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DEFINING THE MAJOR LINEAGES OF RED ALGAE (RHODOPHYTA)[1]

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Previous phylogenetic studies of the Rhodophyta have provided a framework for understanding red algal phylogeny, but there still exists the need for a comprehensive analysis using a broad sampling of taxa and sufficient phylogenetic information to clearly define the major lineages. In this study, we determined 48 sequences of the PSI P700 chl *a* apoprotein A1 (*psa*A) and *rbc*L coding regions and established a robust red algal phylogeny to identify the major clades. The tree included most of the lineages of the Bangiophyceae (25 genera, 48 taxa). Seven well-supported lineages were identified with this analysis with the Cyanidiales having the earliest divergence and being distinct from the remaining taxa; i.e. the Porphyridiales 1–3, Bangiales, Florideophyceae, and Compsopogonales. We also analyzed data sets with fewer taxa but using seven proteins or the DNA sequence from nine genes to resolve inter-clade relationships. Based on all of these analyses, we propose that the Rhodophyta contains two new subphyla, the Cyanidiophytina with a single class, the Cyanidiophyceae, and the Rhodophytina with six classes, the Bangiophyceae, Compsopogonophyceae, Florideophyceae, Porphyridiophyceae *classis nov.* (which contains *Porphyridium, Flintiella*, and *Erythrolobus*), Rhodellophyceae, and Stylonematophyceae *classis nov.* (which contains *Stylonema, Bangiopsis, Chroodactylon, Chroothece, Purpureofilum, Rhodosorus, Rhodospora*, and *Rufusia*). We also describe a new order, Rhodellales, and a new family, Rhodellaceae (with *Rhodella, Dixoniella*, and *Glaucosphaera*).

Key index words: **Bangiophyceae; Compsopogonophyceae; Cyanidiophyceae; Florideophyceae; Porphyridiophycae; red algal lineages; Rhodellophyceae; Rhodophyta; Stylonematophyceae**

Abbreviations: **BPP, Bayesian posterior probabilities; ML, maximum likelihood; MP, maximum parsimony; PsaA, PSI P700 chlorophyll *a* apoprotein A1; PsaB, PSI P700 chlorophyll *a* apoprotein A2; PsbA, PSII reaction center protein D1; PsbC, PSII 44 KD apoprotein; PsbD, PSII D2 reaction center protein; TBR, tree bisection-reconnection**

The red algae (Rhodophyta) are a distinct eukaryotic lineage whose members are united in phylogenetic analyses of nuclear, plastid, and mitochondrial genes (Freshwater et al. 1994, Ragan et al. 1994, Van de Peer and De Wachter 1997, Burger et al. 1999, Yoon et al. 2002b, 2004). Rhodophytes lack chl *b* and *c* but contain allophycocyanin, phycocyanin, and phycoerythrin in the form of phycobilisomes on unstacked thylakoids. The plastid in these taxa is bound by two membranes and produces floridean starch that is deposited in the cytoplasm. All members of this group lack flagella and centrioles in all stages of the life history (Gabrielson et al. 1990, Graham and Wilcox 2000). It is believed that the red algal plastid originated from a cyanobacterial primary endosymbiosis and this organelle shares a common ancestry with green and glaucophyte algae

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(Bhattacharya and Medlin 1995, Delwiche et al. 1995, Cavalier-Smith 1998, McFadden 1999, Bhattacharya et al. 2004, Rodríguez-Ezpeleta et al. 2005). These three "primary" plastid-containing groups are considered to be taxonomically united in the kingdom Plantae (Cavalier-Smith 1998) or Archaeplastida (Adl et al. 2005).

Traditionally, the phylum Rhodophyta has been divided into two classes (or subclasses), Bangiophyceae (Bangiophycidae) and Florideophyceae (Florideophycidae) (Garbary and Gabrielson 1990). However, recent studies have concluded that the Florideophyceae form a monophyletic group with the order Bangiales (Oliveira and Bhattacharya 2000, Müller et al. 2001, Yoon et al. 2002b, Saunders and Hommersand 2004). The Bangiophyceae, which has in the past been divided into six orders (Bangiales, Cyanidiales, Compsopogonales, Erythropeltidales, Porphyridiales, and Rhodochaetales), is now considered to form a series of radiations that define the ancestral lineages of the red algae (Gabrielson et al. 1985, Freshwater et al. 1994, Ragan et al. 1994). Comprehensive phylogenetic studies of the Bangiophyceae (Oliveira and Bhattacharya 2000, Müller et al. 2001, 2003, West et al. 2005) using the plastid and nucleus-encoded small subunit (SSU) rDNA and plastid *rbc*L show furthermore that the Porphyridiales are paraphyletic and comprise at least three independent lineages. This result is generally consistent with previous morphological studies that show great differences in plastid ultrastructure and variable vegetative and reproductive anatomy (Gabrielson et al. 1985, 1990, Garbary and Gabrielson 1990, Müller et al. 2001). However, all of these previous studies are characterized either by broad taxon sampling with a single gene (Müller et al. 2001) or narrow sampling with multiple genes (Yoon et al. 2002b, 2004). For this reason, whereas the identity of the major red algal lineages has been relatively firmly established, their interrelationships remain unclear. Recently, Saunders and Hommersand (2004) proposed a new red algal taxonomic scheme based on previous molecular phylogenies and ultrastructural characters (e.g. Golgi–ER association). Their taxonomic system is a large step forward but still contains the paraphyletic class Rhodellophyceae that includes both unicellular and pseudofilamentous forms (i.e. Porphyridiales Kylin ex Skuja 1939, Stylonematales K. Drew 1956, and "Porphyridiales 1" *sensu* Müller et al. 2001).

In this study, we determined 48 sequences from the PSI P700 chl *a* apoprotein A1 (*psa*A) and *rbc*L coding regions with broad taxonomic sampling. We included most of the lineages of Bangiophyceae (25 genera, 48 taxa) in the analyses because these taxa represent the ancestral pool of red algae. In addition, we analyzed data sets with fewer taxa but using seven proteins or DNA sequence from nine genes to resolve inter-clade relationships. The combination of all of these phylogenetic studies was then used to advance our understanding of the higher-level taxonomic relationships within the red algae.

MATERIALS AND METHODS

Taxon sampling and sequencing. Forty-eight red algal taxa were used to infer the phylogeny of the Bangiophyceae (Table 1). The data set included all bangiophycean orders and 25 genera from the different phylogenetic lineages (Garbary and Gabrielson 1990, Müller et al. 2001). Our alignment also included 10 green algae, two glaucophytes, and three cyanobacteria as the outgroup (Bhattacharya and Medlin 1995, Moreira et al. 2000). We obtained algal cultures from the Culture Collection of Algae & Protozoa (CCAP), Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), the Dipartimento di Biologia Vegetale (DBV) culture collection at the University of Naples, the Sammlung von Algenkulturen (SAG) at the University of Göttingen, and the Culture Collection of Algae at the University of Texas at Austin (UTEX). Some of the bangiophytes were collected in the field and/or maintained in the private collection of F. D. Ott.

The algal cells were frozen in liquid nitrogen and ground with glass beads using a glass rod and/or Mini-BeadBeater™ (Biospec Products Inc., Bartlesville, OK, USA). Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Santa Clarita, CA, USA). PCR were carried out using specific primers for each plastid gene (Yoon et al. 2002a). Because introns were found in the *psa*A gene of some red algae, the RT-PCR method was used to isolate cDNA for these coding regions (H. S. Yoon et al. unpublished data). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and were used for direct sequencing using the BigDye™ Terminator Cycle Sequencing Kit (PE-Applied Biosystems, Norwalk, CT, USA), and an ABI-3700 at the Roy J. Carver Center for Comparative Genomics at the University of Iowa. Some PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) before sequencing.

Phylogenetic analyses. We primarily used amino acid sequences in the phylogenetic analysis in order to minimize potentially misleading phylogenetic signal because of DNA mutation bias at the third positions of codons (Sanderson et al. 2000, Pinto et al. 2003) or because of heterogeneous codon usage (Inagaki et al. 2004). The protein sequences were manually aligned using SeqPup (Gilbert 1995). Two data sets were used in the phylogenetic analyses and the alignments are available from D. Bhattacharya. In the first data set, we generated a concatenated alignment of seven plastid-encoded proteins (7PEP; a total of 2564 aa): PsaA (465 aa), PSI P700 chlorophyll *a* apoprotein A2 (PsaB, 422 aa), PSII reaction center protein D1 (PsbA, 319 aa), PSII 44 KD apoprotein (PsbC, 334 aa), PSII D2 reaction center protein (PsbD, 296 aa), RbcL (405 aa), and TufA (323 aa), from 16 bangiophytes, and from 15 outgroup taxa including green and glaucophyte algae and cyanobacteria. Because the *rbc*L gene of the green and glaucophyte algae are of a cyanobacterial origin, whereas those in the red algae and red algal-derived plastids are of proteobacterial origin (Valentin and Zetsche 1990, Delwiche and Palmer 1996), the evolutionarily distantly related green and glaucophyte *rbc*L sequences were coded as missing data in the phylogenetic analyses. In the second amino acid data set, we combined only PsaA and RbcL (2PEP), and added 33 more bangiophytes to the alignment (now a total of 49 bangiophytes). The Cyanidiales, which is the earliest diverging clade of red algae (Yoon et al. 2002b, 2004), was used as the outgroup for this data set. In addition to the amino acid data, we generated a DNA alignment that included nuclear SSU rDNA (18S; 1486 bp) and plastid SSU rDNA (16S; 1285 bp) sequences resulting in a nine-gene DNA sequence data set (9GDS; 10,463 bp) that excluded the green and glaucophyte algae. Three additional Cyanidiales species were included in the DNA alignment and used as the outgroup for this data set.

TABLE 1. Sample information and GenBank accession numbers for taxa included in the phylogenetic analyses.

Taxa	Species	Source	*rbc*L	*psa*A	*psa*B	*psb*A	*psb*C	*psb*D	*tuf*A	18S rDNA	16S rDNA
Rhodophyta, Bangiophyceae											
Bangiales	*Bangia atropurpurea* (Roth) C Agardh	SAG 33.94 FD Ott O458 (fresh-water)	AY119770	AY119698	AY391374	AY119734	AY876202	AY876227	AF545587	D88387	AF545616
	Bangia fuscopurpurea (Dillwyn) Lyngbye[a]	SAG 59.81 (marine)[a]	AY119771	AY119699							
	Bangia sp. (*maxima* form)	Bolinas, CA	**DQ308423**	**DQ308441**							
	Porphyra leucosticta Thuret	SAG 55.88	**DQ308424**	**DQ308442**							
	Porphyra purpurea (Roth) C. Agardh	GenBank	NC_000925	NC_000925	NC_000925	NC_000925	NC_000925	NC_000925	NC_000925	AF362362	NC_000925
Compsopogonales	*Boldia erythrosiphon* Herndon	Black River, Ontario, Canada	AF078121	Missing							
	Compsopogon coeruleus (Balbis ex C. Agardh) Montagne	SAG 36.94 FD Ott O798	AF087116	AY119701	AY391375	AY119737	AY876203	AY876228	AF545589	AF342748	AF1707139
	C. coeruleus (syn. =*C. hookeri*)	FD Ott O646	**DQ308425**	**DQ308443**							
	C. coeruleus (syn. =*C. oishii*)	FD Ott O1123	**DQ308426**	**DQ308444**							
	C. coeruleus (syn. =*Compsopogonopsis leptoclados*)	Alabama, USA	AF087115	**DQ308445**							
Erythropeltidales	*Erythrotrichia carnea* (Dillwyn) J. Agardh	UTEX LB 1425 FD Ott MO14	AF087118	AY119703							
	Erythrocladia irregularis Rosenvinge	UTEX LB 1419 FD Ott MO360	AF087117	**DQ308446**							
Rhodochaetiales	*Rhodochaete parvula* Thuret	UTEX LB 2715	AY119777	AY119707	AY391389	AY119743	Missing	Missing	AF545601	AF139462	AF545623
Cyanidiales	*Cyanidioschyzon merolae* Luca, Taddei et Varano	DBV 201 JAVA	AY119765	AY119693	AY391376	AY119729	NC_004799	NC_004799	AF545590	AF441376	AF545617
	Cyanidium caldarium (Tilden) Geitler	RK1 (Shimotsuke, Japan)	NC_001840	NC_001840	NC_001840	NC_001840	NC_001840	NC_001840	NC_001840	AB090833	NC_001840
	Cyanidium caldarium (Tilden) Geitler	DBV 019 SIPE	AY541297	AY541281							
	Cyanidium sp.	Monte Rotaro, Italy	AY391368	AY391362	AY391377	AY391365	Missing	Missing	AY391371	Missing	AY391359
	Cyanidium sp.	Sybil Cave, Naples, Italy	AY391369	AY391363	AY391378	AY391366	AY876204	AY876229	AY391372	Missing	AY391360
	Galdieria daedala Sentsova	IPPAS P508	AY541302	AY541283							
	Galdieria maxima Sentsova	IPPAS P507	AY391370	AY391364	AY391379	AY391367	AY876205	AY876230	AY391373	AB090832	AY391361
	Galdieria partita Sentsova	IPPAS P500	AB18008	AY541284							
	Galdieria sulphuraria (Galdieri) Merola	SAG 108.79	AY119767	AY119695	AY391380	AY119731	AY876206	AY876231	AF545591	AF342747	AF170718
	Galdieria sulphuraria (Galdieri) Merola	UTEX 2393	AF233069	AY541285							
	Galdieria sulphuraria-DBV	DBV 009 VTNE	AY119768	AY119696	AY391381	AY119732	AY876207	AY876232	AF545592	Missing	AF545618
	Galdieria sulphuraria-DBV	DBV 012 BNTE	AY541310	AY541288							
Porphyridiales											
P1	*Dixoniella grisea* (Geitler) Scott, Broadwater, Saunders, Thomas et Gabrielson	SAG 39.94, FD Ott O113	AY119773	AY119702	AY391383	AY119738	Missing	Missing	AF545595	L26187	AF545621
P1	*Glaucosphaera vacuolata* Korshikov	UTEX LB 1662	**DQ308427**	**DQ308447**							

TABLE 1 (Continued).

Taxa	Species	Source	*rbc*L	*psa*A	*psa*B	*psb*A	*psb*C	*psb*D	*tuf*A	18S rDNA	16S rDNA
P1	*Rhodella maculata* Evans	CCMP 736	**DQ308428**	**DQ308448**							
P1	*Rhodella violacea* (Kornmann) Wehrmeyer	SAG 115.79	AY119776	AY119706	AY391386	AY119742	**DQ308461**	**DQ308462**	AF545598	AF168624	AF545622
P2	*Bangiopsis subsimplex* (Montagne) Schmitz	PR21 (Puerto Rico)	AY119772	AY119700	AY391382	AY119736	Missing	Missing	AF545594	AF168627	AF545620
P2	*Chroodactylon ornatum* (C. Agardh) Basson (syn. =*C. ramosum)*	SAG 103.79, FD Ott MO447	**DQ308429**	**DQ308449**							
P2	*Chroothece mobilis* Pascher et Petrová	FD Ott O1363	**DQ308430**	**DQ308450**							
P2	*Kyliniella latvica* Skuja	FD Ott O506	**DQ308431**	**DQ308451**							
P2	*Purpureofilum apyrenoidigerum* West et Zuccarello	CCAP 1383/1	**DQ308432**	**DQ308452**							
P2	*Rhodospora sordida* Geitler	UTEX LB 2616 FD Ott O1515	**DQ308433**	**DQ308453**							
P2	*Rhodosorus marinus* Geitler	SAG 116.79 FD Ott MO360	AY119778	AY119708	Missing	AY119744	AY876208	AY876233	AF545595	AF342750	AF170719
P2	*Rhodosorus* sp.	CCMP 1530	**DQ308434**	**DQ308454**							
P2	*Rufusia pilicola* Wujek et Timpano	FD Ott O7031	**DQ308435**	**DQ308455**							
P2	*Stylonema alsidii* (Zanardini) Drew	SAG 2.94	AY119779	AY119709	AY391388	AY119745	Missing	Missing	AF545600	AF168633	AF170714
P2	*S. alsidii* (syn. =*Goniotrichum elegans*)	UTEX LB 1957	**DQ308436**	**DQ308456**							
P3	*Erythrolobus coxiae* Baca, Wolf et Cox	FD Ott O530	**DQ308437**	**DQ308457**							
P3	*E. coxae* (syn. =*E. fottii*)	UTEX LB 2545	**DQ308438**	**DQ308458**							
P3	*Flintiella sanguinaria* Ott	SAG 40.94 FD Ott O340	AY119774	AY119704	AY391384	AY119740	**DQ308463**	**DQ308464**	AF545596	AF342749	AF170719
P3	*Porphyridium aerugineum* Geitler	SAG 1380-2	AY119775	AY119705	AY391385	AY119741	AY876209	AY876234	AF545597	AF168623	X17597
P3	*Porphyridium purpureum* (Bory de Saint-Vincent) Ross in Drew et Ross (syn.= *P. cruentum*)	CCMP 1328	**DQ308439**	**DQ308459**							
P3	*Porphyridium sordidum* Geitler	FD Ott O250	**DQ308440**	**DQ308460**							
Rhodophyta, Florideophyceae											
	Chondrus crispus Stackhouse	Nova Scotia, Canada	U02984	AY119710	AY391390	AY119746	AY876210	AY876235	AF545602	Z14140	Z29521
	Palmaria palmata (L.) Kuntze	Maine, USA	U28421	AY119711	AY391391	U28165	AY876211	AY876236	AF545603	Z14142	Z18289
	Thorea violacea Bory de Saint-Vincent	SAG 51.94 FD Ott O282	AF029160	AY119712							
Viridiplantae	*Arabidopsis thaliana* (L.) Heynhold	GenBank	NA	NC_000932	NC_000932	NC_000932	NC_000934	NC_000935	X52256		
	Anthoceros formosae	GenBank	NA	NC_004543	NC_004543	NC_004543	NC_004543	NC_004543	NA		
	Chaetosphaeridium globosum (Nordstedt) Klebahn	GenBank	NA	NC_004115	NC_004115	NC_004115	NC_004115	NC_004115	NA		
	Chlamydomonas reinhardtii Dangeard	GenBank	NA	NC_005353	NC_005353	NC_005353	NC_005353	NC_005353	NC_005353		
	Chlorella vulgaris Beijerinck	GenBank	NA	NC_001865	NC_001865	NC_001865	NC_001865	NC_001865	NC_001865		
		GenBank	NA	NC_001319	NC_001319	NC_001319	NC_001319	NC_001319	NA		

TABLE 1 (Continued).

Taxa	Species	Source	*rbc*L	*psa*A	*psa*B	*psb*A	*psb*C	*psb*D	*tuf*A	18S rDNA	16S rDNA
	Marchantia polymorpha L.										
	Mesostigma viride Lauterborn	GenBank	NA	NC_002186	NC_002186	NC_002186	NC_002186	NC_002186	NC_002186		
	Pinus thunbergiana Franco	GenBank	NA	NC_001631	NC_001631	NC_001631	NC_001631	NC_001631	NA		
	Psilotum nudum (L.) Beauvois	GenBank	NA	NC_003386	NC_003386	NC_003386	NC_003386	NC_003386	NA		
	Zea mays L.	GenBank	NA	NC_001666	NC_001666	NC_001666	NC_001666	NC_001666	NA		
Glaucophyta	*Cyanophora paradoxa* Korshikov	GenBank	NA	NC_001675	NC_001675	NC_001675	NC_001675	NC_001675	NC_001675		
	Glaucocystis nostochinearum Itzigsohn	UTEX B 1929	NA	AY876195	AY876199	AY876201	AY876296	AY876252	Missing		
Cyanobacteria	*Nostoc* sp. PCC 7120	GenBank	NA	NC_003272	NC_003272	NC_003272	NC_003272	NC_003272	NC_003272		
	Synechocystis sp. PCC 6803	GenBank	NA	NC_000911	NC_000911	NC_000911	NC_000911	NC_000911	NC_000911		
	Thermosynechococcus elongatus BP-1	GenBank	NA	NC_004113	NC_004113	NC_004113	NC_004113	NC_004113	NC_004113		

P1, porphyridiales-(1); P2, porphyridiales-(2); P3, porphyridiales-(3).
The accession numbers of sequences determined in this study are shown in bold text.
[a]Müller et al. (2003).

All protein phylogenies were reconstructed under maximum likelihood (ML) using proml in the PHYLIP V3.6b program package (Felsenstein 2002). The trees were inferred with the JTT + Γ evolutionary model and global rearrangements with four random addition replicates (Jones et al. 1992). The α values for the γ distribution for the different data sets were calculated using TREE-PUZZLE V5.2 (Schmidt et al. 2002). To assess the stability of monophyletic groups in the ML trees, we calculated bootstrap support values using PHYML V2.4.3 (Guindon and Gascuel 2003) and unweighted maximum parsimony (MP) using PAUP*V4.0b10 (Swofford 2004). We also calculated Bayesian posterior probabilities (BPP) using MrBayes (V3.0b4, Huelsenbeck and Ronquist 2001). In the Bayesian inference of the amino acid data, we used the WAG + Γ model with Metropolis-coupled Markov chain Monte Carlo from a random starting tree. These analyses were run for 1,000,000 generations with trees sampled each 200 cycles. Four chains were run simultaneously of which three were heated and one was cold, with the initial 20,000 cycles (200 trees) being discarded as the "burn in." A consensus tree was made with the remaining 1800 phylogenies to determine the posterior probabilities at the different nodes. In the MP analyses, 1000 bootstrap replicates were analyzed (Felsenstein 1985) with 10 heuristic searches with random-addition-sequence starting trees and tree bisection-reconnection (TBR) branch rearrangements. For the ML bootstrap analysis (500 replicates), we used the JTT + Γ evolutionary model.

For the DNA data set, we used ML and BPP approaches. We used a site-specific GTR model to incorporate different rates in the genes at the three codon positions. This approach appears to be superior to using a single set of rate parameters for protein-coding DNA sequences (Shapiro et al. 2005). Five different rates were used to account for among-site rate variation (0.50931, 18S; 0.43367, 16S; 0.60518, 1st codon; 0.16149, 2nd codon; 2.80154, 3rd codon). For the ML analysis, global rearrangements and random sequence addition replicates (five rounds) were used with TBR branch swapping. The unweighted MP analysis was performed as described above.

RESULTS AND DISCUSSION

The seven major lineages of red algae. Seven well-supported lineages were identified within the red algae (Figs. 1 and 2). The Cyanidiales was the earliest divergence in the plastid protein tree and was distinct from the remaining red algal lineages (i.e. the Porphyridiales-(1), Porphyridiales-(2), Porphyridiales-(3), Bangiales, Florideophyceae, and Compsopogonales). The Porphyridiales-(2) and the Compsopogonales were united in a clade (> 95% BPP, 55% MP; Fig. 2), whereas the Porphyridiales-(1) grouped together with the monophyletic clade of Bangiales + Florideophyceae (> 95% BPP, 76% ML; Fig. 1). The existence of these seven red algal lineages in the plastid trees is consistent with analyses of nuclear SSU rDNA (Müller et al. 2001), plastid SSU rDNA (Oliveira and Bhattacharya 2000), and multi-gene plastid data set (Yoon et al. 2002b, 2004).

Although we used an extensive concatenated data set of 7PEP (2564 aa) in our analyses, the lineage relationships were weakly supported except for the early divergence point of the Cyanidiales and the Bangiales + Florideophyceae sister group relationship. We then added 18S and 16S rDNA sequences to the seven-

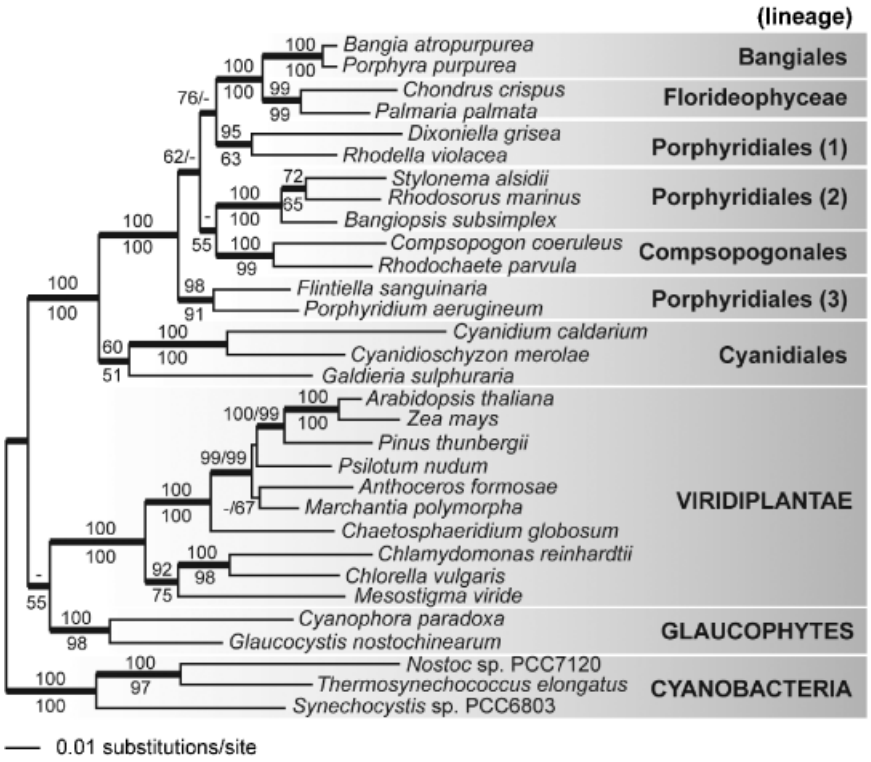

FIG. 1. Bangiophycean red algal phylogeny inferred using the maximum likelihood (ML) method and the combined plastid protein sequences of PSI P700 chlorophyll *a* apoprotein A1, PSI P700 chlorophyll *a* apoprotein A2, PSII reaction center protein D1, PSII 44 KD apoprotein, PSII D2 reaction center protein, RbcL, and TufA. The results of a ML bootstrap analysis are shown above the branches, whereas the values below the branches result from a maximum parsimony bootstrap analysis. The thick branches represent >95% Bayesian posterior probability.

gene DNA sequence data set (9GDS; total 10,463 bp) in an attempt to increase the phylogenetic resolution. Three additional Cyanidiales species were added to test the interrelationship of these taxa and this clade was used to root the tree. The ML phylogeny tree inferred from these data using the site-specific GTR model was similar to the 7PEP tree, except for the position of the Porphyridiales-(3) lineage (Fig. 2). Similar to the 7PEP phylogeny, the Compsopogonales and Porphyridiales-(2) grouped together (55% ML) and the Porphyridiales-(1) showed a weak sister group relationship (>95% BPP, 53% ML) to the Bangiales and Florideophyceae. Our analyses highlight the inherent difficulties in resolving the intra-lineage relationships of the red algae (except for the position of the Cyanidiales and the monophyly of the Bangiales + Florideophyceae). This may reflect a rapid radiation of the red algal lineages or poor resolving power of plastid sequences.

There are two important fossils to interpret the evolutionary history of the red algae. One is the well-preserved *Bangia*-like multicellular filamentous fossil, *Bangiomorpha pubescens* (Butterfield 2000). This fossil was found from the approximately 1200 million year old (Ma) Hunting Formation in Somerset Island, Canada, and contains putative spores that indicate sexual differentiation. The second fossil is of Corallinales (florideophytes) that contain typical reproductive structures from the 599 Ma Doushantuo Formation, China (Xiao et al. 1998, 2004). Xiao et al. (2004) suggested the florideophyte—bangiophyte divergence to have occurred in the Neoproterozoic or earlier. Based on these fossils and our phylogeny, we postulate that after the divergence of the Cyanidiales, the five major red algal lineages radiated around 1200 Ma, likely over a relatively short evolutionary time period. Thereafter,

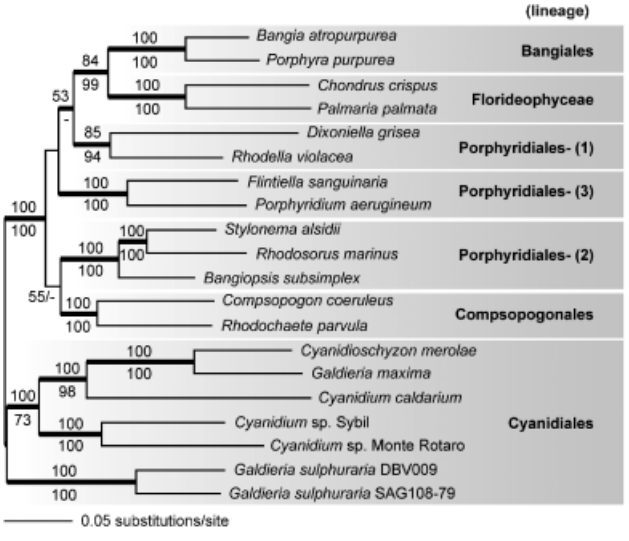

FIG. 2. Bangiophycean red algal phylogeny inferred using the maximum likelihood (ML) method and the combined DNA sequences of 18S rDNA, 16S rDNA, PSI P700 chlorophyll *a* apoprotein A1, PSI P700 chlorophyll *a* apoprotein A2, PSII reaction center protein D1, PSII 44 KD apoprotein, PSII D2 reaction center protein, *rbc*L, and *tuf*A. The results of a ML bootstrap analysis are shown above the branches, whereas the values below the branches result from a maximum parsimony bootstrap analysis. The thick branches represent >95% Bayesian posterior probability.

the Florideophyceae diverged from the ancestor of the Bangiales before 599 Ma when the coralline algae diverged from the stem of florideophytes. Yoon et al. (2004) estimated divergence times using molecular clock methods and proposed the following dates for the split of the red algae (1449–1513 Ma), Cyanidiales (1350–1416 Ma), and florideophytes (around 800 Ma, see Fig. 3 in Yoon et al. 2004).

Novel findings of our study. Porphyridiales-(2): Porphyridiales-(2) is comprised of mostly pseudofilamentous, filamentous, or colonial multicellular taxa (*Bangiopsis, Chroodactylon, Chroothece, Kyliniella, Purpureofilum*, and *Stylonema*), but also includes unicellular forms such as *Rhodosorus, Rhodospora*, and *Rufusia* (Fig. 3). Most members of this lineage contain sorbitol/digeneaside or only sorbitol as a low molecular weight carbohydrate (Karsten et al. 2003, West et al. 2005). In addition all species of Porphyridiales-(2) contain putative group II introns in the *psa*A gene at conserved positions (H. S. Yoon et al. unpublished data). These ribozymes are unique and are potentially a diagnostic character for this lineage. Group II introns are common in the green algae and in green algal-derived plastids [e.g. *Euglena* and *Chlamydomonas* (Odom et al. 2004, Perron et al. 2004, Sheveleva and Hallick 2004)] but apparently are rare in the red algae. Our preliminary phylogenetic analyses of the Pophyridiales-(2) group II introns show that sequences at the homologous genic site 229 in *psa*A differ significantly in sequence from other introns present in *Flintiella* [position 219, Porphyridiales-(3)] and *Rhodella* [position 91, Porphyridiales-(1)]. It is likely that the introns at position 229 share a single origin (H. S. Yoon et al. unpublished data). This finding suggests that these mobile elements invaded the *psa*A gene (and potentially other sequences) in the plastid genome of the common ancestor of Porphyridiales-(2) and have been maintained in some taxa.

Rufusia pilicola diverges first in the Porphyridiales-(2). This poorly studied species was isolated from sloth's hair by one of the authors (F. D. Ott). The material came from the coastal city of Limón, Costa Rica (provided by Dr. Arroyo). The other species of Porphyridiales-(2) occur in a variety of habitats, including marine, freshwater, and in caves. *Bangiopsis* and *Kyliniella* make a well-supported monophyletic group with *Purpureofilum* that is sister to two *Stylonema* strains. The SAG stain of *S. alsidii* formed a well supported a clade with the UTEX strain. The *S. alsidii* UTEX strain had been recognized as *Goniotrichum elegans* (Chauvin) V. May 1965, whereas the SAG strain was renamed from *Goniotrichum alsidii*. Because these two *S. alsidii* strains show a high sequence divergence (79 substitutions among 2610 bp) in the *psa*A and *rbc*L genes, a more detailed monographic study of *Stylonema*, including examination of type specimens is required to verify the species delimitation.

Porphyridiales-(3): The Porphyridiales-(3) lineage includes the three unicellular genera *Erythrolobus*, *Flintiella*, and *Porphyridium*. The three species of

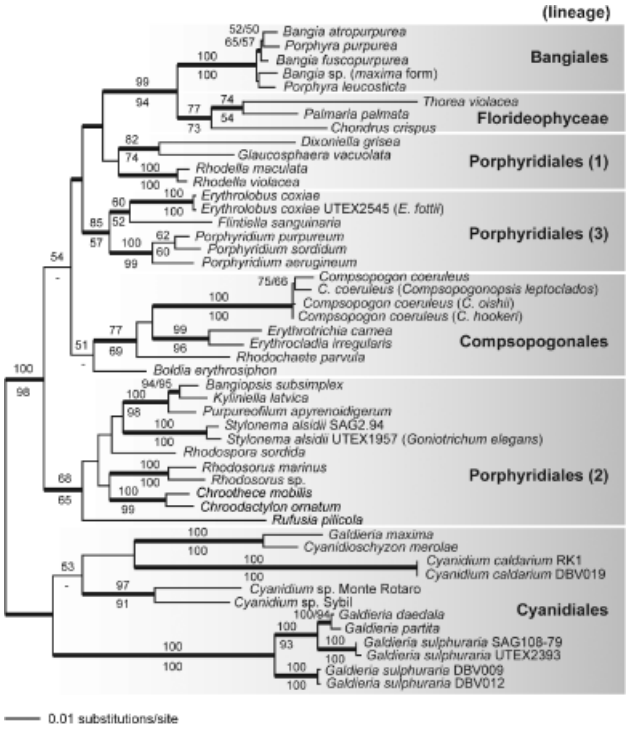

FIG. 3. Bangiophycean red algal phylogeny inferred using the maximum likelihood (ML) method and the combined plastid protein sequences of PSI P700 chlorophyll *a* apoprotein A1 and RbcL. The results of a ML bootstrap analysis are shown above the branches, whereas the values below the branches result from a maximum parsimony bootstrap analysis. The thick branches represent >95% Bayesian posterior probability.

Porphyridium are phylogenetically distinct from the *Flintiella* and *Erythrolobus* clade. It was previously reported that *Flintiella* and *Porphyridium* share the association of the golgi with the mitochondria and ER (Scott et al. 1992) and floridoside as a low molecular weight carbohydrate (Karsten et al. 2003).

Compsopogonales: This lineage consists of one freshwater order Compsopogonales and two marine orders Erythropeltidales and Rhodochaetales. *Sahlingia*, *Chlidophyllon*, and *Pyrophyllon* are included in this lineages (Kornmann 1989, Nelson et al. 2003). All of these species are multicellular, though with varied morphologies. Although our two-gene phylogeny (Fig. 3) did not support the monophyly of *Compsopogon* spp. with *Boldia erythrosiphon* (as would be expected; Rintoul et al. 1999, Müller et al. 2001), the nuclear SSU rDNA and the plastid multi-gene analyses show a sister group relationship of the Erythropeltidales and Rhodochaetales (Zuccarello et al. 2000, Müller et al. 2001, Yoon et al. 2002b) with the Compsopogonales and *B. erythrosiphon*. We were, however unable to isolate the *psa*A coding region from *B. erythrosiphon* and only used the *rbc*L sequence in this analysis. Rintoul et al. (1999) suggested that *Composopogon coeruleus* and *Compsopogoniopsis leptocladus* should be recognized as the single species,

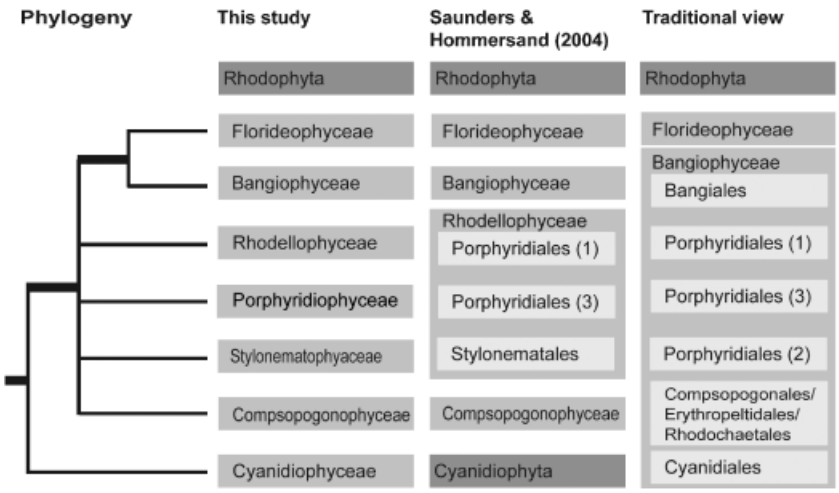

FIG. 4. Red algal phylogeny and alternative taxonomic schemes.

C. coeruleus, based on the identity in the sequence of SSU rDNA and *rbc*L.

Taxonomic conclusions. A classification system for a group of organisms should reflect its phylogeny. A reliable phylogeny ideally uses both broad taxon sampling and sufficient phylogenetic information. This study used analyses of seven- and nine-gene data sets with selected taxa, and a two-gene data set that encompassed most of the bangiophycean genera. Although many clade interrelationships are still unresolved, we clearly identified seven red algal lineages that should be recognized at the class-level. The Cyanidiophyceae diverged first in red algal evolution and is separated from the remainder of the red algal lineages. Hence, we propose the creation of two new subphyla, Cyanidiophytina, for this grouping, and Rhodophytina that encompasses the rest of the classes. Saunders and Hommersand (2004) treated the Cyanidiophyceae as the phylum Cyanidiophyta with another phylum Rhodophyta under the subkingdom Rhodoplantae. However, we believe that it is not necessary to divide the red algae into two phyla because this group, including the cyanidiophytes, is phylogenetically distinct in eukaryotic trees (Rodríguez-Ezpeleta et al. 2005) and they share important biochemical features with other red algae such as the biosynthesis of floridean starch in the cytoplasm (Barbier et al. 2005). In addition, all rhodophytes share an important synapomorphy, a plastid bound by two membranes that lacks chl *b* or *c*. Rather, our data indicate that it is appropriate to divide the rhodophytes into two subphyla, maintaining the traditional characteristics of the phylum as a whole (Fig. 4). Under this taxonomic scheme the subphylum Rhodophytina contains six classes: Bangiophyceae, Compsopogonophyceae, Florideophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae. The interrelationships among the classes of the subphylum Rhodophytina are unclear except for the monophyly of the Bangiophyceae and the Florideophyceae. We did not find any phylogenetic evidence to unite the Porphyridiales into a single clade (as suggested by Saunders and Hommersand 2004). This (negative) evidence does not disprove Porphyridiales monophyly. However, it suggests that if all three or a subset of the lineages share a monophyletic origin, the branch that unites them may prove difficult to establish unless a great deal more (or with higher phylogenetic resolution) sequence data is brought to bear on this issue. Our trees do, however, demonstrate an ancient split of taxa formerly classified in the Porphyridiales. For these reasons, we chose to recognize this group as three independent clades within the Rhodophytina.

Phylum Rhodophyta Wettstein 1901

Subphylum 1: Cyanidiophytina H. S. Yoon, K. M. Müller, R. G. Sheath, F. D. Ott et D. Bhattacharya, subphylum nov.

Rhodophyta unicellularis, forma globosa vel elliptica; habitatio thermae acidae; cum paries crassus vel paries absens; heterotrophicus vel autotophicus; cellulae divisionem vel endospora.

Unicellular red algae, spherical or elliptical in shape, inhabiting in acidic and high temperature environment; thick cell wall or lack of cell wall; facultative heterotrophs or obligate photoautotrophs; cell division or endospore formation.

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

Order Cyanidiales Christensen 1962
Family 1 Cyanidiaceae Geitler 1935
Genera *Cyanidium, Cyanidioschyzon*
Family 2 Galdieriaceae Merola, Castaldo, De Luca, Gambardella, Musacchio et Taddei 1981
Genus *Galdieria*

Subphylum 2: Rhodophytina

Unicellular, pseudofilamentous or multicellular red algae; various plastid morphologies and organellar associations; life histories unknown or where known biphasic or triphasic.

Class 1: Bangiophyceae Wettstein 1901

Order Bangiales Nägeli 1847
Family Bangiaceae Engler 1892
Genera *Bangia*, *Dione*, *Minerva*, *Porphyra*, *Pseudobangia*

Class 2: Compsopogonophyceae G. W. Saunders et Hommersand 2004

Order 1 Compsopogonales Skuja 1939
Family 1 Boldiaceae Herndon 1964
Genus *Boldia*
Family 2 Compsopogonaceae Schmitz in Engler et Prantl 1896
Genus *Compsopogon*
Order 2 Erythropeltidales Garbary, Hansen et Scagel 1980
Family Erythrotrichiaceae G. M. Smith 1933
Genera *Erythrotrichia, Chlidophyllon, Erythrocladia, Pyrophyllon, Sahlingia*
Order 3 Rhodochaetales Bessey 1907
Family Rhodochaetaceae Schmitz in Engler et Prantl 1896
Genus *Rhodochaete*

Class 3: Florideophyceae Cronquist 1960
Multiple orders.

Class 4: Porphyridiophyceae H. S. Yoon, K. M. Müller, R. G. Sheath, F. D. Ott et D. Bhattacharya, class nov.

Rhodophyta unicellularis; chloroplasti singularis et ramosi vel stellaris cum pyrenoides; Golgi cum reticulo endoplasmatico et mitochondrion consociatus; cellulae cum floridoside; reproductio a divisio cellulosa.
Unicellular red algae with a single branched or stellate plastid with or without pyrenoid; Golgi association with mitochondria and ER; cells with floridoside as a low molecular weight carbohydrate; reproduction by cell division.
Order Porphyridiales Kylin ex Skuja 1939
Family Porphyridiaceae Skuja 1939
Genera *Porphyridium*, *Erythrolobus*, *Flintiella*

Class 5: Rhodellophyceae Cavalier-Smith 1998

Order Rhodellales ord. nov. H. S. Yoon, K. M. Müller, R. G. Sheath, F. D. Ott et D. Bhattacharya, order nov.
Rhodophyta unicelluaris; chloroplasti singularis et lobati cum pyrenoides in centro vel a centro; Golgi cum reticulo endoplasmatico et nucleus consociatus; cum mannitol; reproductio a divisio cellulosa.
Unicellular red algae; a single highly lobed plastid with eccentric or centric pyrenoid, Golgi association with nucleus and ER; contains mannitol; reproduction by cell division.
Family Rhodellaceae fam. nov. H. S. Yoon, K. M. Müller, R. G. Sheath, F. D. Ott et D. Bhattacharya, fam. nov.
Characters as for order
Genera *Rhodella, Dixoniella, Glaucosphaera*

Class 6: Stylonematophyceae H.S. Yoon, K.M. Müller, R.G. Sheath, F.D. Ott et D. Bhattacharya, classis nov.

Rhodophyta unicelluaris, pseudofilamentosis vel filamentosis; chloroplasti cum morphologiae variabilis et cum vel sine pyrenoides; Golgi cum reticulo endoplasmatico et mitochondrion consociatus; reproductio a division cellulosa vel monospora.
Unicellular or pseudofilamentous or filamentous red algae; various plastid morphologies with or without pyrenoid; Golgi association with mitochondria and ER; reproduction by cell division or monospores.
Order Stylonematales Drew 1956
Family Stylonemataceae Drew 1956
Genera *Stylonema*, *Bangiopsis*, *Chroodactylon*, *Chroothece*, *Purpureofilum*, *Rhodosorus*, *Rhodospora*, *Rufusia*

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