* | AF488391 |
| | | | *Eupogodon* | *spinellus* | AF488392 |
| | | | *Heterosiphonia* | *plumosa* | AF488396 |
| | | | *Heterosiphonia* | *gunniana* | AF488393 |
| | | | *Heterosiphonia* | *japonica* | AF488394 |
| | | | *Heterosiphonia* | *muelleri* | AF488395 |
| | | | *Heterosiphonia* | *pulchra* | AF488397 |
| | | | *Dasysiphonia* | *chejuensis* | AF488388 |
| | | | *Dictyurus* | *occidentalis* | AF488389 |
| | | | *Dictyurus* | *purpurascens* | AF488390 |
| | | | *Rhodoptilum* | *plumosum* | AF488398 |
| | | | *Thuretia* | *australasica* | AF488399 |
| | | | *Thuretia* | *quercifolia* | AF488400 |
| | | | *Halodictyon* | *mirabile* | AF488407 |
| | | Delesseriaceae | *Caloglossa* | *leprieurii* | AF488401 |
| | | | *Caloglossa* | *cf ogasawaraensis* | AF251514 |
| | | | *Hypoglossum* | *hypoglossoides* | AF488405 |
| | | | *Phycodrys* | *rubens* | L26198 |
| | | | *Sorella* | *repens* | AF488406 |
| | | | *Cryptopleura* | *ramosa* | AF488402 |
| | | | *Cryptopleura* | *crispa* | AY617139 |
| | | | *Delesseria* | *serrulata* | AF488403 |
| | | | *Hemineura* | *frondosa* | AF488404 |
| | | | *Gonimophyllum* | *skottsbergii* | AY617137 |
| | | | *Melanamansia* | *mamillaris* | AF203889 |
| | | | *Melanamansia* | *glomerata* | AF251512 |
| | | Inkyuleeaceae | *Inkyuleea* | *ballioides* | AF236789 |
| | | | *Inkyuleea* | *mariana* | AF236792 |
| | | Rhodomelaceae | *Rhodomela* | *confervoides* | AY617145 |
| | | | *Rhodomela* | *confervoides* | L26203 |
| | | | *Neurymenia* | *fraxinifolia* | AF107042 |
| | | | *Neurymenia* | *fraxinifolia* | AF339899 |
| | | | *Laurencia* | *filiformis* | AF203894 |
| | | | *Bostrychia* | *moritziana* | AF203893 |
| | | | *Bostrychia* | *radicans* | AY617138 |
| | | | *Heterocladia* | *australis* | AF203890 |
| | | | *Heterocladia* | *caudata* | AF203891 |
| | | | *Heterocladia* | *umbellata* | AF203892 |
| | | | *Micropeuce* | *strobiliferum* | AF203896 |
| | | | *Murrayella* | *periclados* | AF203887 |
| | | | *Sonderella* | *linearis* | AF203888 |
| | | | *Pleurostichidium* | *falkenbergii* | AF251511 |
| | | | *Halopithys* | *incurva* | AF251513 |
| | | | *Osmundaria* | *prolifera* | AF339900 |
| | | | *Osmundaria* | *fimbriata* | AY237286 |
| | | | *Osmundaria* | *spiralis* | AY237287 |
| | | | *Osmundaria* | *colensoi* | AY237285 |
| | | | *Protokuetzingia* | *australasica* | AF339901 |
| | | | *Neorhodomela* | *larix* | AY617140 |
| | | | *Lembergia* | *allanii* | AF373215 |

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|---|---|---|---|---|---|
| | | | *Lophothalia* | *hormoclados* | AF373216 |
| | | | *Boergeseniella* | *fruticulosa* | AF427526 |
| | | | *Enelittosiphonia* | *stimpsonii* | AF427527 |
| | | | *Womersleyella* | *setacea* | AF427537 |
| | | | *Kuetzingia* | *canaliculata* | AY237283 |
| | | | *Rytiphlaea* | *tinctoria* | AY237284 |
| | | | *Aneurianna* | *dentata* | AY237280 |
| | | | *Odonthalia* | *floccosa* | AY617141 |
| | | | *Odonthalia* | *washintoniensis* | AY617142 |
| | | | *Lenormandia* | *prolifera* | AF203895 |
| | | | *Lenormandia* | *muelleri* | AF203897 |
| | | | *Lenormandia* | sp. | AF339897 |
| | | | *Lenormandia* | sp. | AF339898 |
| | | | *Lenormandia* | *angustifolia* | AF339892 |
| | | | *Lenormandia* | *latifolia* | AF339893 |
| | | | *Lenormandia* | *pardalis* | AF339894 |
| | | | *Lenormandia* | *smithiae* | AF339895 |
| | | | *Lenormandia* | *spectabilis* | AF339896 |
| | | | *Lenormandia* | *marginata* | AY237281 |
| | | | *Neosiphonia* | *savatieri* | AF203885 |
| | | | *Neosiphonia* | *japonica* | AF427528 |
| | | | *Neosiphonia* | *japonica* | AB219908 |
| | | | *Neosiphonia* | sp. | AB219867 |
| | | | *Neosiphonia* | sp. | AB219868 |
| | | | *Neosiphonia* | sp. | AB219869 |
| | | | *Neosiphonia* | sp. | AB219870 |
| | | | *Neosiphonia* | sp. | AB219871 |
| | | | *Neosiphonia* | sp. | AB219872 |
| | | | *Neosiphonia* | sp. | AB219877 |
| | | | *Neosiphonia* | sp. | AB219881 |
| | | | *Neosiphonia* | sp. | AB219882 |
| | | | *Neosiphonia* | sp. | AB219883 |
| | | | *Neosiphonia* | sp. | AB219884 |
| | | | *Neosiphonia* | sp. | AB219885 |
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| | | | *Neosiphonia* | sp. | AB219889 |
| | | | *Neosiphonia* | sp. | AB219890 |
| | | | *Neosiphonia* | sp. | AB219891 |
| | | | *Neosiphonia* | sp. | AB219892 |
| | | | *Neosiphonia* | sp. | AB219893 |
| | | | *Neosiphonia* | sp. | AB219894 |
| | | | *Neosiphonia* | sp. | AB219895 |
| | | | *Neosiphonia* | sp. | AB219897 |
| | | | *Neosiphonia* | sp. | AB219898 |
| | | | *Neosiphonia* | sp. | AB219902 |
| | | | *Neosiphonia* | sp. | AB219903 |
| | | | *Neosiphonia* | sp. | AB219904 |
| | | | *Neosiphonia* | sp. | AB219905 |
| | | | *Neosiphonia* | sp. | AB219909 |
| | | | *Neosiphonia* | sp. | AB219918 |
| | | | *Neosiphonia* | sp. | AB219920 |
| | | | *Neosiphonia* | sp. | AB219921 |
| | | | *Neosiphonia* | sp. | AB219922 |
| | | | *Neosiphonia* | sp. | AB219923 |
| | | | *Neosiphonia* | sp. | AB219925 |
| | | | *Neosiphonia* | sp. | AB219927 |
| | | | *Neosiphonia* | sp. | AB219928 |
| | | | *Neosiphonia* | sp. | AB219929 |
| | | | *Polysiphonia* | *lanosa* | AF203886 |
| | | | *Polysiphonia* | *harveyi* | AF427531 |
| | | | *Polysiphonia* | *lanosa* | AY617143 |

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|---|---|---|---|---|---|
| | | | *Polysiphonia* | *elongata* | AF427529 |
| | | | *Polysiphonia* | *fucoides* | AF427530 |
| | | | *Polysiphonia* | *morrowii* | AF427532 |
| | | | *Polysiphonia* | *nigra* | AF427534 |
| | | | *Polysiphonia* | *pacifica* | AF427533 |
| | | | *Polysiphonia* | *stricta* | AF427535 |
| | | | *Polysiphonia* | *virgata* | AF427536 |
| | | | *Polysiphonia* | *howei* | AY237282 |
| | | | *Polysiphonia* | *paniculata* | AY617144 |
| | | | *Polysiphonia* | sp. | AB219858 |
| | | | *Polysiphonia* | sp. | AB219859 |
| | | | *Polysiphonia* | sp. | AB219860 |
| | | | *Polysiphonia* | sp. | AB219873 |
| | | | *Polysiphonia* | sp. | AB219874 |
| | | | *Polysiphonia* | sp. | AB219875 |
| | | | *Polysiphonia* | sp. | AB219876 |
| | | | *Polysiphonia* | sp. | AB219878 |
| | | | *Polysiphonia* | sp. | AB219879 |
| | | | *Polysiphonia* | sp. | AB219880 |
| | | | *Polysiphonia* | sp. | AB219887 |
| | | | *Polysiphonia* | sp. | AB219888 |
| | | | *Polysiphonia* | sp. | AB219900 |
| | | | *Polysiphonia* | sp. | AB219906 |
| | | | *Polysiphonia* | sp. | AB219910 |
| | | | *Polysiphonia* | sp. | AB219913 |
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| | | | *Polysiphonia* | sp. | AB219915 |
| | | | *Polysiphonia* | sp. | AB219916 |
| | | | *Polysiphonia* | sp. | AB219919 |
| | | | *Polysiphonia* | sp. | AB219926 |
| | | | *Bostrychiocolax* | *australis* | AY617125 |
| | | | *Choreocolax* | *polysiphoniae* | AY617126 |
| | | | *Dawsoniocolax* | *bostrychiae* | AY617127 |
| | | | *Harveyella* | *mirabilis* | AY617128 |
| | | | *Harveyella* | *mirabilis* | AY617130 |
| | | | *Harveyella* | *mirabilis* | AY617131 |
| | | | *Harveyella* | *mirabilis* | AY617129 |
| | | | *Leachiella* | *pacifica* | AY617132 |
| | | | *Leachiella* | *pacifica* | AY617133 |
| | | Sarcomeniaceae | *Platysiphonia* | *victoriae* | AF488408 |
| | | Wrangeliaceae | *Griffithsia* | *globulifera* | L26192 |
| | | | *Griffithsia* | *monilis* | U32565 |
| | | | *Anotrichium* | *furcellatum* | U32561 |
| | | | *Ptilota* | *serrata* | DQ022756 |
| | | | *Ptilota* | *serrata* | AF203884 |
| | | | *Halurus* | *flosculosus* | AF488381 |
| | | | *Plumaria* | *plumosa* | AF488382 |
| | | | *Plumaria* | *plumosa* | DQ022758 |
| | | | *Neoptilota* | *densa* | DQ022757 |
| | Corallinales | Corallinaceae | *Metagoniolithon* | *chara* | U60743 |
| | | | *Metagoniolithon* | *radiatum* | U61250 |
| | | | *Metagoniolithon* | *stelliferum* | U61251 |
| | | | *Neogoniolithon* | *spectabile* | AY234238 |
| | | | *Metamastophora* | *flabellata* | AY234239 |
| | | | *Metamastophora* | *flabellata* | AY234240 |
| | | | *Amphiroa* | *fragilissima* | U60744 |
| | | | *Amphiroa* | sp. | U62116 |
| | | | *Amphiroa* | sp. | U62115 |
| | | | *Amphiroa* | *hancockii* | AY234233 |
| | | | *Amphiroa* | *tribulus* | AY234234 |
| | | | *Lithothrix* | *aspergillum* | U61249 |

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|---|---|---|---|---|---|
| | | | *Jania* | *crassa* | U62113 |
| | | | *Jania* | *rubens* | U61259 |
| | | | *Corallina* | *officinalis* | L26184 |
| | | | *Corallina* | *elongata* | U60946 |
| | | | *Cheilosporum* | *sagittatum* | U60745 |
| | | | *Bossiella* | *orbigniana* | U60746 |
| | | | *Bossiella* | *californica* | U60945 |
| | | | *Calliarthron* | *cheilosporioides* | U60943 |
| | | | *Calliarthron* | *tuberculosum* | U60944 |
| | | | *Haliptilon* | *roseum* | U60947 |
| | | | *Spongites* | *yendoi* | U60948 |
| | | | *Serraticardia* | *macmillanii* | U62114 |
| | | | *Arthrocardia* | *filicula* | U61258 |
| | | | *Hydrolithon* | *onkodes* | AY234237 |
| | | | *Hydrolithon* | *samoense* | AY234236 |
| | | | *Hydrolithon* | *pachydermum* | AY234235 |
| | | | *Hydrolithon* | *gardineri* | DQ628992 |
| | | | *Hydrolithon* | *gardineri* | DQ628991 |
| | | | *Hydrolithon* | *cf onkodes* | DQ628996 |
| | | | *Hydrolithon* | *cf onkodes* | DQ628997 |
| | | | *Lithophylloideae* | sp. | DQ628980 |
| | | | *Lithophyllum* | *kotschyanum* | U62117 |
| | | | *Lithophyllum* | *kotschyanum* | DQ628974 |
| | | | *Lithophyllum* | *cf kotschyanum* | DQ628976 |
| | | | *Lithophyllum* | *insipidum* | DQ628978 |
| | | | *Lithophyllum* | *insipidum* | DQ628979 |
| | | | *Lithophyllum* | *insipidum* | DQ628977 |
| | | | *Mastophoroideae* | sp. | DQ629004 |
| | | | *Mastophoroideae* | sp. | DQ629005 |
| | | | *Pneophyllum* | *conicum* | DQ628988 |
| | | | *Pneophyllum* | *conicum* | DQ628983 |
| | | | *Pneophyllum* | *conicum* | DQ628985 |
| | | | *Pneophyllum* | *conicum* | DQ628986 |
| | | | *Pneophyllum* | *conicum* | DQ628987 |
| | | | *Pneophyllum* | *cf conicum* | DQ628994 |
| | | | *Pneophyllum* | *cf conicum* | DQ628989 |
| | | | *Lithothamnion* | *glaciale* | U60738 |
| | | | *Lithothamnion* | *tophiforme* | U60739 |
| | | | *Lithothamnion* | sp. | DQ629010 |
| | | Hapalidiaceae | *Choreonema* | *thuretii* | AY221254 |
| | | | *Phymatolithon* | *laevigatum* | U60740 |
| | | | *Phymatolithon* | *lenormandii* | U60741 |
| | | | *Clathromorphum* | *compactum* | U60742 |
| | | | *Clathromorphum* | *parcum* | U61252 |
| | | | *Mastophoropsis* | *canaliculata* | U62118 |
| | | | *Leptophytum* | *acervatum* | U62119 |
| | | | *Leptophytum* | *ferox* | U62120 |
| | | | *Leptophytum* | *foveatum* | AF031470 |
| | | | *Synarthrophyton* | *patena* | U61255 |
| | | | *Melobesioideae* | sp. | DQ628972 |
| | | | *Mesophyllum* | *engelhartii* | U61256 |
| | | | *Mesophyllum* | *erubescens* | U61257 |
| | | | *Mesophyllum* | *erubescens* | DQ629011 |
| | | | *Mesophyllum* | *erubescens* | DQ629012 |
| | | Sporolithaceae | *Heydrichia* | *woelkerlingii* | U61253 |
| | | | *Heydrichia* | *homalopasta* | AF411629 |
| | | | *Sporolithon* | *durum* | AF411627 |
| | | | *Sporolithon* | *durum* | U61254 |
| | | | *Sporolithon* | *durum* | AF411626 |
| | | | *Sporolithon* | sp. | DQ629015 |
| | | Unknown | *Corallinales* | sp. | AY247408 |

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| | Gelidiales | Gelidiaceae | *Pterocladiella* | *bartlettii* | EF191192 |
| | | | *Pterocladiella* | *caerulescens* | EF191193 |
| | | | *Ptilophora* | *diversifolia* | EF191194 |
| | | | *Ptilophora* | *scalarimosa* | EF191195 |
| | | | *Pterocladiella* | *melanoidea* | U60341 |
| | | | *Suhria* | *vittata* | AF515300 |
| | | | *Capreolia* | *implexa* | U60344 |
| | | | *Ptilophora* | *pinnatifida* | U60345 |
| | | | *Ptilophora* | *subcostata* | U60348 |
| | | | *Acanthopeltis* | *japonica* | AB017664 |
| | | | *Acanthopeltis* | *japonica* | AB017665 |
| | | | *Yatabella* | *hirsuta* | AB017666 |
| | | | *Gelidium* | *amansii* | DQ316994 |
| | | | *Gelidium* | *caulacantheum* | U60343 |
| | | | *Gelidium* | *floridanum* | U60351 |
| | | | *Gelidium* | *latifolium* | U60350 |
| | | | *Gelidium* | *latifolium* | Y11953 |
| | | | *Gelidium* | *americanum* | U60347 |
| | | | *Gelidium* | *pusillum* | AB017663 |
| | | | *Gelidium* | *pusillum* | U60352 |
| | | | *Gelidium* | *pusillum* | U60355 |
| | | | *Gelidium* | *pusillum* | U32564 |
| | | | *Gelidium* | *serrulatum* | U60340 |
| | | | *Gelidium* | *sesquipedale* | U60354 |
| | | | *Gelidium* | *sesquipedale* | Y11959 |
| | | | *Gelidium* | *vagum* | AB017671 |
| | | | *Gelidium* | *vagum* | L26190 |
| | | | *Gelidium* | *divaricatum* | AB017662 |
| | | | *Gelidium* | *elegans* | AB017670 |
| | | | *Gelidium* | *australe* | DQ343660 |
| | | | *Pterocladiella* | *capillacea* | AB017672 |
| | | | *Pterocladiella* | *capillacea* | U60346 |
| | | | *Pterocladiella* | *capillacea* | Y11957 |
| | | | *Pterocladiella* | *capillacea* | Y11960 |
| | | | *Pterocladiella* | *caerulescens* | AB031301 |
| | | | *Pterocladiella* | *caloglossoides* | AB031302 |
| | | | *Pterocladiella* | *nana* | AB031303 |
| | | Gelidiellaceae | *Parviphycus* | *tenuissimus* | EF191191 |
| | | | *Gelidiella* | *pannosa* | AB031300 |
| | | | *Onikusa* | *pristoides* | U60353 |
| | | | *Onikusa* | *japonicum* | AB017667 |
| | | | *Onikusa* | sp. | AB017668 |
| | | | *Gelidiella* | *acerosa* | U60342 |
| | | | *Gelidiella* | *ligulata* | AB017669 |
| | | Pterocladiaceae | *Pterocladia* | *lucida* | U60349 |
| | | | *Pterocladia* | *lucida* | Y11958 |
| | | | *Aphanta* | *pachyrrhiza* | EF191190 |
| | Gigartinales | Acrotylaceae | *Acrotylus* | *australis* | AY437647 |
| | | | *Amphiplexia* | *hymenocladioides* | AY437648 |
| | | | *Antrocentrum* | *nigrescens* | AY437649 |
| | | | *Calviclonium* | *ovatum* | AY437650 |
| | | | *Hennedya* | *crispa* | AY437652 |
| | | Areschougiaceae | *Erythroclonium* | *angustatum* | AY437655 |
| | | | *Rhabdonia* | *verticillata* | AY437656 |
| | | | *Areschougia* | *congesta* | U09613 |
| | | | *Callophycus* | *oppositifolius* | AY437654 |
| | | Calosiphonaceae | *Schmitzia* | sp. | AY437660 |
| | | | *Schmitzia* | sp. | AY437659 |
| | | | *Caulacanthus* | *okamurae* | AY437663 |
| | | | *Catenella* | *caespitosa* | AY437661 |
| | | | *Catenella* | *nipae* | AY437662 |

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|---|---|---|---|---|---|
| | | Corynocystaceae | *Corynocystis* | *prostrata* | AY437651 |
| | | Cruoriaceae | *Cruoria* | *pellita* | AY437664 |
| | | Cubiculosporaceae | *Cubiculosporum* | *koronicarpis* | AY437665 |
| | | Cystocloniaceae | *Hypnea* | *charoides* | AY437682 |
| | | | *Hypnea* | *ramentacea* | AY437683 |
| | | | *Calliblepharis* | *jubata* | AY437669 |
| | | | *Calliblepharis* | *ciliata* | AY437668 |
| | | | *Calliblepharis* | *celatospora* | AY437667 |
| | | | *Calliblepharis* | *planicaulis* | AY437670 |
| | | | *Craspedocarpus* | *ramentaceus* | AY437671 |
| | | | *Rhodophyllis* | *volans* | AF515299 |
| | | | *Rhodophyllis* | *multipartita* | AY437676 |
| | | | *Stictosporum* | *nitophylloides* | AY437677 |
| | | | *Austroclonium* | *charoides* | AY437666 |
| | | | *Cystoclonium* | *purpureum* | AY437672 |
| | | | *Fimbrifolium* | *dichotomum* | AY437674 |
| | | | *Gloiophyllis* | *barkeriae* | AY437675 |
| | | | *Erythronaema* | *ceramioides* | AY437673 |
| | | Dicranemataceae | *Dicranema* | *revolutum* | AY437678 |
| | | Dumontiaceae | *Dilsea* | *californica* | AF317094 |
| | | | *Dilsea* | *californica* | U33126 |
| | | | *Dilsea* | *carnosa* | AF317096 |
| | | | *Dilsea* | *socialis* | AF317097 |
| | | | *Dasyphloea* | *insignis* | U09614 |
| | | | *Dumontia* | *contorta* | AF317099 |
| | | | *Dumontia* | *alaskana* | AF317101 |
| | | | *Dumontia* | *simplex* | AF317104 |
| | | | *Dudresnaya* | *capricornica* | AF317098 |
| | | | *Rhodopeltis* | *borealis* | AF317119 |
| | | | *Farlowia* | *mollis* | U33129 |
| | | | *Weeksia* | *coccinea* | AF317120 |
| | | | *Constantinea* | *subulifera* | AF317092 |
| | | | *Cryptosiphonia* | *woodii* | AF317093 |
| | | | *Gibsmithia* | *dotyi* | AF317108 |
| | | | *Hyalosiphonia* | *caespitosa* | AF317109 |
| | | | *Kraftia* | *dichotoma* | AF317110 |
| | | | *Neodilsea* | *borealis* | AF317112 |
| | | | *Neodilsea* | *natashae* | AF317113 |
| | | | *Neodilsea* | *yendoana* | AF317114 |
| | | | *Orculifilum* | *denticulatum* | AF317115 |
| | | | *Pikea* | *californica* | AF317116 |
| | | Endocladiaceae | *Gleiopeltis* | *furcata* | U33130 |
| | | | *Endocladia* | *muricata* | U33127 |
| | | Furcellariaceae | *Furcellaria* | *lumbricalis* | Z14141 |
| | | Gainiaceae | *Gainia* | *mollis* | AF317107 |
| | | Gigartinaceae | *Mazzaella* | *laminarioides* | AF515287 |
| | | | *Rhodoglossum* | *gigartinoides* | AY437679 |
| | | | *Sarcothalia* | *crassifolia* | U09615 |
| | | | *Chondrus* | *ocellatus* | DQ316985 |
| | | | *Chondrus* | *nipponicus* | DQ316987 |
| | | | *Chondrus* | *nipponicus* | DQ316986 |
| | | | *Chondrus* | *pinnulatus* | DQ316989 |
| | | | *Chondrus* | *yendoi* | DQ316988 |
| | | | *Chondrus* | *armatus* | DQ316990 |
| | | | *Chondrus* | sp. | DQ316991 |
| | | | *Chondrus* | sp. | DQ316992 |
| | | | *Chondrus* | *crispus* | DQ317001 |
| | | | *Chondrus* | *crispus* | DQ316997 |
| | | | *Chondrus* | *crispus* | DQ316996 |
| | | | *Chondrus* | *crispus* | DQ316998 |
| | | | *Chondrus* | *crispus* | DQ317000 |

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|---|---|---|---|---|---|
| | | | *Chondrus* | *crispus* | Z14140 |
| | | | *Chondrus* | *crispus* | DQ316993 |
| | | | *Chondrus* | *crispus* | DQ316999 |
| | | | *Chondrus* | *crispus* | DQ317002 |
| | | Gloiosiphoniaceae | *Gloiosiphonia* | *capillaris* | AY437680 |
| | | Kallymeniaceae | *Callophyllis* | *rangiferina* | U33123 |
| | | | *Kallymenia* | *tasmanica* | AF317111 |
| | | | *Erythrophyllum* | *delesserioides* | AF317105 |
| | | | *Euthora* | *cristata* | AY437684 |
| | | | *Pugetia* | *fragilissima* | EF033588 |
| | | Mychodeaceae | *Mychodea* | *carnosa* | U33135 |
| | | | *Mychodea* | *marginifera* | AY437685 |
| | | | *Mychodea* | *pusilla* | AY437686 |
| | | | *Mychodea* | *acanthymenia* | EF033589 |
| | | Mychodeophyllaceae | *Mychodeophyllum* | *papillitectum* | AY437687 |
| | | Nizymeniaceae | *Nizymenia* | *australis* | U09616 |
| | | Peyssonneliaceae | *Sonderopelta* | *coriacea* | AY437689 |
| | | | *Polystrata* | *fosliei* | AB231326 |
| | | | *Polystrata* | *dura* | AB231454 |
| | | | *Polystrata* | *dura* | AB231325 |
| | | | *Peyssonnelia* | sp. | AY437688 |
| | | | *Peyssonnelia* | *inamoena* | AB231312 |
| | | | *Peyssonnelia* | *distenta* | AB231313 |
| | | | *Peyssonnelia* | *harveyana* | AB231314 |
| | | | *Peyssonnelia* | *rumoiana* | AB231315 |
| | | | *Peyssonnelia* | *armorica* | AB231316 |
| | | | *Peyssonnelia* | *rosenvingii* | AB231317 |
| | | | *Peyssonnelia* | *meridionalis* | AB231318 |
| | | | *Peyssonnelia* | *japonica* | AB231319 |
| | | | *Peyssonnelia* | *squamaria* | AB231320 |
| | | | *Peyssonnelia* | *atropurpurea* | AB231321 |
| | | | *Peyssonnelia* | *dubyi* | AB231322 |
| | | | *Peyssonnelia* | *immersa* | AB231323 |
| | | | *Peyssonnelia* | *caulifera* | AB231324 |
| | | | *Peyssonnelia* | *capensis* | DQ465989 |
| | | | *Peyssonnelia* | *capensis* | DQ343662 |
| | | | *Peyssonnelia* | *novae hollandiae* | DQ343663 |
| | | | *Peyssonnelia* | *rubra* | DQ629018 |
| | | | *Peyssonnelia* | *rubra* | DQ629016 |
| | | | *Peyssonnelia* | sp. | DQ629017 |
| | | Phacelocarpaceae | *Phacelocarpus* | *peperocarpos* | U09617 |
| | | Phyllophoraceae | *Gymnogongrus* | sp. | AY437690 |
| | | | *Stenogramme* | *interrupta* | AY437691 |
| | | | *Schottera* | *nicaeensis* | U33137 |
| | | | *Mastocarpus* | *stellatus* | DQ317003 |
| | | | *Mastocarpus* | *stellatus* | L26195 |
| | | Polyidaceae | *Polyides* | *rotundus* | AF317117 |
| | | Rhizophyllidaceae | *Portieria* | *hornemanii* | AF317118 |
| | | Rissoellaceae | *Rissoella* | *verruculosa* | AY437693 |
| | | Sarcodiaceae | *Sarcodia* | *montagneana* | AY437695 |
| | | | *Sarcodia* | *marginata* | DQ343667 |
| | | | *Sarcodia* | sp. | AY437694 |
| | | | *Sarcodia* | *ciliata* | DQ343666 |
| | | | *Trematocarpus* | *fragilis* | AY437696 |
| | | Schmitziellaceae | *Schmitziella* | *endophloea* | AY437697 |
| | | Solieriaceae | *Eucheuma* | *denticulata* | U25439 |
| | | | *Wurdemannia* | *miniata* | AY437699 |
| | | | *Tikvahiella* | *candida* | AY437658 |
| | | | *Eucheuma* | *isiforme* | U25438 |
| | | | *Sarcodiotheca* | *gaudichaudii* | U33062 |
| | | | *Sarcodiotheca* | *gaudichaudii* | U43550 |

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|---|---|---|---|---|---|
| | | | *Sarcodiotheca* | *furcata* | U43553 |
| | | | *Solieria* | *robusta* | AY437657 |
| | | | *Kappaphycus* | *alvarezii* | U25437 |
| | | | *Gardneriella* | *tuberifera* | U43554 |
| | | | *Betaphycus* | *speciosum* | AY437653 |
| | | Sphaerococcaceae | *Sphaerococcus* | *coronopifolius* | U09622 |
| | | Tichocarpaceae | *Tichocarpus* | *crinitus* | AY437698 |
| | Gracilariales | Gracilariaceae | *Melanthalia* | *obtusata* | L26215 |
| | | | *Curdiea* | *flabellata* | L26207 |
| | | | *Curdiea* | *codiodes* | AY204130 |
| | | | *Curdiea* | *codiodes* | AY204131 |
| | | | *Curdiea* | sp. | AY204132 |
| | | | *Gracilariophila* | *oryzoides* | U43555 |
| | | | *Gracilariophila* | *oryzoides* | U43556 |
| | | | *Gracilariophila* | *oryzoides* | U43557 |
| | | | *Hydropuntia* | *pauciramosa* | AF468887 |
| | | | *Gracilaria* | sp. | AF468886 |
| | | | *Gracilaria* | *tenuistipitata* | DQ316995 |
| | | | *Gracilaria* | *lemaneiformis* | M54986 |
| | | | *Gracilaria* | *lemaneiformis* | X54263 |
| | | | *Gracilaria* | *lemaneiformis* | L26214 |
| | | | *Gracilaria* | *cornea* | AF468891 |
| | | | *Gracilaria* | *cornea* | AF468892 |
| | | | *Gracilaria* | *cornea* | L26212 |
| | | | *Gracilaria* | *pacifica* | L26206 |
| | | | *Gracilaria* | sp. | L26216 |
| | | | *Gracilaria* | *cuneata* | AF468905 |
| | | | *Gracilaria* | *caudata* | AF468888 |
| | | | *Gracilaria* | *caudata* | AF468889 |
| | | | *Gracilaria* | *caudata* | AF472415 |
| | | | *Gracilaria* | *caudata* | AY204133 |
| | | | *Gracilaria* | *curtissiae* | AF468901 |
| | | | *Gracilaria* | *crassissima* | AF468893 |
| | | | *Gracilaria* | *cervicornis* | AF468897 |
| | | | *Gracilaria* | *domingensis* | AF468902 |
| | | | *Gracilaria* | *domingensis* | AF468903 |
| | | | *Gracilaria* | *beckeri* | AY204141 |
| | | | *Gracilaria* | *capensis* | AY204153 |
| | | | *Gracilaria* | *salicornia* | AY204142 |
| | | | *Gracilaria* | *foliifera* *var angustissima* | AF468895 |
| | | | *Gracilaria* | *aff tepocensis* | AF468894 |
| | | | *Gracilaria* | *aff lacinulata* | AF468896 |
| | | | *Gracilaria* | *aff mammillaris* | AF468900 |
| | | | *Gracilaria* | *aff mammillaris* | AF468904 |
| | | | *Gracilaria* | sp. | AF468898 |
| | | | *Gracilaria* | sp. | AF468899 |
| | | | *Gracilaria* | sp. | AF468890 |
| | | | *Gracilaria* | *cliftonii* | AY617146 |
| | | | *Gracilaria* | *aculeata* | AY204143 |
| | | | *Gracilaria* | *canaliculata* | AY204154 |
| | | | *Gracilaria* | *corticata* | AY204134 |
| | | | *Gracilaria* | *corticata* | AY204135 |
| | | | *Gracilaria* | *corticata* | AY204136 |
| | | | *Gracilaria* | *corticata* *var cylindrica* | DQ409339 |
| | | | *Gracilaria* | *corticata* *var corticata* | DQ409340 |
| | | | *Gracilaria* | *denticulata* | AY204137 |
| | | | *Gracilaria* | *denticulata* | AY204138 |
| | | | *Gracilaria* | *denticulata* | AY204139 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Gracilaria* | *denticulata* | AY204140 |
| | | | *Gracilaria* | *millardetii* | AY204144 |
| | | | *Gracilaria* | *millardetii* | AY204145 |
| | | | *Gracilaria* | *vermiculophylla* | AY465828 |
| | | | *Gracilaria* | *tikvahiae* | M33640 |
| | | | *Gracilaria* | *chilensis* | L26217 |
| | | | *Gracilaria* | *gracilis* | AY204146 |
| | | | *Gracilaria* | *gracilis* | AY204147 |
| | | | *Gracilaria* | *gracilis* | AY204148 |
| | | | *Gracilaria* | *gracilis* | AY204149 |
| | | | *Gracilaria* | *gracilis* | AY204150 |
| | | | *Gracilaria* | *gracilis* | AY204151 |
| | | | *Gracilaria* | *gracilis* | AY204152 |
| | | | *Gracilaria* | *gracilis* | L26213 |
| | | | *Gracilaria* | *gracilis* | L26205 |
| | | | *Gracilaria* | *gracilis* | L26210 |
| | | | *Gracilaria* | *gracilis* | L26211 |
| | | | *Gracilaria* | *gracilis* | M33638 |
| | | | *Gracilaria* | *gracilis* | L26218 |
| | | | *Gracilaria* | *gracilis* | L26180 |
| | | | *Gracilaria* | *gracilis* | L26179 |
| | | | *Gracilaria* | *gracilis* | AY617147 |
| | | | *Gracilariopsis* | *lemaneiformis* | AY617149 |
| | | | *Gracilariopsis* | *tenuifrons* | AF468884 |
| | | | *Gracilariopsis* | *longissima* | AY204158 |
| | | | *Gracilariopsis* | *longissima* | AY204159 |
| | | | *Gracilariopsis* | *longissima* | AY204160 |
| | | | *Gracilariopsis* | *longissima* | AY204161 |
| | | | *Gracilariopsis* | *longissima* | AY617148 |
| | | | *Gracilariopsis* | sp. | AF468885 |
| | | | *Gracilariopsis* | *funiculus* | AY204155 |
| | | | *Gracilariopsis* | *funiculus* | AY204156 |
| | | | *Gracilariopsis* | *funiculus* | AY204157 |
| | | | *Gracilariopsis* | sp. | L26208 |
| | | | *Gracilariopsis* | sp. | M33639 |
| | | | *Gracilariopsis* | sp. | L26209 |
| | | | *Gracilariopsis* | sp. | L26256 |
| | | | *Gracilariopsis* | sp. | L26178 |
| | | Pterocladiophilaceae | *Holmsella* | *pachyderma* | AY617134 |
| | | | *Holmsella* | *pachyderma* | AY617135 |
| | | | *Holmsella* | *australis* | AY617136 |
| | Halymeniales | Halymeniaceae | *Corynomorpha* | *clavata* | AY437700 |
| | | | *Carpopeltis* | *phyllophora* | U33124 |
| | | | *Prionitis* | *lyallii* | EF033591 |
| | | | *Cryptonemia* | *undulata* | U33125 |
| | | | *Halymenia* | *plana* | U33133 |
| | | | *Polyopes* | *constrictus* | AY437705 |
| | | | *Polyopes* | *tenuis* | AY437706 |
| | | | *Zymurgia* | *chondriopsidea* | AF515304 |
| | | | *Epiphloea* | *bullosa* | AY437701 |
| | | | *Norrissia* | *setchellii* | AY437703 |
| | | | *Isabbottia* | *ovalifolia* | EF033590 |
| | | | *Grateloupia* | *filicina* | U33132 |
| | | | *Grateloupia* | *filicina* | L26191 |
| | | | *Grateloupia* | *intestinalis* | AY437702 |
| | | | *Pachymenia* | *carnosa* | AF515289 |
| | | | *Pachymenia* | *cf orbicularis* | AY437704 |
| | | Tsengiaceae | *Tsengia* | *laingii* | AF515302 |
| | | Tsengiaceae | *Tsengia* | *lanceolata* | AF515288 |
| | | Tsengiaceae | *Tsengia* | *comosa* | AF515303 |
| | Hildenbrandiales | Hildenbrandiaceae | *Apophlaea* | *lyallii* | AF076996 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Apophlaea* | *sinclairii* | AF534424 |
| | | | *Hildenbrandia* | *occidentalis* | AF534412 |
| | | | *Hildenbrandia* | *occidentalis* | AF108402 |
| | | | *Hildenbrandia* | *crouanii* | AF534416 |
| | | | *Hildenbrandia* | *crouanii* | AF534417 |
| | | | *Hildenbrandia* | *crouanii* | AF534419 |
| | | | *Hildenbrandia* | *lecannellieri* | AF534418 |
| | | | *Hildenbrandia* | *lecannellieri* | AF534420 |
| | | | *Hildenbrandia* | *dawsonii* | AF534413 |
| | | | *Hildenbrandia* | *patula* | AF534421 |
| | | | *Hildenbrandia* | *angolensis* | AF534415 |
| | | | *Hildenbrandia* | *angolensis* | AF207833 |
| | | | *Hildenbrandia* | *angolensis* | AF108416 |
| | | | *Hildenbrandia* | *angolensis* | AF108404 |
| | | | *Hildenbrandia* | *angolensis* | AF108418 |
| | | | *Hildenbrandia* | *angolensis* | AF108415 |
| | | | *Hildenbrandia* | *angolensis* | AF108417 |
| | | | *Hildenbrandia* | *rivularis* | AY028810 |
| | | | *Hildenbrandia* | *rivularis* | AY028811 |
| | | | *Hildenbrandia* | *rivularis* | AY028812 |
| | | | *Hildenbrandia* | *rivularis* | AY028813 |
| | | | *Hildenbrandia* | *rivularis* | AF208827 |
| | | | *Hildenbrandia* | *rivularis* | AF208822 |
| | | | *Hildenbrandia* | *rivularis* | AF208825 |
| | | | *Hildenbrandia* | *rivularis* | AF208823 |
| | | | *Hildenbrandia* | *rivularis* | AF208824 |
| | | | *Hildenbrandia* | *rivularis* | AF208830 |
| | | | *Hildenbrandia* | *rivularis* | AF208829 |
| | | | *Hildenbrandia* | *rivularis* | AF208816 |
| | | | *Hildenbrandia* | *rivularis* | AF208818 |
| | | | *Hildenbrandia* | *rivularis* | AF208817 |
| | | | *Hildenbrandia* | *rubra* | AF534422 |
| | | | *Hildenbrandia* | *rubra* | AJ880422 |
| | | | *Hildenbrandia* | *rubra* | AF108413 |
| | | | *Hildenbrandia* | *rubra* | AF108410 |
| | | | *Hildenbrandia* | *rubra* | AF208826 |
| | | | *Hildenbrandia* | *rubra* | AF208821 |
| | | | *Hildenbrandia* | *rubra* | AF108411 |
| | | | *Hildenbrandia* | *rubra* | AF208819 |
| | | | *Hildenbrandia* | *rubra* | AF208828 |
| | | | *Hildenbrandia* | *rubra* | AF208820 |
| | | | *Hildenbrandia* | *rubra* | AF208831 |
| | | | *Hildenbrandia* | *rubra* | AF108399 |
| | | | *Hildenbrandia* | *rubra* | AF108414 |
| | | | *Hildenbrandia* | *rubra* | AF108406 |
| | | | *Hildenbrandia* | *rubra* | AF108408 |
| | | | *Hildenbrandia* | *rubra* | AF108400 |
| | | | *Hildenbrandia* | *rubra* | UNP00680 |
| | | | *Hildenbrandia* | *rubra* | AF108401 |
| | | | *Hildenbrandia* | *rubra* | AF108409 |
| | | | *Hildenbrandia* | *rubra* | AF108412 |
| | | | *Hildenbrandia* | *rubra* | AF076995 |
| | | | *Hildenbrandia* | *rubra* | L19345 |
| | Nemaliales | Galaxauraceae | *Galaxaura* | *marginata* | AF006090 |
| | | Liagoraceae | *Cumagloia* | *andersonii* | DQ343669 |
| | | | *Nemalion* | *helminthoides* | L26196 |
| | Nemastomatales | Nemastomataceae | *Predaea* | *aurora* | AF515296 |
| | | | *Predaea* | *kraftiana* | AF515297 |
| | | | *Predaea* | *weldii* | AF515298 |
| | | | *Adelophycus* | *corneus* | AF515285 |
| | | | *Nemastoma* | *dichotomum* | DQ343664 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | Schizymeniaceae | *Schizymenia* | *dubyi* | U33136 |
| | | | *Schizymenia* | *pacifica* | EF033592 |
| | | | *Titanophora* | *weberae* | AF515301 |
| | | | *Platoma* | *cyclocolpa* | AF515292 |
| | | | *Platoma* | *cf cyclocolpa* | AF515295 |
| | | | *Platoma* | sp. | AF515294 |
| | | | *Platoma* | sp. | AF515293 |
| | | | *Wetherbeella* | *australica* | AF515290 |
| | | | *Wetherbeella* | *foliosa* | AF515291 |
| | Palmariales | Palmariaceae | *Halosaccion* | *glandiforme* | L26193 |
| | | | *Devaleraea* | *ramentacea* | L26186 |
| | | | *Palmaria* | *palmata* | AY029384 |
| | | | *Palmaria* | *palmata* | AY029385 |
| | | | *Palmaria* | *palmata* | AY029386 |
| | | | *Palmaria* | *palmata* | AY029387 |
| | | | *Palmaria* | *palmata* | AY029388 |
| | | | *Palmaria* | *palmata* | AY029389 |
| | | | *Palmaria* | *palmata* | AY029391 |
| | | | *Palmaria* | *palmata* | AY029392 |
| | | | *Palmaria* | *palmata* | Z14142 |
| | | | *Palmaria* | *palmata* *var sarniiensis* | AY029390 |
| | | | *Palmaria* | *palmata* *var sarniiensis* | AY029393 |
| | | Rhodophysemataceae | *Meiodiscus* | *spetsbergensis* | U23814 |
| | | | *Rhodophysema* | *elegans* | U23817 |
| | | | *Rhodothamniella* | *floridula* | U23818 |
| | | | *Camontagnea* | *oxyclada* | AF079794 |
| | Pihiellales | Pihiellaceae | *Pihiella* | *liagoraciphila* | AY301992 |
| | Plocamiales | Plocamiaceae | *Plocamiocolax* | *pulvinata* | U09618 |
| | | | *Plocamiocolax* | *pulvinata* | U43552 |
| | | | *Plocamium* | *mertensii* | AY437709 |
| | | | *Plocamium* | *pacificum* | DQ343665 |
| | | | *Plocamium* | *cartilagineum* | U09619 |
| | | | *Plocamium* | *cartilagineum* | AY437708 |
| | | | *Plocamium* | *cartilagineum* | U43551 |
| | | Pseudoanemoniaceae | *Hummbrella* | *hydra* | AY437692 |
| | Rhodogorgonales | Rhodogorgonaceae | *Rhodogorgon* | *carriebowensis* | AF006089 |
| | Rhodymeniales | Champiaceae | *Champia* | sp. | EF192579 |
| | | | *Gastroclonium* | *ovatum* | AF085265 |
| | | | *Champia* | *affinis* | U23951 |
| | | | *Chylocladia* | *verticillata* | AF085263 |
| | | | *Dictyothamnion* | *saltatum* | AF085264 |
| | | Faucheaceae | *Gloioderma* | *fruticulosa* | U33131 |
| | | | *Fauchea* | *laciniata* | AF085266 |
| | | | *Fauchea* | *repens* | DQ790750 |
| | | | *Fauchea* | *repens* | AF085267 |
| | | | *Faucheopsis* | *coronata* | AF085268 |
| | | | *Webervanbossea* | *splachnoides* | AF085269 |
| | | | *Gloiocladia* | *furcata* | DQ790749 |
| | | Fryeellaceae | *Fryeella* | *gardneri* | AF085273 |
| | | | *Hymenocladiopsis* | *crustigena* | AF085274 |
| | | Hymenocladiaceae | *Erythrymenia* | *minuta* | AF085272 |
| | | | *Hymenocladia* | *chondricola* | AF117128 |
| | | | *Asteromenia* | *peltata* | AY880245 |
| | | | *Asteromenia* | *peltata* | AF085253 |
| | | | *Asteromenia* | *peltata* | AY437710 |
| | | | *Asteromenia* | *bermudensis* | DQ343651 |
| | | | *Asteromenia* | *anastomosans* | DQ343652 |
| | | Lomentariaceae | *Lomentaria* | *baileyana* | L26194 |
| | | | *Lomentaria* | *australis* | U33134 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Ceratodictyon* | *spongiosum* | AF117127 |
| | | | *Gelidiopsis* | *variabilis* | AF085270 |
| | | | *Gelidiopsis* | *intricata* | EF033594 |
| | | | *Semnocarpa* | *minuta* | AF085271 |
| | | Rhodymeniaceae | *Rhodymenia* | *stenoglossa* | AF085262 |
| | | | *Rhodymenia* | *leptophylla* | U09621 |
| | | | *Botryocladia* | *ebriosa* | AF085255 |
| | | | *Botryocladia* | *sonderi* | AF085256 |
| | | | *Botryocladia* | *leptopoda* | DQ343160 |
| | | | *Cordylecladia* | *erecta* | U23952 |
| | | | *Halichrysis* | *micans* | DQ343655 |
| | | | *Halichrysis* | *concrescens* | DQ343654 |
| | | | *Cephalocystis* | *furcellata* | U23949 |
| | | | *Cephalocystis* | *leucobotrys* | U23950 |
| | | | *Erythrocolon* | *podagricum* | U23953 |
| | | | *Epymenia* | *wilsonis* | U33128 |
| | | | *Chrysymenia* | *ornata* | AF085257 |
| | | | *Chrysymenia* | *wrightii* | AF117129 |
| | | | *Coelarthrum* | *opuntia* | AF085258 |
| | | | *Gloiosaccion* | *brownii* | AF085259 |
| | | | *Irvinea* | *ardreana* | AF085254 |
| | | | *Leptosomia* | *rosea* | AF085260 |
| | | | *Sparlingia* | *pertusa* | AF085261 |
| | | | *Maripelta* | *rotata* | DQ343159 |
| | | | *Drouetia* | *coalescens* | DQ343653 |
| | Sebdeniales | Sebdeniaceae | *Sebdenia* | *flabellata* | U33138 |
| | | | *Lesleigha* | sp. | AF515286 |
| | | | *Lesleigha* | sp. | AY437707 |
| | | | *Crassitegula* | *walsinghamia* | AY964057 |
| | Thoreales | Thoreaceae | *Thorea* | *violacea* | AF506274 |
| | | | *Thorea* | *violacea* | AF506275 |
| | | | *Thorea* | *violacea* | AF026042 |
| | | | *Thorea* | *violacea* | AF342744 |
| | | | *Thorea* | sp. | AF420253 |
| | | | *Thorea* | *hispida* | AF506273 |
| | | | *Nemalionopsis* | *tortuosa* | AF342743 |
| | | | *Nemalionopsis* | *shawii* | AF506272 |
| Porphyridiophyceae | Compsopogonales | Compsopogonaceae | *Compsopogonopsis* | *leptoclados* | AF087123 |
| | | | *Compsopogon* | *coeruleus* | AY617150 |
| | | | *Compsopogon* | *coeruleus* | AF087124 |
| | | | *Compsopogon* | *coeruleus* | AF087127 |
| | | | *Compsopogon* | *coeruleus* | AF087128 |
| | | | *Compsopogon* | *coeruleus* | AF342748 |
| | | | *Compsopogon* | *hookeri* | AJ880416 |
| | Erytropeltidales | Erythrotrichiaceae | *Erythropeltidales* | CSW318 | AY126430 |
| | | | *Erythropeltidales* | MEK258 | AY126433 |
| | | | *Erythropeltidales* | LHF907 | AY126432 |
| | | | *Smithora* | *naiadum* | AF087129 |
| | | | *Smithora* | *naiadum* | AF087126 |
| | | | *Pyrophyllon* | *cameronii* | AY126429 |
| | | | *Pyrophyllon* | *subtumens* | AY126434 |
| | | | *Chlidophyllon* | *kaspar* | AY126431 |
| | | | *Erythrocladia* | sp. | L26188 |
| | | | *Erythrocladia* | sp. | AY617154 |
| | | | *Erythrotrichia* | *carnea* | AY617155 |
| | | | *Erythrotrichia* | *carnea* | AJ880417 |
| | | | *Erythrotrichia* | *carnea* | L26189 |
| | Porphyridiales | Porphyridiaceae | *Flintiella* | *sanguinaria* | AF168621 |
| | | | *Flintiella* | *sanguinaria* | AF342749 |
| | | | *Porphyridium* | *aerugineum* | AF168623 |
| | | | *Porphyridium* | *aerugineum* | AJ421145 |

| Class | Order | Family | Genus | Species | Genbank Accession # |
|---|---|---|---|---|---|
| | | | *Porphyridum* | *aerugineum* | L27635 |
| | | | *Porphyridium* | *sordidum* | AF168630 |
| | | | *Porphyridium* | *purpureum* | AJ880418 |
| | | | *Porphyridium* | *purpureum* | AB045584 |
| | | | *Porphyridium* | sp. | AB183597 |
| Rhodellophyceae | Rhodellales | Rhodellaceae | *Rhodella* | *violacea* | AB045604 |
| | | | *Rhodella* | *violacea* | AB045580 |
| | | | *Rhodella* | *violacea* | AF168624 |
| | | | *Rhodella* | *maculata* | AJ880419 |
| | | | *Rhodella* | *maculata* | AB045608 |
| | | | *Rhodella* | *maculata* | U21217 |
| | | | *Rhodella* | sp. | AB045591 |
| | | | *Rhodella* | sp. | AB045594 |
| | | | *Rhodella* | sp. | AB045598 |
| | | | *Rhodella* | *cyanea* | AB045605 |
| | | | *Rhodella* | sp. | AB183619 |
| | | | *Rhodella* | sp. | AB183652 |
| | | | *Rhodella* | sp. | AB183648 |
| | Rhodellales/ | Rhodellaceae/ | *Dixoniella* | *grisea* | AB045596 |
| | Dixoniellales | Glaucosphaeraceae | *Dixoniella* | *grisea* | AB045581 |
| | | | *Dixoniella* | *grisea* | L26187 |
| Stylonematophyceae | Stylonematales | Stylonemataceae | *Bangiopsis* | *subsimplex* | AF168627 |
| | | | *Bangiopsis* | sp. | AY766363 |
| | | | *Goniotrichiopsis* | *sublittoralis* | AF168629 |
| | | | *Chroodactylon* | *ornatum* | AF168628 |
| | | | *Chroodactylon* | *ramosum* | AF342751 |
| | | | *Stylonema* | *alsidii* | AF168632 |
| | | | *Stylonema* | *alsidii* | AF168633 |
| | | | *Stylonema* | *alsidii* | AY617153 |
| | | | *Stylonema* | *alsidii* | L26204 |
| | | | *Stylonema* | *cornu cervi* | AF168622 |
| | | | *Rhodosorus* | *marinus* | AF168625 |
| | | | *Rhodosorus* | *marinus* | AF342750 |
| | | | *Rhodosorus* | sp. | AF168626 |
| | | | *Purpureofilum* | *apyrenoidigerum* | AY617151 |
| Chlorophyceae | Chlorellales | Chlorellaceae | *Chlorella* | *vulgaris* | AB162910 |
| | Sphaeropleales | Ankistrodesmaceae | *Ankistrodesmus* | *bibraianus* | Y16938 |
| | | | *Monoraphidium* | *dybowskii* | Y16939 |
| Trebouxiophyceae | Chlorellales | Chlorellaceae | *Parachlorella* | *kessleri* | AB162911 |
| Ulvophyceae | Trentepohliales | Trentepohliaceae | *Trentepohlia* | *iolithus* | AY220983 |

# Bibliography

Adl, S.M., A.G.B. Simpson, M.A. Farmer, R.A. Andersen, O.R. Anderson, J.R. Barta, S.S. Browser, G. Brugerolle, R.A. Fensome, S. Fredericq, T.Y. James, S. Karpov, P. Kugrens, J. Krug, C.E. Lane, L.A. Lewis, J. Lodge, D.H. Lynn, D.G. Mann, R.M. McCourt, L. Mendoza, O. Moestrup, S.E. Mozley-Standridge, T.A. Nerad, C.A. Shearer, A.V. Smirnov, F.W. Spiegel, and M.F.J.R. Taylor. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology*. **52**: 399-451.

Ali, A.B., J. Wuyts, R. De Wachter, A. Meyer, and Y. Van de Peer. 1999. Construction of a variability map for eukaryotic large subunit ribosomal RNA. *Nucleic Acids Research*. **27**: 2825.

Allen, G.C., M.A. Flores-Vergara, S. Krasynanski, S. Kumar, and W.F. Thompson. 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols.* **1**: 2320-2325.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J.H. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. **25**: 3389-3402.

Asamizu, E., M. Nakajima, Y. Kitade, N. Saga, Y. Nakamura, and S. Tabata. 2003. Comparison of RNA expression profiles between the two generations of *Porphyra yezoensis* (Rhodophyta), based on expressed sequence tag frequency analysis. *Journal of Phycology*. **39**: 923-930.

Baldauf, S.L., A.J. Roger, I. Wenk-Siefert, and W.F. Doolittle. 2000. A Kingdom-Level Phylogeny of Eukaryotes Based on Combined Protein Data. *Science*. **290**: 972-977.

Ban, N., P. Nissen, J. Hansen, P.B. Moore, and T.A. Steitz. 2000. The Complete Atomic Structure of the Large Ribosomal Subunit at 2.4 A Resolution. *Science*. **289**: 905-920.

Batters, E.A.L. 1892. On *Schmitziella*; a new genus of endophytic algae, belonging to the order Corallinaceae. *Annals of Botany*. **6**: 185-194.

Bhattacharya, D., and L. Medlin. 1995. The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions. *Journal of Phycology*. **31**: 489–498.

Bornet, B., E. Antoine, M. Bardouil, and C.M. Baut. 2004. ISSR as new markers for genetic characterization and evaluation of relationships among phytoplankton. *Journal of Applied Phycology*. **16**: 285-290.

Bornet, B., and M. Branchard. 2001. Nonanchored inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter*. **19**: 209-215.

Broadwater, S.T., Scott, J.L., Goss, S.P.A. and Saunders, B.D. 1995. Ultrastructure of vegetative organization and cell division in *Glaucoshphaeara vacuolata* Korshikov (Porphyridiales, Rhodophyta). *Phycologia*. **34**: 352-361.

Brodie, J., P.K. Hayes, G.L. Barker, L.M. Irvine, and I. Bartsch. 1998. A reappraisal of Porphyra and Bangia (Bangiophycidae, Rhodophyta) in the northeast Atlantic based on the rbcL-rbcS intergenic spacer. *Journal of Phycology*. **34**: 1069-1074.

Broom, J.E., W.A. Jones, D.F. Hill, G.A. Knight, and W.A. Nelson. 1999. Species recognition in New Zealand Porphyra using 18S rDNA sequencing. *Journal of Applied Phycology*. **11**: 421-428.

Broom, J.E.S., T.J. Farr, and W.A. Nelson. 2004. Phylogeny of the *Bangia* flora of New Zealand suggests a southern origin for *Porphyra* and *Bangia* (Bangiales, Rhodophyta). *Molecular Phylogenetics and Evolution*. **31**: 1197-1207.

Broom, J.E., S.C. Lindstrom, W.A. Nelson, J. Brodie, M.D.J. Lynch, M.S. Hwang, H.G. Choi, M. Miyata, N. Kikuchi, M.C. Oliveira, T. Farr, C. Neefus, A. Mols-Mortensen, D. Milstein, K. Mü ller. 2011. A new look at an ancient order: generic revision of the Bangiales. *Journal of Phycology*. In press.

Buckley, T.R. 2002. Model misspecification and probabilistic tests of topology: evidence from empirical data sets. *Systematic Biology*. **51**: 509.

Butterfield, N.J. 2000. *Bangiomorpha pubescens* n. gen., n. sp.: implications for the evolution of sex, multicellularity, and the Mesoproterozoic/Neoproterozoic radiation of eukaryotes. *Paleobiology*. **26**: 386-404.

Butterfield, N.J. 2001. Paleobiology of the late Mesoproterozoic (ca. 1200 Ma) Hunting Formation, Somerset Island, arctic Canada. *Precambrian Research*. **111**: 235-256.

Butterfield, N.J., A.H. Knoll, and K. Swett. 1990. A Bangiophyte red alga from the Proterozoic of Arctic Canada. *Science*. **250**: 104-107.

Caetano-Anollés, G. 2001. Novel strategies to study the role of mutation and nucleic acid structure in evolution. *Plant Cell, Tissue and Organ Culture*. **67**: 115-132.

Caetano-Anollés, G. 2002a. Tracing the evolution of RNA structure in ribosomes. *Nucleic Acids Research*. **30**: 2575.

Caetano-Anollés, G. 2002b. Evolved RNA Secondary Structure and the Rooting of the Universal Tree of Life. *Journal of Molecular Evolution*. **54**: 333-345.

Cannone, J.J., S. Subramanian, M.N. Schnare, J.R. Collett, L.M.D. Souza, Y. Du, B. Feng, N. Lin, L.V. Madabusi, N. Pande, Z. Shang, N. Yu, and R.R. Gutell. 2002. The Comparative RNA Web (CRW) Site: An online database of comparative sequence and structure information for ribsomal intron and other RNAs. *Bioinformatics*. **3**: 1-31.

Cavalier-Smith, T. 1998. A revised six-kingdom system of life. *Biological Reviews*. **73**: 203-266.

Chapman, D.J. 1974. Taxonomic status of *Cyanidium caldarium* the Porphyridiales and Goniotrichales. *Nova Hedwigia*. **25**: 673-682.

Choi, Han-Gu, G.T. Kraft, Hyung-Seop Kim, M.D. Guiry and G.W. Saunders. 2008. Phylogenetic relationships among lineages of the Ceramiaceae (Ceramiales, Rhodophyta) based on nuclear small subunit rDNA sequence data. *Journal of Phycology* **44**: 1033-1048

Chu, K.H., J. Qi, Z.G. Yu, and V. Anh. 2004. Origin and Phylogeny of Chloroplasts Revealed by a Simple Correlation Analysis of Complete Genomes. *Molecular Biology and Evolution*. **21**: 200-206.

Cole, J.R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, and J.M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*. **37**: D141-D145.

Den Hartog, C. 1972. The effect of the salinity tolerance of algae on their distribution, as exemplified by Bangia. p. 274. *In* Proceedings of the Seventh International Seaweed Symposium, Sapporo, Japan, August 8-12, 1971.

Dornburg, A., F. Santini, and M.E. Alfaro. 2008. The Influence of Model Averaging on Clade Posteriors: An Example Using the Triggerfishes (Family Balistidae). *Systematic Biology*. **57**: 905-919.

Dunn, C.W., A. Hejnol, D.Q. Matus, K. Pang, W.E. Browne, S.A. Smith, E. Seaver, G.W. Rouse, M. Obst, G.D. Edgecombe, M.V. Sorensen, S.H.D. Haddock, A. Schmidt-Rhaesa, A. Okusu, R.M. Kristensen, W.C. Wheeler, M.Q. Martindale, and G. Giribet. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*. **452**: 745-749.

Ebach, M.C., and C. Holdrege. 2005. DNA barcoding is no substitute for taxonomy. *Nature*. **434**: 697–697.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*. **32**: 1792-1797.

Ellegren, H. 2004. Microsatellites: Simple sequences with complex evolution. *Nature Reviews Genetics*. **5**: 435-445.

Engel, C.R., R. Wattier, C. Destombe, and M. Valero. 1999. Performance of non-motile male gametes in the sea: analysis of paternity and fertilization success in a natural population of a red seaweed, *Gracilaria gracilis*. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **266**: 1879-1886.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software

package for population genetics data analysis. *Evolutionary Bioinformatics Online*. **1**: 47-50.

Fox, G.E., and C.R. Woese. 1975. 5S RNA secondary structure. *Nature*. **256**: 505-507.

Fredericq, R., and Ramirez, M.E. 1996. Systematic studies of the Antarctic species of the Phyllophoraceae (Gigartinales, Rhodophyta) based on *rbc*L sequence analysis. *Hydrobiologia*. **326–327**: 137–143.

Freshwater, D.W., S. Fredericq, B.S. Butler, M.H. Hommersand, and M.W. Chase. 1994. A Gene Phylogeny of the Red Algae (Rhodophyta) Based on Plastid Rbcl. *Proceedings of the National Academy of Sciences of the United States of America*. **91**: 7281-7285.

Gabrielson, P.W., D.J. Garbary, and R.F. Scagel. 1985. The nature of the ancestral red alga: inferences from a cladistic analysis. *Biosystems*. **18**: 335-346.

Gabrielson, P.W., D.J. Garbary, M.R. Sommerfeld, R.A. Townsend, and P.L. Tyler. 1990. Phylum Rhodophyta. p. 102-118. *In* Margulis, L., Corliss, J.O., Melkonian, M. (eds.), Handbook of Protoctista. Jones and Bartlett Publishers, Boston, Mass.

Galtier, N., M. Gouy, and C. Gautier. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Bioinformatics*. **12**: 543.

Garbary, D.J., and P.W. Gabrielson. 1990. Taxonomy and evolution. p. 477-498. *In* Cole, K.M., Sheath, R.G. (eds.), Biology of the red algae. Cambridge University Press, Cambridge, U.K.

Garbary, D.J., G.I. Hansen, and R.F. Scagel. 1980. A revised classification of the Bangiophyceae (Rhodophyta). *Nova Hedwigia*. **33**: 145-166.

Gardner, N.L. 1927. New Rhodophyceae from the Pacific coast of North America. II. *University of California Publications in Botany*. **13**: 235-249.

Gascuel, O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution*. **14**: 685.

Geesink, R. 1973. Experimental investigations on marine and freshwater *Bangia* (Rhodophyta) from the Netherlands. *Journal of Experimental Marine Biology and Ecology*. **11**: 239–247.

Gouy, M., S. Guindon, and O. Gascuel. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*. **27**: 221-224.

Graham, L.E., and L.W. Wilcox. 2000. Algae. Prentice-Hall, Inc., Upper Saddle River, NJ.

Guindon, S., and O. Gascuel. 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology*. **52**: 696-704.

Guiry, M.D., and E.N. Dhonncha. 2002. AlgaeBase. World-wide Web electronic publication www.algaebase.org [6 February 2003].

Gupta, M., Y.S. Chyi, J. Romeroseverson, and J.L. Owen. 1994. Amplification of Dna Markers from Evolutionarily Diverse Genomes using Single Primers of Simple-Sequence Repeats. *Theoretical and Applied Genetics*. **89**: 998-1006.

Gutell, R.R., B. Weiser, C.R. Woese, and H.F. Noller. 1985. Comparative anatomy of 16-S-like ribosomal RNA. *Progress in Nucleic Acids Research and Molecular Biology*. **32**:

155-216.

Hall, M.M., and M.L. Vis. 2000. Phylogeography and within-stream genetic variation of *Batrachospermum helmentosum* (Rhodophyta) using inter-simple sequence repeats. *Journal of Phycology*. **36(suppl.)**: 27-28.

Hall, M.M., and M.L. Vis. 2002. Genetic variation in *Batrachospermum helminthosum* (Batrachospermales, Rhodophyta) among and within stream reaches using intersimple sequence repeat molecular markers. *Phycological Research*. **50**: 155-162.

Harper, J.T., and G.W. Saunders. 2001a. The application of sequences of the ribosomal cistron to the systematics and classification of the florideophyte red algae (Florideophyceae, Rhodophyta). *Cahiers de Biologie Marine*. **42**: 25-38.

Harper, J.T., and G.W. Saunders. 2001b. Molecular systematics of the Florideophyceae (Rhodophyta) using nuclear large and small subunit rDNA sequence data. *Journal of Phycology*. **37**: 1073-1082.

Harvey, W. 1853. Nereis Boreali-Americana. II. Rhodospermae. *Smithsonian Contributions Knowledge*. **5**: 1-128.

Hayden, H.S., J. Blomster, C.A. Maggs, P.C. Silva, M.J. Stanhope, and J.R. Waaland. 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology*. **38**: 277-294.

Hebert, P.D., A. Cywinska, S.L. Ball, and J.R. Dewaard. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, Series B: Biological Sciences*. **270**: 313–321.

Higgs, P.G. 2000. RNA secondary structure: physical and computational aspects. *Quarterly Reviews of Biophysics*. **33**: 199-253.

Hoek, C.V.D., D.G. Mann, and H.M. Jahns. 1995. Algae, an introduction to phycology. Cambridge University Press, Cambridge, U.K.

Hong, Y., and A. Chuah. 2003. A format for databasing and comparison of AFLP fingerprint profiles. *BMC Bioinformatics*. **4**: 7.

Houliston G.J. and H.M. Chapman. 2004. Reproductive strategy and population variability in the facultative apomict *Hieracium pilosella* (Asteraceae). *American Journal of Botany*. **91**: 37 – 44.

Huelsenbeck, J.P., and B. Rannala. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Systematic Biology*. **53**: 904-913.

Huelsenbeck, J.P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*. **17**: 754-755.

Johansen, H.W. 1969. Morphology and systematics of coralline algae with species reference to *Calliarthron*. *University of California Publications in Botany*. **49**: vi q 1–98.

Jukes, T.H., and C.R. Cantor. 1969. Evolution of protein molecules. *Mammalian Protein Metabolism*. **3**: 21–132.

Keeling, P.J. 2004. Diversity and evolutionary history of plastids and their hosts. *American Journal of Botany*. **91**: 1481-1493.

Klein, A.S., A.C. Mathieson, C.D. Neefus, D.F. Cain, H.A. Taylor, B.W. Teasdale, A.L. West, E.J. Hehre, J. Brodie, C. Yarish, and A.L. Wallace. 2003. Identification of north-western Atlantic *Porphyra* (Bangiaceae, Bangiales) based on sequence variation in nuclear SSU and plastid *rbc*L genes. *Phycologia*. **42**: 109-122.

Krayesky, D.M., J.N. Norris, P.W. Gabrielson, D. Gabriel, and S. Fredericq. 2009. A new order of red algae based on the Peyssonneliaceae, with an evaluation of the ordinal classification of the Florideophyceae (Rhodophyta). *Proceedings of the Biological Society of Washington*. **122**: 364–391.

Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt, and D.H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America*. **102**: 8369.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*. **5**: 150.

Kylin, H. 1956. Die Gattungen der Rhodophyceen. pp. i-xv, 1-673, 458 figs. Lund: C.W.K. Gleerups.

Lagercrantz, U., H. Ellegren and L. Andersson. 1993. The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research*. **21**: 1111–1115.

Le Gall L, J.L. Dalen and G.W. Saunders. Phylogenetic analyses of the red algal order Rhodymeniales supports recognition of the Hymenocladiaceae fam. nov., Fryeellaceae fam. nov., and *Neogastroclonium* gen. nov. *Journal of Phycology*. **44**: 1556-1571.

Lewis, L.A., and R.M. McCourt. 2004. Green algae and the origin of land plants. *American Journal of Botany*. **91**: 1535-1556.

Lim, B.L., H. Kawai, H. Hori, and S. Osawa. 1986. Molecular Evolution of 5s Ribosomal-Rna from Red and Brown-Algae. *Japanese Journal of Genetics*. **61**: 169-176.

Lindstrom, S.C., and K.M. Cole. 1992. The *Porphyra lanceolata-P. pseudolanceolata* (Bangiales, Rhodophyta) Complex Unmasked - Recognition of New Species Based on Isozymes, Morphology, Chromosomes and Distributions. *Phycologia*. **31**: 431-448.

Lindstrom, S.C., and S. Fredericq. 2003. *rbc*L gene sequences reveal relationships among north-east Pacific species of *Porphyra* (Bangiales, Rhodophyta) and a new species, *P. aestivalis. Phycological Research*. **51**: 211-224.

Lindstrom, S. 2008a. Cryptic diversity, biogeography and genetic variation in Northeast Pacific species of *Porphyra sensu lato* (Bangiales, Rhodophyta). *Journal of Applied Phycology*. **20**: 951-962.

Lindstrom, S. 2008b. Cryptic diversity and phylogenetic relationships within the *Mastocarpus papillatus* species complex (Rhodophyta, Phyllophoraceae). *Journal of Phycology*. **44**: 1300-1308.

Lynch, M.D.J., R.G. Sheath, and K.M. Müller. 2008. Phylogenetic position and ISSR-estimated intraspecific genetic variation of *Bangia maxima* (Bangiales, Rhodophyta). *Phycologia*. **47**: 599-613.

Maddison, W., and D. Maddison. Mesquite: a modular system for evolutionary analysis.

Magne, F. 1989. Classification et phylogénie des rhodophycées= Classification and phylogeny of Rhodophyta. *Cryptogamie. Algologie*. **10**: 101–115.

McFadden, G.I., and G.G.V. Dooren. 2004. Evolution: Red Algal Genome Affirms a Common Origin of All Plastids. *Current Biology*. **14**: R514-R516.

Merrill, J.E. 1993. Development of nori markets in the western world. *Journal of Applied Phycology*. **5**: 149–154.

Müller, K.M., J.J. Cannone, R.R. Gutell, and R.G. Sheath. 2001. A structural and phylogenetic analysis of the group IC1 introns in the order Bangiales (Rhodophyta). *Molecular Biology and Evolution*. **18**: 1654-1667.

Müller, K.M., J.J. Cannone, and R.G. Sheath. 2005. A molecular phylogenetic analysis of the Bangiales (Rhodophyta) and description of a new genus and species, *Pseudobangia kaycoleia*. *Phycologia*. **44**: 146-155.

Müller, K.M., K.M. Cole, and R.G. Sheath. 2003. Systematics of *Bangia* (Bangiales, Rhodophyta) in North America. II. Biogeographical trends in karyology: Chromosome numbers and linkage with gene sequence phylogenetic trees. *Phycologia*. **42**: 209-219.

Müller, K.M., M.C. Oliveira, R.G. Sheath, and D. Bhattacharya. 2001a. Ribosomal DNA phylogeny of the Bangiophycidae (Rhodophyta) and the origin of secondary plastids. *American Journal of Botany*. **88**: 1390-1400.

Müller, K.M., M.C. Oliveira, R.G. Sheath, and D. Bhattacharya. 2001b. Ribosomal DNA phylogeny of the Bangiophycidae (Rhodophyta) and the origin of secondary plastids.

*American Journal of Botany*. **88**: 1390-1400.

Müller, K.M., R.G. Sheath, M.L. Vis, T.J. Crease, and K.M. Cole. 1998. Biogeography and systematics of *Bangia* (Bangiales, Rhodophyta) based on the Rubisco spacer, *rbc*L gene and 18S rRNA gene sequences and morphometric analyses. 1. North America. *Phycologia*. **37**: 195-207.

Müller, K.M., A.R. Sherwood, C.M. Pueschel, R.R. Gutell, and R.G. Sheath. 2002. A proposal for a new red algal order, the thoreales. *Journal of Phycology*. **38**: 807-820.

Mumford, T.F., and A. Miura. 1988. Porphyra as food: cultivation and economics. Algae and Human Affairs, Cambridge University Press, Cambridge. 87–117.

Necchi, O. Jr. and M.R. Zucchi. 1997. Taxonomy and distribution of Thorea (Thoreaceae, Rhodophyta) in Brazil. *Algological Studies.* **84**: 83-90.

Nelson, W.A. 2007. *Bangiadulcis* gen. nov.: a new genus for freshwater filamentous Bangiales (Rhodophyta). *Taxon*. **56**: 883–886.

Nelson, W.A., T.J. Farr, and J.E.S. Broom. 2005. *Dione* and *Minerva*, two new genera from New Zealand circumscribed for basal taxa in the Bangiales (Rhodophyta). *Phycologia*. **44**: 139-145.

Nelson, W.A., T.J. Farr, and J.E.S. Broom. 2006. Phylogenetic relationships and generic concepts in the red order Bangiales: challenges ahead. *Phycologia*. **45**: 249-259.

Niwa, K., N. Kikuchi, and Y. Aruga. 2005. Morphological and molecular analysis of the endangered species *Porphyra tenera* (Bangiales, Rhodophyta). *Journal of Phycology*. **41**: 294-304.

Niwa, K., N. Kikuchi, M. Iwabuchi, and Y. Aruga. 2004. Morphological and AFLP variation of *Porphyra yezoensis* Ueda form. *narawaensis* Miura (Bangiales, Rhodophyta). *Phycological Research*. **52**: 180-190.

Niwa, K., A. Kobiyama, and Y. Aruga. 2005. Confirmation of cultivated *Porphyra tenera* (Bangiales, Rhodophyta) by polymerase chain reaction restriction fragment length polymorphism analyses of the plastid and nuclear DNA. *Phycological Research*. **53**: 296-302.

Oates, B.R. and K.M. Cole. 1992. Chloroplast division polarity, a marker of cell division planes during morphogenesis in *Bangia vermicularis* Harvey (Rhodophyceae). *Protoplasma*. **169**: 155–167.

Oliveira, M.C., and D. Bhattacharya. 2000. Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. *American Journal of Botany*. **87**: 482-492.

Oliveira, M.C., J. Kurniawan, C.J. Bird, E.L. Rice, C.A. Murphy, R.K. Singh, R.R. Gutell, and M.A. Ragan. 1995. A preliminary investigation of the order Bangiales (Bangiophycidae, Rhodophyta) based on sequences of nuclear small-subunit ribosomal RNA genes. *Phycological Research*. **43**: 71-79.

Page, R.D.M. 1996. TreeView: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*. **12**: 357-358.

Paradis, E., J. Claude, and K. Strimmer. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*. **20**: 289.

Park, E.J., S. Fukuda, H. Endo, Y. Kitade, and N. Saga. 2007. Genetic polymorphism within *Porphyra yezoensis* (Bangiales, Rhodophyta) and related species from Japan and Korea detected by cleaved amplified polymorphic sequence analysis. European *Journal of Phycology*. **42**: 29-40.

Patrone, L.M., S.T. Broadwater and J.L. Scott. 1991. Ultrastructure of vegetative and dividing cells of the unicellular red algae *Rhodella violacea* and *Rhodella maculata. Journal of Phycology*. **27**: 742-753.

Perez, T., J. Albornoz, and A. Dominguez. 1998. An evaluation of RAPD fragment reproducibility and nature. *Molecular Ecology*. **7**: 1347–1357.

Posada, D., and K.A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*. **14**: 817-818.

Posada, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*. **25**: 1253-1256.

Powell, W., M. Morgante, C. Andre, J.W. McNicol, G.C. Machray, J.J. Doyle, S.V. Tingey, and J.A. Rafalski. 1995. Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. *Current Biology*. **5**: 1023-1029.

Provasoli, L. and I.J. Pintner. 1980. Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of Phycology*. **16**: 196 – 201.

Pruesse, E., C. Quast, K. Knittel, B.M. Fuchs, W. Ludwig, J. Peplies, and F.O. Glockner. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*. **35**:

7188-7196.

Pueschel, C.M. 1989. An expanded survey of the ultrastructure of red algal pit plugs. *Journal of Phycology*. **25**: 625–636.

Pueschel, C.M., G.W. Saunders and J.A. West. 2000. Affinities of the freshwater red alga *Audouinella macrospora* (Florideophyceae, Rhodophyta) and related forms based on SSU rRNA gene sequence analysis and pit plug ultrastructure. *Journal of Phycology*. **36**: 433–439.

R Development Core Team. 2006. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Ragan, M.A. 1998. On the delineation and higher-level classification of algae. *European Journal of Phycology*. **33**: 1-15.

Ragan, M.A., C.J. Bird, E.L. Rice, R.R. Gutell, C.A. Murphy, and R.K. Singh. 1994. A molecular phylogeny of marine red algae (Rhodophyta) based on the nuclear small-subunit rRNA gene. *Proceedings of the National Academy of Sciences of the United States of America*. **91**: 7276-7280.

Rambaut, A. FigTree: Tree Figure Drawing Tool. Institute of Evolutionary Biology, University of Edinburgh.

Rintoul, T.L., R.G. Sheath, and M.L. Vis. 1999. Systematics and biogeography of the Compsopogonales (Rhodophyta) with emphasis on the freshwater families in North America. *Phycologia*. **38**: 517-527.

Robba, L., S.J. Russell, G.L. Barker, and J. Brodie. 2006. Assessing the use of the

mitochondrial cox1 marker for use in DNA barcoding of red algae (Rhodophyta). *American Journal of Botany*. **93**: 1101.

Roberts, D.W. 2006. labdsv: Laboratory for Dynamic Synthetic Vegephenomenology. R package version.: 1–2.

Roux, O., M. Gevrey, L. Arvanitakis, C. Gers, D. Bordat, and L. Legal. 2007. ISSR-PCR: Tool for discrimination and genetic structure analysis of Plutella xylostella populations native to different geographical areas. *Molecular Phylogenetics and Evolution*. **43**: 240–250.

Saunders, G.W. 1993. Gel Purification of Red Algal Genomic Dna - an Inexpensive and Rapid Method for the Isolation of Polymerase Chain Reaction-Friendly Dna. *Journal of Phycology*. **29**: 251-254.

Saunders, G.W. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical Transactions of the Royal Society B-Biological Sciences*. **360**: 1879-1888.

Saunders, G.W., and M.H. Hommersand. 2004. Assessing red algal supraordinal diversity and taxonomy in the context of contemporary systematic data. *American Journal of Botany*. **91**: 1494-1507.

Saunders, G.W., and G.T. Kraft. 1997. A molecular perspective on red algal evolution: focus on the Florideophycidae. *Plant Systematics and Evolution*. **11(suppl.)**: 115-138.

Savill, N.J., D.C. Hoyle, and P.G. Higgs. 2001. RNA Sequence Evolution With Secondary Structure Constraints Comparison of Substitution Rate Models Using Maximum-Likelihood Methods. *Genetics*. **157**: 399-411.

Schneider, C.W., and M.J. Wynne. 2007. A synoptic review of the classification of red algal genera a half century after Kylin's "Die Gattungen der Rhodophyceen". *Botanica Marina*. **50**: 197–249.

Scott, J.L., S.T. Broadwater, B.D. Saunders, J.P. Thomas and P.W. Gabrielson. 1992. Ultrastructure of vegetative organization and cell division in the unicellular red alga *Dixoniella grisea* gen. nov. (Rhodophyta) and a consideration of the genus *Rhodella*. *Journal of Phycology*. **28**: 649:660.

Seifert, K.A., R.A. Samson, J.R. deWaard, J. Houbraken, C.A. Lévesque, J.M. Moncalvo, G. Louis-Seize, and P.D. Hebert. 2007. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *Proceedings of the National Academy of Sciences of the United States of America*. **104**: 3901.

Sheath, R.G., and K.M. Cole. 1984. Systematics of *Bangia* (Rhodophyta) in North America. I. Biogeographic trends in morphology. *Phycologia*. **23**: 383-396.

Silva, P.C., and W.A. Nelson. 2008. History of the typification of conserved and rejected names, including an account of the typification of *Bangia* Lyngb.(Bangiaceae, Rhodophyta). *Taxon*. **57**: 1351–1354.

Soltis, P.S., D.E. Soltis, and M.W. Chase. 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature*. **402**: 402-404.

Sosa, P.A. and S.C. Lindstrom. 1999. Isozymes in macroalgae (seaweeds): genetic differentiation, genetic variability and applications in systematics. *European Journal of Phycology*. **34**: 427-442.

Sosa, P.A., M. Valero, F. Batista, and M.A. Gonzalez-Perez. 1998. Genetic structure of natural populations of *Gelidium* species: A re-evaluation of results. *Journal of Applied Phycology*. **10**: 279-284.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*. **22**: 2688.

Stiller, J.W., and B.D. Hall. 1997. The origin of red algae: Implications for plasmid evolution. *Proceedings of the National Academy of Sciences of the United States of America*. **94**: 4520-4525.

Stiller, J.W., and J.R. Waaland. 1993. Molecular Analysis Reveals Cryptic Diversity in *Porphyra* (Rhodophyta). *Journal of Phycology*. **29**: 506-517.

Svedelius, N. 1911. Rhodophyceae (Bangiales und Florideae). In Die Naturlichen Pflanzenfamilien. Nachtrage. Vol. 1. Part 2 (Lief 243–244). Edited by A. Engler and K. Prantl. W. Engelmann, Leipzig. pp. 193–284.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*. **24**: 1596-1599.

Tan, I.H., J. Blomster, G. Hansen, E. Leskinen, C.A. Maggs, D.G. Mann, H.J. Sluimam, and M.J. Stanhope. 1999. Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds, *Ulva* and *Enteromorpha*. *Molecular Biology and Evolution*. **16**:

1011-1018.

Tappan, H. 1976. Possible eucaryotic algae (Bangiophycidae) among early Proterozoic microfossils. *Geological Society of America Bulletin*. **87**: 633-639.

Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Some mathematical questions in biology—DNA sequence analysis. **17**: 57–86.

Toplin, J.A., T.B. Norris, C.R. Lehr, T.R. McDermott, and R.W. Castenholz. 2008. Biogeographic and Phylogenetic Diversity of Thermoacidophilic Cyanidiales in Yellowstone National Park, Japan, and New Zealand. *Applied and Environmental Microbiology*. **74**: 2822-2833.

Van de Peer, Y., J. Jansen, P. De Rijk, and R. De Wachter. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research*. **25**: 111-116.

Van de Peer, Y., S.A. Rensing, U.G. Maier, and R. De Wachter. 1996. Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. *Proceedings of the National Academy of Sciences of the United States of America*. **93**: 7732-7736.

Verbruggen, H., C.A. Maggs, G.W. Saunders, L. Le Gall, H.S. Yoon, and O. De Clerck. 2010. Data mining approach identifies research priorities and data requirements for resolving the red algal tree of life. *BMC Evolutionary Biology*. **10**: 16.

Vis, M.L. 1999. Intersimple sequence repeats (ISSR) molecular markers to distinguish gametophytes of *Batrachospermum boryanum* (Batrachospermales, Rhodophyta). *Phycologia*. **38**: 70-73.

Waterhouse, A.M., J.B. Procter, D.M.A. Martin, M. Clamp, and G.J. Barton. 2009. Jalview Version 2 - a multiple sequence alignment editor and analysis workbench. *Bioinformatics*. **25**:1189-1191.

Weng, M.L., B. Liu, D.M. Jin, Q.K. Yang, G. Zhao, J.H. Ma, P. Xu, D.L. Duan, and B. Wang. 2005. Identification of 27 *Porphyra* lines (Rhodophyta) by DNA fingerprinting and molecular markers. *Journal of Applied Phycology*. **17**: 91-97.

White, T.J., T. Bruns, S. Lee, J. Taylor, and others. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*. **18**: 315–322.

Wimberly, B.T., D.E. Brodersen, W.M. Clemons Jr, R.J. Morgan-Warren, A.P. Carter, C. Vonrhein, T. Hartsch, and V. Ramakrishnan. 2000. Structure of the 30S ribosomal subunit. *Nature*. **407**: 327-339.

Winker, S., and C.R. Woese. 1991. A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Systematic and Applied Microbiology*. **14**: 305-310.

Woelkerling, W.J. and L.M. Irvine. 1982. The genus *Schmitziella* Bornet et Batters (Rhodophyta): Corallinaceae or Acrochaetiaceae? *British Phycological Journal*. **17**: 275-295.

Woese, C.R., and N.R. Pace. 1993. 4 Probing RNA Structure, Function, and History by Comparative Analysis. *Cold Spring Harbor Monograph Archive*. **24**: 91-117.

Woese, C.R. 1987. Bacterial evolution. *Microbiology and Molecular Biology Reviews*. **51**:

221.

Woese, C.R., and G.E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*. **74**: 5088-5090.

Wolfe, A.D., Q. Qiang and S.R. Kephart. 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. *Molecular Ecology*. **7**: 1107–1125.

Wright J.T., G.C. Zuccarello and P.D. Steinberg. 2000. Genetic structure of the subtidal red alga *Delisea pulchra*. *Marine Biology.* **136**: 439-448.

Wuyts, J., P. De Rijk, Y. Van de Peer, G. Pison, P. Rousseeuw, and R. De Wachter. 2000. Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. *Nucleic Acids Research*. **28**: 4698.

Wuyts, J., G. Perrière, and Y. Van De Peer. 2004. The European ribosomal RNA database. *Nucleic Acids Research.* **32**(Database issue): D101-103.

Yokoyama, A., Sato, K. and Hara, Y. 2004. The generic delimitation of *Rhodella* (Porphyridiales, Rhodophyta) with emphasis on ultrastructure and molecular phylogeny. *Hydrobiologia*. **512**: 177-183

Yokoyama, A., J.L. Scott, G.C. Zuccarello, M. Makiko Kajikawa, Y. Hara and J.A. West. 2009. *Corynoplastis japonica* gen. et sp. nov. and Dixoniellales ord. nov. (Rhodellophyceae, Rhodophyta) based on morphological and molecular evidence. *Phycological Research*. 57: 278-289.

Yoon, H.S., J.D. Hackett, and D. Bhattacharya. 2006. A genomic and phylogenetic perspective on endosymbiosis and algal origin. *Journal of Applied Phycology*. **18**: 475-481.

Yoon, H.S., J.D. Hackett, F.M.V. Dolah, T. Nosenko, L. Lidie, and D. Bhattacharya. 2005. Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. *Molecular Biology and Evolution*. **22**: 1299-1308.

Yoon, H.S., Y.D. Hackett, C. Ciniglia, G. Pinto, and D. Bhattacharya. 2004. A molecular timeline for the origin of photosynthetic eukaryotes. *Molecular Biology and Evolution*. **21**: 809-818.

Yoon, H.S., K.M. Müller, R.G. Sheath, F.D. Ott, and D. Bhattacharya. 2006a. Defining the major lineages of red algae (Rhodophyta). *Journal of Phycology*. 42(2): 482-492.

Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain-Reaction Amplification. *Genomics*. **20**: 176-183