6 Molecular systematics of red algae: building future structures on firm foundations

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ABSTRACT

Red algal systematics has a solid morphological foundation, based on analyses of female reproductive structures and post-fertilization development by Kylin and other workers. Recognition of the value of pit-plug ultrastructure was a catalyst leading to refinement of the Kylinian ordinal classification. Molecular approaches to systematics have further advanced our understanding of the red algae at every level and led to the proposal of several new orders. Species diversity in particular has traditionally been underestimated due to the presence of cryptic and pseudo-cryptic species.

A literature review covering the last two decades shows that relatively few molecular markers have been employed for studies of red algal systematics. The general trend was toward the use of multiple markers until five years ago when an increased emphasis on lower-level taxonomy (molecular identification) led to greater reliance on single markers. In the future, we predict that there will be relatively few new orders proposed, and the emphasis will change to hypothesis-driven, less descriptive molecular studies in concert with morphological, ultrastructural, and biochemical analyses.

INTRODUCTION TO THE RED ALGAE (RHODOPHYTA)

An ancient and diverse group

The Rhodophyta (red algae) are eukaryotes, and the great majority of the species are marine, photosynthetic, and macroscopic. Red algae are an ancient lineage (Xiao et al., 1998; Yoon et al., 2004), including what is generally believed to be one of the oldest taxonomically resolved eukaryotic fossils, the 1.2 billion year old *Bangiomorpha pubescens* Butterfield (Butterfield, 2000). The Rhodophyta have evolved a diverse range of modifications in cellular organization and general morphology (Pueschel, 1990).

Unique combination of features

The red algae are distinguishable amongst eukaryotic lineages by a combination of biochemical and ultrastructural features. The most striking of these is that they lack flagella as well as centrioles or other 9 + 2 structures at any stage of their life histories (Pueschel, 1990; Ragan and Gutell, 1995); sex and spore dispersal therefore have to be accomplished without the benefit of flagellar propulsion. Instead, red algal sperm sport gothic-style mucilaginous appendages that affect their hydrodynamic properties and contain cell recognition proteins that attach specifically to the sessile female gametes—carpogonia (Broadwater et al., 1991; Kim et al., 1996).

Whereas green algae and plants store starch in the chloroplasts, the red algal polysaccharide reserves of floridean starch are in the cytoplasm. An important ultrastructural feature is that red algal plastids (term used for non-green chloroplasts) have unstacked thylakoid membranes and lack an encircling endoplasmic reticulum membrane. Chlorophyll *a* is the only chlorophyll, and accessory red/blue phycobilin pigments, predominantly the red-coloured phycoerythrin, occur in stalked phycobilisomes on thylakoids (van den Hoek et al., 1995). Although none of the characteristics listed here is unique to the Rhodophyta, the red algae represent the only group of organisms in which all are found, so that "in practice there is little difficulty in distinguishing what is or is not a red alga" (Ragan and Gutell, 1995).

From the early twentieth century until very recently, red algae were divided into two groups (Bangiophyceae and Florideophyceae) based on morphological, anatomical, and life-history differences (Dixon, 1973), but the taxonomic rank to which these groups were assigned fluctuated (Gabrielson et al., 1985; Garbary and Gabrielson, 1990; Murray and Dixon, 1992). In order to introduce these groups, we refer to the morphologically defined classes as Bangiophyceae or Florideophyceae "in the traditional sense"; a more recent classification is provided below in the section on molecular markers and higher-level systematics.

Bangiophyceae: relatively simple morphologies

The smaller class Bangiophyceae has mainly been defined rather unsatisfactorily by the absence of characters confined to the Florideophyceae (e.g. tetrasporangia, filamentous gonimoblasts) or by the presence of characters (e.g. single star-shaped plastids) that are found only in some members of the class. Compared to the florideophytes, the bangiophytes are generally morphologically simple and have traditionally been considered to contain the most primitive red algal forms (Müller et al., 2001). Their life histories are mostly poorly known but appear to be diverse (Brodie and Irvine, 2003).

The best-known genus, *Porphyra*, which is commercially cultivated for nori, displays a heteromorphic life history, the two phases of which are so morphologically dissimilar that they were linked only by culture studies (Drew, 1954). The blade-like haploid gametophytic phase gives rise to a microscopic shell-boring diploid conchocelis phase. In some species at least, meiosis takes place during germination of spores formed by the conchocelis phase (Mitman and van der Meer, 1994; Brodie and Irvine, 2003). Apart from the Bangiales, the Bangiophyceae are mostly asexual (Brodie and Irvine, 2003).

Diverse, complex florideophyceae

The more complex Florideophyceae (e.g. the valuable carrageen-producing "Irish Moss" *Chondrus crispus* Stackhouse) exhibit an enormous diversity of morphological structures and complicated haplo-diploid life histories. Haploid and diploid phases are morphologically closely similar if not identical (isomorphic) in some species, whereas other life histories such as that of the Japanese invasive species *Bonnemaisonia hamifera* Hariot are heteromorphic like *Porphyra*. The cryptic phase, which may be either the gametophyte (unisexual or bisexual) or the sporophyte, is crustose, filamentous, or boring, whereas the more conspicuous phase is erect, often resembling leaves or twigs. These intricate life histories involve specialized meiotic sporangia (tetrasporangia), characteristic of the Florideophyceae, that release four haploid tetraspores. Uniquely, in most Florideophyceae, the immediate product of fertilization is not the diploid sporophyte, but a hemi-parasitic diploid tissue (the "gonimoblast") surrounded by female nutritive tissue, collectively called the "cystocarp" (or carposporophyte). This stage, representing a clonal growth strategy compensating for the lack of motile sperm in the red algae (Searles, 1980), releases numerous genetically identical diploid spores that give rise to sporophytes.

Ordinal classification has firm foundations

Florideophyceae: the enduring legacy of Schmitz and Kylin

Until the impact of molecular data, the ordinal classification of the Florideophyceae was still based on the monumental posthumous treatise of Kylin (1956), which incorporated his revisions of the earlier schemes of Schmitz (1889) and Oltmanns (1898). This classification depended mainly on characteristics of female reproductive anatomy before and after fertilization. Six orders of Florideophyceae (Nemalionales, Gelidiales, Cryptonemiales, Gigartinales, Rhodymeniales, and Ceramiales) were recognized (Figure 6.4; Papenfuss, 1966). In the last four of these Kylinian orders, the gonimoblast originates from the auxiliary cell, a cytoplasm-rich cell into which is injected the diploid zygotic nucleus, or a diploid nucleus produced after one or several divisions of the zygotic nucleus (Dixon, 1973). The Ceramiales was generally considered the most advanced of these orders because the auxiliary cell is produced only after fertilization. The Nemaliales was characterized by the direct development of the gonimoblast from the zygote in the absence of an auxiliary cell, and the Gelidiales had been separated from it because although auxiliary cells were present they did not initiate gonimoblasts (see Garbary and Gabrielson, 1990).

Bangiophyceae: fewer, less robust orders

Kylin's (1956) ordinal classification of bangiophytes was less well developed than that for the florideophytes because of the scarcity of morphological characters (Dixon, 1973). In particular, the group lacks cystocarps, whereas the Schmitz–Kylin classification was erected essentially on the basis of the complex female reproductive characters of the florideophytes. The revision by Garbary et al. (1980) incorporated observations on life histories and ultrastructure in addition to new morphological characters and distinguished four orders by a combination of these traits. Erythropeltidales (features: multicellular; vegetative reproduction by monosporangia) was erected as a new order because of similarities in monosporangial formation in three families (Erythropeltidaceae, Boldiaceae, and Compsopogonaceae). The Porphyridiales circumscribed unicellular species that are free living or held

together by a mucilaginous matrix. The Rhodochaetales contained filamentous algae with pit connections and sexual reproduction by formation of a single diploid carpospore following fertilization. The most distinctive and species-rich order, the Bangiales, included all species that exhibited sexual reproduction and in which the conchocelis sporophyte generation formed pit connections.

Pit-plug ultrastructure: a crucial catalyst

The most significant contribution to red algal systematics after Kylin's morphological synthesis and prior to the application of molecular markers came in a series of papers by Curt Pueschel, starting with Pueschel and Cole (1982), which reported on his ultrastructural studies of pit-plugs. In florideophytes and some bangiophytes, cross-wall formation after cell division is incomplete, leaving a membrane-lined pore in the central region (Pueschel, 1990). Tubular membranes appear in this region, then a homogeneously granular protein mass (the plug core) is deposited around the tubules, followed by the disappearance of the tubules. The pit-plug either consists only of the core or acquires additional features, such as carbohydrate domes and cap membranes, which are continuous with the cell membrane. Although some previous workers had doubted the possible systematic value of pit-plugs because of observed intraspecific variation (Duckett and Peel, 1978), Pueschel and Cole (1982) showed that a combination of three characters, the presence or absence of the inner and outer cap layers, and the shape of the outer cap (domed or plate-like) was useful in distinguishing among higher taxa of florideophytes. Features of pit-plugs introduced a new age of criteria for ordinal relationships in the florideophytes; two new orders (Batrachospermales and Hildenbrandiales) were segregated, and four previously described orders (e.g. the Palmariales, originally based on tetrasporangial ontogeny) were confirmed in the analyses of Pueschel and Cole (1982). Later, Trick and Pueschel (1991) demonstrated that plate-like and domed outer cap layers were chemically similar and probably homologous, and proposed that the domed state (found in the Corallinales and some Acrochaetiales) is ancestral, plate-like outer caps being derived.

MOLECULAR TOOLS AND THEIR CONTRIBUTION TO SYSTEMATICS

Pioneer papers

The first molecular phylogenetic studies that included red algae appeared in the mid-1980s. Analyses of 5S ribosomal sequences of representative prokaryotic and eukaryotic organisms found the red algae to be the most ancient eukaryote lineage (Hori et al., 1985; Lim et al., 1986; Hori and Osawa, 1987). Despite the small size and evolutionary constraints on this gene which make it inappropriate for this kind of phylogenetic analyses, trees were broadly congruent with later 18S trees (Ragan and Gutell, 1995). In Hori and Osawa's tree, the Palmariales was found to branch more basally within the Florideophyceae than the Gigartinales, Gelidiales, and Gracilariales.

Other early molecular studies used a variety of markers. Goff and Coleman (1988) employed restriction fragment length polymorphism (RFLP) analysis of plastid DNA for a geographical study of *Gracilariopsis andersonii* (Grunow) Dawson (as *Gracilaria sjoestedtii*), showing populations over a 2000 km range to be remarkably genetically homogeneous. The same technique was used to link the dissociated (apomictic) phases of the heteromorphic life history in *Gymnogongrus* (now *Ahnfeltiopsis*) (Parsons et al., 1990). The phylogenetic position of *Gracilariopsis* in the eukaryotes was determined from 18S nrDNA sequences (Bhattacharya et al., 1990). In red algae the large (*rbc*L) and small (*rbc*S) subunits of the ribulose biphosphate carboxylase/oxygenase (rubisco) gene are organised as a co-transcribed operon including a short highly variable intergenic spacer (the rubisco spacer) (Kostrzewa et al., 1990). This spacer was exploited for several early species-level studies that distinguished between populations of *Ahnfeltiopsis* (as *Gymnogongrus*) *devoniensis* (Greville) P.C. Silva et DeCew and closely related species (Maggs et al., 1992) and amongst

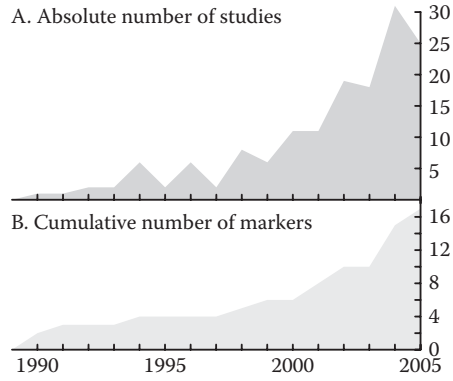

FIGURE 6.1 (A) Absolute number of red algal molecular phylogenetic studies per year through time. (B) Cumulative number of red algal molecular phylogenetic markers through time.

members of the *Gracilaria verrucosa* species complex (Destombe and Douglas, 1991). Bird et al. (1992) carried out the first molecular study at the family to species level in red algae, using using the SSU nrDNA to determine phylogenetic relationships between members of the Gracilariaceae.

Molecular phylogenetic markers

Since the early 1990s, the numbers both of phylogenetic studies and markers used have increased steadily (Figure 6.1A and Figure 6.1B). Phylogenetic studies have been carried out at all taxonomic levels across most of the spectrum of red algal biodiversity. Our examination of 156 red algal molecular systematic papers published between 1990 and early 2006 revealed that a wide array of nuclear ribosomal, plastid, and mitochondrial markers is currently available for red algal phylogenetics (Table 6.1). The number of molecular phylogenetic markers used has gradually increased

TABLE 6.1
Red Algal Phylogenetic Markers and Their Frequency of Use in 156 Screened Studies

Genome	Marker	Type	Ref.	Frequency
Nuclear	5S	Ribosomal DNA	Hori et al. (1985)	3
	18S	Ribosomal DNA	Bhattacharya et al. (1990)	62
	28S	Ribosomal DNA	Freshwater and Bailey (1998)	21
	ITS region	Two ribosomal spacers	Steane et al. (1991)	17
	Actin	Gene	Hoef-Emden et al. (2005)	1
Mitochondrial	*cox*1	Gene	Saunders (2005)	1
	*cox*2-3	Intergenic spacer	Zuccarello et al. (1999)	11
Plastid	16S	Ribosomal DNA	Olson et al. (2005)	3
	*rbc*L	Gene	Freshwater et al. (1994)	77
	*rbc*S	Gene	Lee et al. (2001)	2
	Rubisco spacer	Intergenic spacer	Destombe and Douglas (1991)	22
	*psa*A	Gene	Yang and Boo (2004)	3
	*psa*B	Gene	Yoon et al. (2004)	1
	*psb*A	Gene	Seo et al. (2003)	4
	*psb*C	Gene	Yoon et al. (2002, 2006)	1
	*psb*D	Gene	Yoon et al. (2002, 2006)	1
	*tuf*A	Gene	Yoon et al. (2004)	1
	URP markers	Genes and spacers	Provan et al. (2004)	1

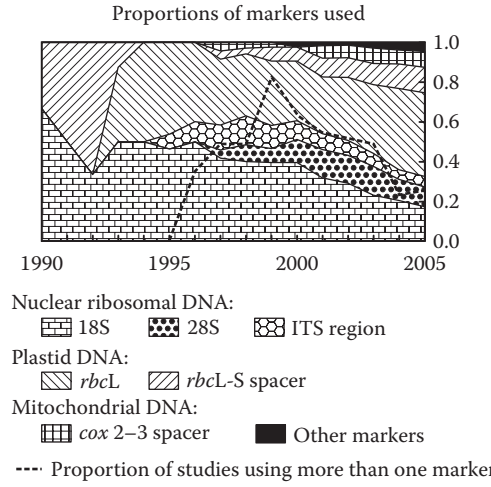

FIGURE 6.2 Use of phylogenetic markers through time, plotted as proportion of total studies. The black line indicates the proportion of studies using more than one marker.

with time (Figure 6.1B). Although many of the markers have been employed for studies at various taxonomic levels, plastid and mitochondrial genes are mostly exploited to resolve the relationships between species belonging to one genus or one family, at higher taxonomic levels plastid or nuclear ribosomal DNA sequences are mostly used, and spacer sequences are almost exclusively used to infer haplotype trees or networks for closely related species and populations. To evaluate the taxonomic level at which markers performed best, Verbruggen et al. (unpublished) reanalysed 104 published phylogenetic datasets using Bayesian methods. The resolution of the markers, measured as the proportion of nodes receiving posterior probability ≥0.90, was evaluated at five taxonomic levels, ranging from intraspecific nodes to nodes above the ordinal level. Whereas the resolution of plastid genes was highest at low taxonomic levels and gradually decreased toward higher ranks, rDNA markers showed the opposite trend.

There are some trends in the use of phylogenetic markers through time (Figure 6.2), such as a gradual decrease in the use of the relatively slowly evolving SSU ribosomal DNA marker in favour of faster-evolving organellar markers (*rbc*L, *rbc*L-S spacer, *cox*2-3 spacer). This trend coincides with a steady increase in the relative number of studies focussing at low taxonomic levels. The most recently developed markers reinforce the latter trend; nearly all of them are highly variable markers for use between the genus and species level (e.g. the mitochondrial *cox*1 gene and *cox*2-3 spacer, and the plastid genes *psb*A, *psa*A, and URP markers). There is also a clear trend in the number of studies using more than one marker. The use of multiple markers shows a sharp increase in the second half of the 1990s to decrease again in the 2000s (Figure 6.2). We interpret this observation as being a result of the rapid widening of the usage of molecular markers in red algal systematics. The first two decades of molecular systematic research, particularly in the pre-PCR age, involved a great deal of pioneering technical work, and researchers experimented with a wide range of different markers. Most of the recent papers are primarily taxonomically centred, and for most systematic questions, adequate data can be acquired from single markers.

Higher taxonomic levels: relationship between Bangiophyceae and Florideophyceae

Two large, high-level molecular phylogenies of the red algae appeared together in 1994, utilizing the nuclear 18S ribosomal gene (Ragan et al., 1994) and the plastid-encoded gene for the large subunit of Rubisco, *rbc*L (Freshwater et al., 1994). Both of these ambitious studies suffered from

various flaws, including comparatively primitive methods of data analysis, underrepresentation of bangiophyte taxa, and long-branch attraction, but they provided broad overviews of relationships among florideophyte orders, generally comparable with more recent trees. Many of the problems were overcome in later studies.

Cavalier-Smith (1998) proposed a division of the red algae into two subphyla. His Rhodellophytina, characterized by relatively simple thalli composed of small uninucleate vegetative cells, encompasses the Porphyridiales, Cyanidiales, and Compsopogonales. Cavalier-Smith's Macrorhodophytina, including the florideophytes and Bangiales, exhibit a much wider range of morphology (Goff and Coleman, 1990; Ragan and Gutell, 1995). Cavalier-Smith's classification has been adapted by Saunders and Hommersand (2004; Figure 6.3), and newer proposals are described below.

Two recent papers specifically address the subdivision of the red algae, and particularly the heterogeneous Bangiophyceae, into monophyletic subphyla and classes (Saunders and Hommersand, 2004; Yoon et al., 2006; summarized in Figure 6.3). Saunders and Hommersand (2004) present a classification based on a tree summarizing the relationships compiled from several ribosomal DNA studies. They split up the traditional bangiophycean diversity by moving the Cyanidiales into their own phylum and placing the remaining orders in three subphyla (Figure 6.3). The subphylum Metarhodophytina, with a single class Compsopogonophyceae, was erected to group the Compsopogonales, Erythropeltidales, and Rhodochaetales. A second, possibly paraphyletic, subphylum Rhodellophytina, with a single class Rhodellophyceae, contains the three porphyridialean orders. Finally, Saunders and Hommersand's third, and convincingly monophyletic, subphylum Eurhodophytina contains the Bangiophyceae s.s. (Bangiales) and the Florideophyceae.

Yoon et al. (2006) define major red algal lineages and infer their relationships on the basis of their analyses of a concatenated alignment of seven plastid protein-coding genes, 18S nuclear rDNA and 16S plastid rDNA. They argue that the Cyanidiales do not deserve recognition as a separate phylum and recognize them as a subphylum within the Rhodophyta. They support Saunders and

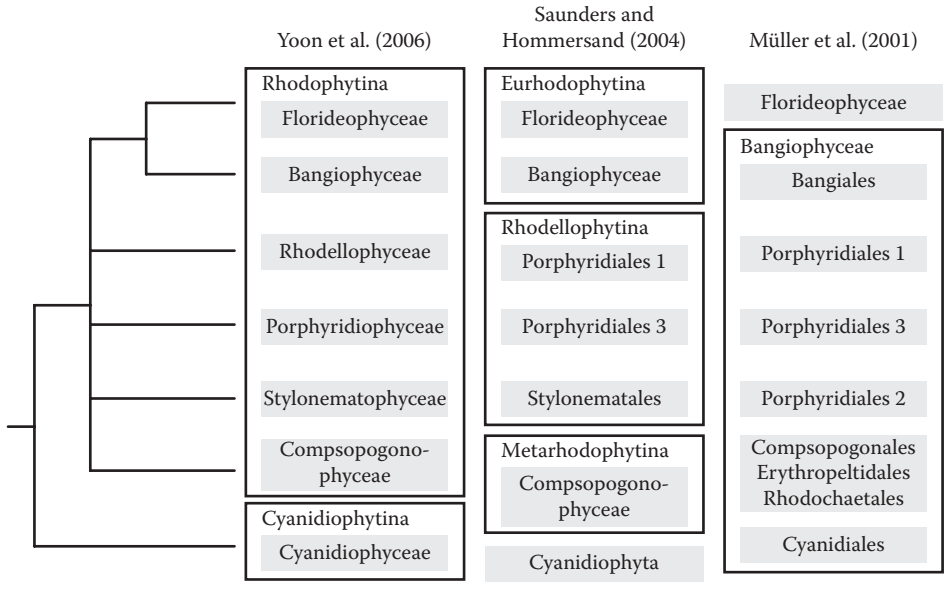

FIGURE 6.3 Phylogeny of the red algae, modified from Yoon et al. (2006), in which branches in front of nodes receiving insufficient confidence have been collapsed. Three current alternative higher-level classification schemes of the red algae into subphyla (boxes) and classes/orders (shaded) are shown, from Yoon et al. (2006, left), Saunders and Hommersand (2004, centre), and Müller et al. (2001, right). Note that in the classification of Saunders and Hommersand (2004), the Cyanidiophyceae were placed in a separate phylum.

Hommersand's Compsopogonophyceae and Bangiophyceae s.s. but raise the three porphyridialean orders to classes (Figure 6.3). In addition to the Cyanidiophytina, they recognize a second subphylum, Rhodophytina, comprising all other classes.

The relationships between the Florideophyceae and the various bangiophycean orders have been obscured until relatively recently. In earlier accounts, the sequences of the bangiophyte unicells *Dixoniella* and *Rhodella* grouped quite strongly with the solidly monophyletic Florideophyceae (Ragan and Gutell, 1995). Addition of a large number of bangiophyte sequences (Oliveira and Bhattacharya, 2000; Müller et al., 2001; Yoon et al., 2006) has repositioned *Dixoniella* and *Rhodella* away from a clade uniting the bangialean and florideophyte algae. The rather perplexing previous position of both genera in Ragan and Gutell's (1995) analyses is now attributed to long-branch attraction (Müller et al., 2001). The branching order among the major red algal lineages is still poorly resolved (Figure 6.3), even based on the nine-marker alignment of Yoon et al. (2006). As long suspected, the Bangiophyceae in the traditional sense is paraphyletic, with the Cyanidiales branching off first, and several orders falling in a polytomy. The link demonstrated between the Bangiales and Florideophyceae supports two of Magne's (1989) primary subdivisions of the Rhodophyta, the Eurhodophycidae (florideophytes + Bangiales) and the Metarhodophycidae (Erythropeltidales, Rhodochaetales, Compsopogonales), although the Porphyridiales, Magne's Archeorhodophycidae, is polyphyletic (Müller et al., 2001). Contrary to earlier beliefs, *Rhodochaete*, the single representative of the order Rhodochaetales, was not resolved as the speculated link between Bangio- and Florideophyceae but grouped closely with Erythropeltidales and Compsopogonales (Zuccarello et al., 2000).

Ordinal systematics of the Florideophyceae

Our analysis shows that there has been a slight increase in phylogenetic studies aiming to infer relationships among orders, and among families within orders, from an average of 2–3 per year in the second half of the 1990s to 5 per year in 2004 and 2005 (although the relative proportion of these studies decreases through time). The two large deep-level molecular phylogenies of the red algae that appeared together in 1994 (Freshwater et al., 1994; Ragan et al., 1994) provided an overview of relationships among florideophyte orders, generally comparable with more recent trees.

If there is one generality among ordinal studies, it is that Kylinian orders were polyphyletic, particularly with respect to taxa lacking recognizable cystocarps, and therefore are being split (Figure 6.4). The number of orders has steadily increased from six in the Kylinian system, via thirteen following the ultrastructural studies in the 1980s, to the mid-twenties in the present molecular phylogenetic age, and a few more families can be expected to be raised to the ordinal level in the near future.

We here give some examples of orders that have been recognized recently as a result of molecular studies, in order to analyse the contribution of molecular data to their recognition as separate orders. Separation of the family Thoreaceae from the rest of the Batrachospermales in 18S and *rbc*L sequence analyses led to the proposal of the Thoreales by Müller et al. (2002). They also carried out the most extensive survey to date of infraspecific variation in pit-plug ultrastructure, which showed that there is some variation in the degree of inflation of the outer cap. The most convincing piece of evidence supporting recognition of the new order is the discovery of unique secondary structure signatures in the 18S gene. However, analyses by Müller et al. (2002) and others show clearly that all the members of the original Nemaliales (apart from Gelidiales and Bonnemaisoniales), i.e. Batrachospermales, Thoreales, Balliales, Acrochaetiales, and Colaconematales (Harper and Saunders, 2002) form a close grouping with the Palmariales and the recently described Balbianiales (Sheath and Müller, 1999). An argument could be made for subsuming some of these orders again into the classical Nemaliales minus Gelidiales and Bonnemaisoniales.

An elegant example of an order recognized by a combination of its molecular phylogenetic position and its morphology is the Pihiellales (Huisman et al. 2003). This order was proposed (with

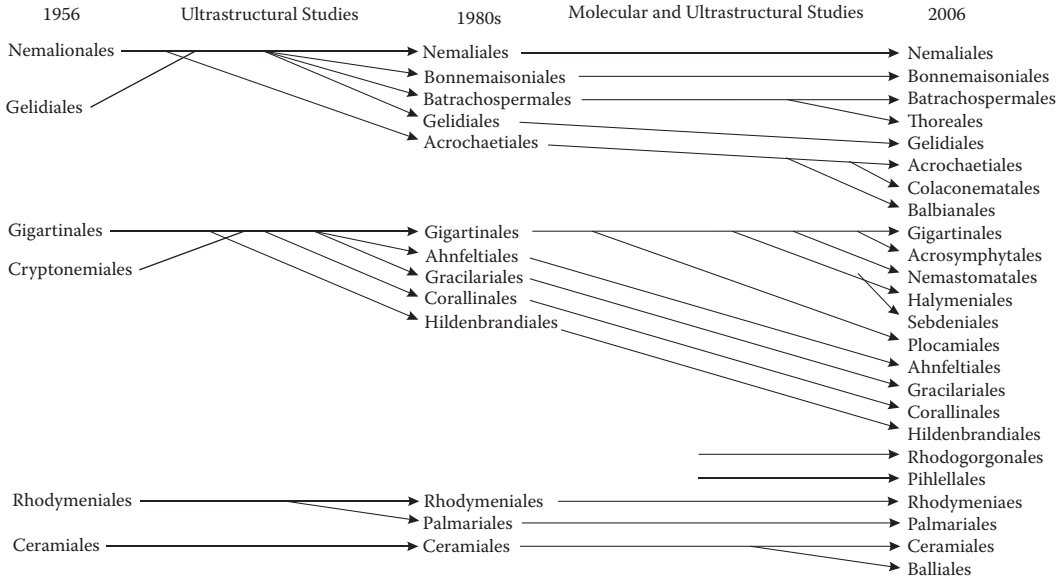

FIGURE 6.4 Changes to the ordinal classification of the florideophytes resulting from ultrastructural and molecular studies. The first column (1956) represents the Kylinian orders, the central column represents the orders recognized toward the end of the 1980s, on the basis of ultrastructural work (mostly pit-plugs), and the right column presents the currently recognized orders resulting from molecular analyses, in many cases supported by ultrastructural data.

its formal diagnosis including a GenBank code), for a previously undescribed minute endo/epiphyte growing on members of the Liagoraceae. Although these organisms were first noticed and illustrated in the mid-nineteenth century, their lack of morphological affinities with any other red alga led to a 150-year hiatus in the process of naming them. Analyses of 18S rDNA showed that the single species *Pihiella liagoraciphila* Huisman, Sherwood et I.A. Abbott was most closely related to *Ahnfeltia*, the sole genus in the Ahnfeltiales (Maggs and Pueschel, 1989), but the extreme morphological differences and the large genetic distance between the two taxa indicate a long evolutionary divergence (Huisman et al., 2003).

Molecular phylogenetics also facilitates reconstruction of the relationships among orders. Whereas single-marker analyses left several of the deeper nodes lacking confidence, recent multigene approaches and more sophisticated analysis techniques have improved the statistical confidence in the relationships among orders despite some conflict among analyses and markers (Harper and Saunders, 2001; Withall and Saunders, 2006). Four main lineages of the Florideophyceae defined by Saunders and Kraft (1997) seem well supported, and a contemporary supraordinal classification for them was proposed by Saunders and Hommersand (2004). These four subclasses are also reasonably well characterized by ultrastructural characters (Table 6.2).

Species-level systematics and DNA barcoding

A large proportion of the phylogenetic studies we have screened (43%) were situated at the genus to species level. More than two-thirds of these studies have been published since 2003, indicating that species-level phylogenetics is an active field. The species-level systematic papers can roughly be subdivided into two classes. Studies belonging to the first class test species boundaries with relatively large sequence alignments in which species are represented by multiple specimens. Such studies usually also employ different types of data (morphology, interfertility) to investigate whether

TABLE 6.2
Monophyletic Supraordinal Lineages Receiving Strong Molecular Support and Defined as Subclasses by Saunders and Hommersand (2004)

Subclass	Orders	Ultrastructural Features
Hildenbrandiophycidae	Hildenbrandiales	Pit-plugs with single cap layer and membrane
Nemaliophycidae	Acrochaetiales, Balbianiales, Balliales, Batrachospermales, Colaconematales, Corallinales, Nemaliales, Palmariales, Rhodogorgonales, Thoreales	Pit-plugs with two cap layers
Ahnfeltiophycidae	Ahnfeltiales, Pihiellales	Pit-plugs naked
Rhodymeniophycidae	Acrosymphytales, Bonnemaisoniales, Ceramiales, Gelidiales, Gigartinales, Gracilariales, Halymeniales, Nemastomatales, Plocamiales, Rhodymeniales, Sebdeniales	Pit-plugs with membranes only (exception: Gelidiales)

different species concepts agree or can be reconciled. The second class of studies at the species level is of a more morphologically descriptive sort. Such studies present morphological descriptions of new species and employ molecular tools to situate the species in the genus.

The single most important conclusion to be drawn from species-level molecular phylogenetic studies is that red algal biodiversity is massively underestimated. Many studies have revealed cryptic and pseudo-cryptic species within morphologically defined species. Cryptic species, sometimes referred to as sibling species, are defined as species that are impossible to distinguish based on morphological characters (Sáez and Lozano, 2005). Pseudo-cryptic species are species that are readily distinguished morphologically once the appropriate characters are considered.

Cryptic diversity in the mangrove- and salt-marsh-dwelling species of *Bostrychia* (Rhodomelaceae) has been addressed by Joe Zuccarello and John West. Their research has shown that despite being morphologically identical, separate reproductively isolated lineages can be identified by plastid and mitochondrial haplotypes (Zuccarello and West, 2003). Moreover, some of these genuine cryptic species occur sympatrically. This situation may be found more commonly amongst the red algae when appropriate studies have been carried out.

Discoveries of pseudo-cryptic species have also been plentiful, and we will give just a few examples. In the genus *Grateloupia*, which has received much attention because of its invasive members (Verlaque et al., 2005), molecular tools unveiled several discrete entities within morphologically circumscribed species (Kawaguchi et al., 2001; De Clerck et al., 2005; Wilkes et al., 2005). These discrete entities can be morphologically recognized, and most of them have been described as separate species. A second genus exemplifying recent discovery of pseudo-cryptic entities within morphologically perceived species is *Plocamium*. Saunders and Lehmkuhl (2005) demonstrated the existence of at least eight divergent cryptic species currently included in *P. cartilagineum*, and defined morphological boundaries of four European species for which their sampling was sufficient. Likewise, Yano and coworkers (Yano et al., 2004, 2006) showed that traditional morphological species boundaries in Japanese *Plocamium* did not correspond with their molecular clusters. They could not find clear-cut morphological characters specific to their molecular entities but demonstrated that colour, bromine concentration, and cell content acidity could be used as identification clues.

A very recent trend is the use of DNA sequences as an identification tool. DNA barcoding, as this technique is commonly known, consists of a first stage in which a large database of sequences is generated from well-documented and accurately identified specimens (Newmaster et al., 2006). At a second stage, the database can be queried using sequences generated from unidentified specimens. The results of such queries allow identification of the unknown specimens (Saunders, 2005;

Robba et al., 2006). Red algal DNA barcoding is currently going through the stage of dataset creation. Molecular datasets of several recent studies of species boundaries in which species are represented by multiple specimens can be used for later querying. Several more such datasets can be expected to appear in the years to come. Examples of specimen identification against a sequence database are still scarce. Rueness (2005) showed newly collected *Gracilaria* specimens from Brittany and Sweden to belong to the invasive species *G. vermiculophylla* using their ITS, *rbc*L, and *cox*2-3 spacer sequences.

When used wisely, DNA barcoding could form an incredible asset to red algal systematics. As outlined above, red algal diversity cannot always be captured using morphological characters, especially in structurally simple genera. In such cases, it is difficult or impossible to figure out which cryptic species corresponds to the type of the morpho-species. One of the great advantages of DNA barcoding is that it can be used to accurately identify cryptic species' haplotype clusters. This is exemplified by the studies of Hughey et al. (2001, 2002), in which the type specimens of several species belonging to gigartinalean genera were sequenced.

Species-level studies most commonly utilize variable plastid (*rbc*L or *rbc*L-*rbc*S spacer) and mitochondrial (*cox*2-3 spacer) markers. Recently, the mitochondrial gene *cox*1, which is used to this purpose in metazoans, has been investigated as a DNA barcoding marker and has yielded encouraging results in several red algal genera (Saunders, 2005; Robba et al., 2006). As a general rule, a single, suitable marker allows recognition of sequence clusters representing species. Fully resolving species-level phylogenies sometimes requires additional markers.

THE FUTURE OF RED ALGAL SYSTEMATICS

In the near future, molecular tools will assist red algal systematics even more than is the case today. From the technical perspective, one can expect the development of additional phylogenetic markers, the possibility of carrying out molecular analyses at very democratic prices, continuous advances in phylogenetic analysis techniques, and easier access to computer systems capable of complex analyses. As a consequence, current gaps in the red algal tree of life can be expected to be filled in relatively rapidly. It can be hoped that these efforts will be accompanied by traditional, morphological taxonomic work, as such descriptive knowledge will aid accurate identification and pave the way toward more advanced research aiming to explain the observed patterns of evolutionary diversification from genetic, physiological, and ecological perspectives. In what follows, we will sketch some perspectives for future research. However, our overview is by no means comprehensive.

Molecular markers

The growing amount of genomic information will facilitate further development of markers for phylogenetics and several other evolutionary questions. Currently, four plastid, three mitochondrial, and two nuclear genomes are known (Table 6.3). Furthermore, the red algal nucleus-derived sequence of the cryptomonad *Guillardia theta* D.R.A. Hill et Wetherbee nucleomorph and three large EST libraries have been determined (Table 6.3); a genome sequencing project of the first florideophyte, *Chondrus crispus* Stackhouse, has started. In our opinion, there is no urgent need for new phylogenetic markers. The existing markers allow inference of relationships at many different taxonomic levels.

The development of high-resolution molecular markers and new analytical methods allows more complex questions to be posed about the influence of dispersal on micro- and macroevolution, as red algal evolutionary studies become more hypothesis driven and ask specific questions. Such questions that will require the development of custom-tailored markers include the following: What is the precise branching order between orders X, Y, and Z? How did this or that gene family evolve in the red algae? What is the contribution of hybrid speciation to red algal diversity? Have speciation events mainly been sympatric or allopatric? How extensive is gene flow among distant populations?

TABLE 6.3
Currently Known Genomic Information*

Class	Species	Type of Information	Size	Ref.
Florideophyceae	*Gracilaria tenuistipitata*	EST	3,000 seq.	P. Nyvall in GenBank
		Plastid genome	184 kb	Hagopian et al. (2004)
	Chondrus crispus	EST	4,056 seq.	Collén et al. (2006)
		Mitochondrial genome	26 kb	Leblanc et al. (1995)
Bangiophyceae	*Porphyra yezoensis*	EST	20,779 seq.	Nikaido et al. (2000) Asamizu et al. (2003)
	Porphyra purpurea	Plastid genome	191 kb	Reith and Munholland (1995)
		Mitochondrial genome	37 kb	Burger et al. (1999)
Bangiophyceae	*Cyanidioschyzon merolae*	Nuclear genome	16.5 Mb	Matsuzaki et al. (2004)
		Plastid genome	150 kb	Matsuzaki et al. (2004)
		Mitochondrial genome	32 kb	Matsuzaki et al. (2004)
	Galdieria sulphuraria	Nuclear genome	70% of ±11 Mb	Barbier et al. (2005)
(Cryptomonad)	*Guillardia theta*	Nucleomorph sequence	550 kb	Douglas et al. (2001)
		Plastid genome	121 kb	Douglas and Penny (1999)

*Classes as defined by Yoon et al. (2006).

What are the ecologically selective causes of speciation? Development of the hypervariable neutral markers (e.g. single nucleotide polymorphisms (SNPs), microsatellites, nuclear introns, and spacers) needed for this kind of research will be easier with genome sequences at hand.

Deep-level phylogenetics

Over the past 25 years, gigantic progress has been made in the delineation of red algal orders and classes. In the near future, we can expect a moderate further increase in the number of orders. The further subdivision of the Gigartinales, which remains heterogeneous, is advocated by some workers. Candidate families for recognition at the ordinal rank are Caulacanthaceae, Calosiphoniaceae, Dumontiaceae, Peyssonneliaceae, Sarcodiaceae, and Sphaerococcaceae. However, the rank at which clades are recognized is a matter of opinion, and it could be argued that a return to more inclusive orders is more practical.

Even though considerable progress has been made in establishing relationships among orders, in part thanks to molecular phylogenetics, many questions remain. In particular, the lack of confidence in nodes connecting classes and orders in molecular phylogenetic trees is troublesome (Withall and Saunders, 2006; Yoon et al., 2006). There is no silver bullet for this problem, if it is solvable at all. Confidence in phylogenetic trees depends on marker and alignment quality, appropriateness of the model used for phylogenetic reconstruction, and other factors.

The first prerequisite is that the chosen markers are suitable for resolving old divergences. Preferably, one would use DNA markers that evolve at relatively slow rates, because fast-evolving markers may show substitutional saturation at the desired taxonomic level, introducing noise into the dataset and reducing topological confidence. Of the currently used markers, 18S and 28S nuclear rDNA seem to be best suited for inference at high taxonomic levels, whereas protein-coding genes tend to deliver less resolution at deep nodes. Plastid 16S rDNA also seems to be a good candidate (Olson et al., 2005). Yoon et al. (2006) used an alignment consisting of seven plastid protein-coding genes, 16S plastid rDNA, and 18S nuclear rDNA, more than 10,000 bases in length, and were still

unable to resolve fully the relationships among red algal classes, illustrating how hard and costly obtaining satisfying resolution can be. Obviously, longer alignments increase the chances of being able to resolve the branching order among a given set of taxa, but the properties of the markers in the alignment are at least as important. As more genomes become available, phylogenomic approaches toward reconstructing the red algal tree of life will gain importance (Reyes-Prieto et al., 2006).

A second issue impacting confidence in trees and their branches is alignment quality. A disappointingly small fraction of molecular phylogenetic papers specify alignment procedures, treatment of gaps and alignment ambiguities, and quality of the resulting alignment. Plastid protein-coding markers can usually be readily aligned, but this is much less the case with ribosomal DNA, especially when one tries to align sequences of highly divergent lineages, as when the focus is on ordinal relationships. Ribosomal DNA sequence alignment should ideally be based on common secondary structure of the corresponding RNA molecules (Wuyts et al., 2004; www.psb.ugent.be/rRNA/). Irrespective of the marker(s) used, alignment quality and combinability of markers in multigene studies should be thoroughly examined.

The third prerequisite for obtaining highly robust phylogenetic trees is the use of appropriate methods for tree inference. Next to maximum parsimony analysis, most phylogenetic analyses are carried out in a likelihood framework, using either true likelihood approaches or Bayesian estimation. Such methods rely on models of base substitution (reviewed in Sullivan and Joyce, 2005). Hence, specification of a model appropriate for the type of data one is analysing is crucial to obtaining correct results.

In addition to those incorporated in Modeltest, specific models are available for protein-coding DNA sequences (Goldman and Yang, 1994; Shapiro et al., 2006), RNA sequences with secondary structure (Telford et al., 2005), and alignments in which rate variation across lineages is obvious (Galtier, 2001). The possibility of uncoupling models across partitions in composite alignments may also result in better model fit.

Clearly, molecular phylogenetic markers are not the only source of information that can be tapped to infer the branching order of the main red algal lineages. The emerging field of evolutionary genomics offers perspectives in this direction. In the near future, we can expect to gain information about gene order in organellar genomes and nuclear gene duplications and losses that can help to infer deep splits (green algal example: Pombert et al., 2005).

Species-level systematics

Although considerable effort has already been made to increase our knowledge of species-level systematics using molecular data (64 studies or 43% of all published studies in the last 15 years), massive amounts of work still need to be done. As more genera are screened using molecular techniques, new species and additional cryptic and pseudo-cryptic species will be discovered. One of the crucial challenges for a stable classification lies in the reconciliation of taxa recognized in sequence alignments with traditional, morphologically defined, species. Therefore, it will be necessary to integrate historic or type material into molecular systematics, as well as to continue morphological studies of living and recently collected material. Bearing this in mind, there is an urgent need for further development and perfection of ancient DNA techniques and their application to algal specimens.

The literature shows a trend toward post hoc morphological characterisation of species following their recognition using molecular data (e.g. Gurgel et al., 2003; De Clerck et al., 2005). Despite statistical analysis of morphological datasets acquired from sequenced specimens being a very powerful tool for post hoc species recognition, as exemplified by the green algal studies of Verbruggen et al. (2005a, 2005b), morphometric analysis is seldom used in concert with molecular tools in red algal systematics, with the notable exceptions of *Plocamium* (Yano et al., 2004) and *Caloglossa* (Kamiya et al., 2003). Such datasets ideally include qualitative and quantitative morphological data, and can also include ecological and physiological features that may aid species recognition (Yano et al., 2006).

Progress in techniques for databasing, querying, and evaluating DNA barcodes will facilitate data management for much of the research outlined above. DNA barcode databases will include and link to many kinds of information, including details on the morphology, geographical origin, and ecology of sequenced specimens, and provide all sorts of online tools to analyse these data (e.g. BOLD: Barcode of Life Data System; www.barcodinglife.org). Obviously, such databases and their analysis tools will be invaluable to future systematists. Hence, we are of the opinion that journals publishing integrative systematic research should investigate procedures for submission of sequence and morphological data to these digital museums of the future. Many present-day molecular markers (e.g. plastid genes, *cox*1, Rubisco, and *cox*2-3 spacers) are suitable for species-level phylogenetic inference and DNA barcoding of red algae (Verbruggen et al., unpublished). Considering that *rbc*L is the marker for which by far most sequences are available on GenBank and that it usually provides higher phylogenetic resolution at the genus to species level than spacer sequences and *cox*1, we advocate its use as the barcoding marker of choice.

Speciation research

A natural next step from descriptive systematic research is trying to find out how taxa came into being. Among other things, studying speciation involves identifying and measuring reproductive isolation and looking for causes of pre- and postzygotic isolation. It encompasses analysis of geographical, ecological, and phylogenetic data, gene flow, drift, and selection in populations. For example, hybrid speciation (when two species form a hybrid that is reproductively isolated from both its parent species: Coyne and Orr, 2004) is a major mechanism of land plant diversification but has hardly been studied in red algae. Although allopatric speciation—speciation of geographically subdivided populations—has traditionally been thought to be the predominant geographic speciation mode, sympatric speciation has been documented for various twigs of the eukaryotic tree of life (e.g. Barluenga et al., 2006; Savolainen et al., 2006).

The influence of dispersal on local- and regional-scale population genetic structure is a very topical subject because of its importance for understanding speciation mechanisms. Reproductive isolation can evolve as a consequence of divergent natural selection on traits between different environments, either in sympatry or allopatry (Schluter, 2001). Confidently assessing the speciation mechanisms that have led to red algal biodiversity will demand integration of experimental systematic, genomic, and molecular cell biological research. Progress has recently been made by the discovery and characterization of "rhodobindin" gamete recognition proteins in *Aglaothamnion* (Kim and Jo, 2005). Although it is too soon to say whether these proteins actually drive speciation or diverge as a by-product of speciation, with possible further divergence through pre-mating reinforcement, discoveries such as this will hopefully spark a wide range of red algal speciation studies.

Making predictions about which red algal taxa will be used as models for speciation studies is difficult. Model taxa should ideally meet the following criteria: undemanding in laboratory culture; known life history that can be readily completed; history of molecular and interfertility testing of species boundaries; accurate information about distribution ranges; a history of genetic, cell-biological, and ecophysiological studies; scope for genetic transformation; and the existence of genomic information. We cannot think of any marine red macrophyte that currently meets all these criteria. Nonetheless, a number of taxa come to mind because they meet at least part of some of the criteria. *Aglaothamnion*, *Bostrychia*, and *Ceramium* score highly as lab rats: their rapid life histories in culture mean that they can be easily crossed and manipulated. Furthermore, rhodobindin genes have been characterized for *Aglaothamnion*. Nuclear genomic information is available for a totally different range of taxa (*Gracilaria*, *Chondrus*, and *Porphyra*), which are much more difficult to manipulate in culture.

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