A *psbA* PHYLOGENY FOR SELECTED RHODOPHYCEAE

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ABSTRACT

Oligonucleotide primers for PCR amplifying and sequencing the plastid-encoded *psbA* gene, which encodes the D1 protein of photosystem II, were developed for red algae. Using these primers *psbA* sequences were determined for 18 different species representing 10 phylogenetically diverse red algal orders. The third codon position of the *psbA* gene is too variable (= homoplasious, mutationally saturated) to be used to estimate phylogenetic relationships among the orders investigated here, whereas amino acid sequences are too conserved. In contrast, analyses including only first and second codon positions yielded trees for red algae that are, with exceptions, congruent with those obtained for other genes. Comparisons of sequence divergence values and phylogenetic measures imply that *psbA* sequences are best suited for use at the rank of family and below.

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INTRODUCTION

The Phylum Rhodophyta (red algae) traditionally has included a single class (Rhodophyceae) and two subclasses, the Bangiophycidae and Florideophycidae (Saunders and Hommersand, 2004). Of these two subclasses the Florideophycidae is considered most advanced. Previous phylogenetic studies based upon analyses of nuclear encoded 18S rRNA genes indicate that orders belonging to the subclass Florideophycidae are divided between two large sister clades (Saunders and Bailey, 1997). One includes 10 orders and is sometimes colloquially referred to as the "two cap-layer clade" because species within each included order possess pit connections with both inner- and outer cap layers (Pueschel, 1990). The second clade of Florideophycidae includes all other orders of this subclass; these species possess pit plugs having only one cap layer (Saunders and Hommersand, 2004).

The division of the Florideophycidae into two clades that may be diagnosed on the basis of pit plug morphology is also supported by a second nuclear marker (26S rRNA) (Harper & Saunders, 2001) as well as plastid-encoded gene sequences for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) (Freshwater et al., 1994; Yoon et al., 2006). The deep divergence between the two clades is now considered a hallmark of florideophyte evolution.

Despite such fundamental progress made over the last decade, the systematics of red algal taxa remains nebulous – especially at rank of genus and above (Saunders & Hommersand, 2004). Most phylogenetic studies of red algae are based upon data obtained from three genes (Olsen et al., 2004). These include DNA sequences for genes encoding the small- (18S) (Bailey et al., 2004; Bailey, 1999; Bailey & Chapman, 1998; Bailey & Freshwater, 1997; Ragan et al., 1994) and large-subunits (26S) (Huisman et al., 2004; Freshwater et al., 1999) of the eukaryotic

ribosome as well as plastid-encoded *rbcL* sequences (Yoon et al., 2006; Bailey & Freshwater, 1997; Freshwater et al., 1994). Although several taxa have been extensively studied, including the Corallinales (Bailey et al., 2004; Bailey, 1999; Bailey & Chapman, 1998), Gelidiales (Freshwater et al., 1999; Patwary et al., 1998; Bailey & Freshwater, 1997), and Gracilariales (Iyer et al., 2004; Gurgel et al., 2003; Bird et. al., 1994), most others have not. Also, relationships among orders within the two- and single-cap layer clades remain the subject of considerable debate (cf. Saunders and Hommersand, 2004; Yoon et al., 2006).

Critical analyses of the nuclear DNA markers (18S and 26S rRNA genes) previously used to investigate red algal evolution reveal a simple but significant problem. Both genes are supposedly conserved via the processes of concerted evolution and their gene products interact with one another; they therefore violate the assumption of independent evolution. One might, therefore, expect 18S and 26S rRNA phylogenies to be congruent or, at the very least, resemble or 'mirror' one another. If one accepts this argument, then it must be conceded that red algal phylogeny is based only on two independently evolving DNA regions; nuclear ribosomal and plastid *rbcL*.

The objective of this investigation was to develop a second protein-encoding plastid gene useful for estimating red algal phylogenies. The *psbA* gene was selected for study and encodes the D1 chlorophyll-binding protein localized to eukaryotic photosystem II (Yang et al., 2004; Zeidner et al., 2003; Trebitsh and Danon, 2001; Bouyoub et al., 1993). Although the gene has been extensively investigated in some other photoautrophs (Kim et al., 2001; Morden and Golden, 1989) (e.g., green plants, in which the protein is a common target of herbicides) (Bouyoub et al., 1993; Ohad and Hirschberg, 1992), its phylogenetic utility in the Rhodophyta is largely unexplored. Because red algae probably originated before extant green algae and long

before extant embryophytes (Saunders and Hommersand, 2004), inferred rates of substitution between these taxa are expected to differ. I have investigated *psbA* sequences among a phylogenetically diverse assemblage of red algal taxa. In order to 'ground truth' my *psbA* investigation I have compared *psbA*-derived phylogenies to trees derived from other genes (Vis et al., 2005; Olsen et al., 2004; Saunders and Bailey, 1997; Saunders and Kraft, 1996; Freshwater et al., 1995). Specifically, I evaluate the use of *psb*A gene sequences for determining "deep divergence" within the Rhodophyta using the pit plug/DNA-derived hypothesis for two separate clades within the Florideophycidae. I also re-investigate relationships among orders placed in the two cap-layer clade.

METHODS

DNA Extraction, PCR Amplification, and Sequencing

Red algal species examined in this study are listed in Table 1. Total cellular DNA for most species was kindly provided by colleagues; otherwise, DNA was extracted as described in Bailey et al. (1998) except that cells were disrupted in buffer using a Mini-Beadbeater™ (Bio Spec Products, Bartlesville, OK). Partial *psbA* sequences were PCR amplified using forward primer *psb*A-1 (5'-ATGACTGCTACTTTAGAAAG-3') in combination with one of two reverse primers, *psb*A-RS (5'-AGAAGCTAAATCTAG-3') or *psb*A-RL (5'-ACCYAAGAAGAAATGTAATG-3'). These primers were designed from an alignment of *psb*A sequences obtained from completely sequenced red algal

Table 1. Species examined in this study and GenBank accession numbers for their *psbA* sequences.

Species	**GenBank Accession Number**
Ahnfeltia fastigiata	TBD
Batrachospermum macrosporum 01	TBD
Batrachospermum macrosporum 04	TBD
Chondrus crispus	TBD
Compospogon coeruleus	TBD
Cumagloia andersonii	TBD
Neosiphonia harveyi	TBD
Palmaria palmata	TBD
Paralemania annulata	TBD
Plocamium cartilaginum	TBD
Polysiphonia fucoids	TBD
Polysiphonia scopulorum	TBD
Polysiphonia sp.	TBD
Polysiphonia subtilissima	TBD
Rhodophysema georgii	TBD
Sirodotia suecica	TBD
Titanoderma pustulatum	TBD
Thorea violacea	TBD
Tuomeya Americana	TBD
Cryptomonas sp.	M1094
Glaucocystis nostochinearum	AY876201

chloroplast genomes available from GenBank. The two reverse primers were used due to the presence of an expansion segment in the *psbA* gene of some red algae. The approximate length and yield of amplification products was determined on 0.8% ethidium bromide-stained agarose gels and PCR products were subsequently cleaned for sequencing according to the manufacturer's specifications for the GeneClean II Kit (Qbiogene, Carlsbad, CA). Cleaned PCR products were sequenced using BigDye cycle sequencing chemistries (v. 3.1, Applied Biosystems, Foster City, CA) and the amplification primers given above. Sequences were determined on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and edited and assembled using Sequencher (GeneCodes Corporation, Ann Arbor, MI).

Phylogenetic Analyses

The 19 *psbA* sequences determined in this study were aligned using CLUSTALX and the alignment was subsequently edited by eye. Homologous sequences for *Cryptomonas sp.* (M1094: Cryptophyceae) and *Glaucocystis nostochinearum* (AY876201: Glaucocystophyta) were selected to root the trees and were simultaneously aligned with the red algal sequences as described above. The data were analyzed as 1) DNA sequences with all codon positions included, 2) DNA sequences with third codon base positions removed, and 3) translated amino acid sequences.

All phylogenetic analyses were performed using PAUP software (Swofford, 2002). Parsimony and maximum likelihood (ML) analyses of the three data sets were conducted using the TBR branch-swapping algorithm and replicated 100 times with random orders of sequence addition. Parsimony bootstrap values (BS; Felsenstein, 1985) were calculated on the basis of 10,000 replicates using the "fast step-wise" addition option, whereas ML bootstrap values were based upon analyses of 100 replicates.

RESULTS

The red algal *psbA* DNA sequence matrix included 19 ingroup taxa; GenBank accession numbers for these sequences are provided in Table 1.

A plot of corrected vs. uncorrected pairwise distance estimates between all ingroup taxa revealed that, among these taxa, the third codon position of the *psbA* gene is mutationally saturated (Fig. 1), and cladistic analyses of the *psb*A matrix including all codon positions yielded trees in which species were placed in highly unorthodox positions. On the other hand, analyses of translated amino acid sequences yielded trees with no resolution. For these reasons the third codon position was excluded in subsequent analyses and the amino acid alignment was not investigated further.

The alignment of first and second codon positions included 638 bases (characters) of which 229 (36%) were parsimony informative. Parsimony analysis of these data yielded two equal length trees of 865 steps (CI=0.47, RI=0.48) the strict consensus of which is shown in Figure 2. The tree yielded by maximum likelihood analysis is shown in Figure 3.

The parsimony and ML trees are, for the purposes of this study, consistent with one another. In Figures 2 and 3, species within orders placed in the two cap-layer clade are depicted using a bold font. In both trees *Thorea violacea* (Thoreales) and *Cumagloia andersonii* (Nemaliales) are positioned outside of a clade that includes all other members of the two cap-layer lineage examined in this study. Both trees also place

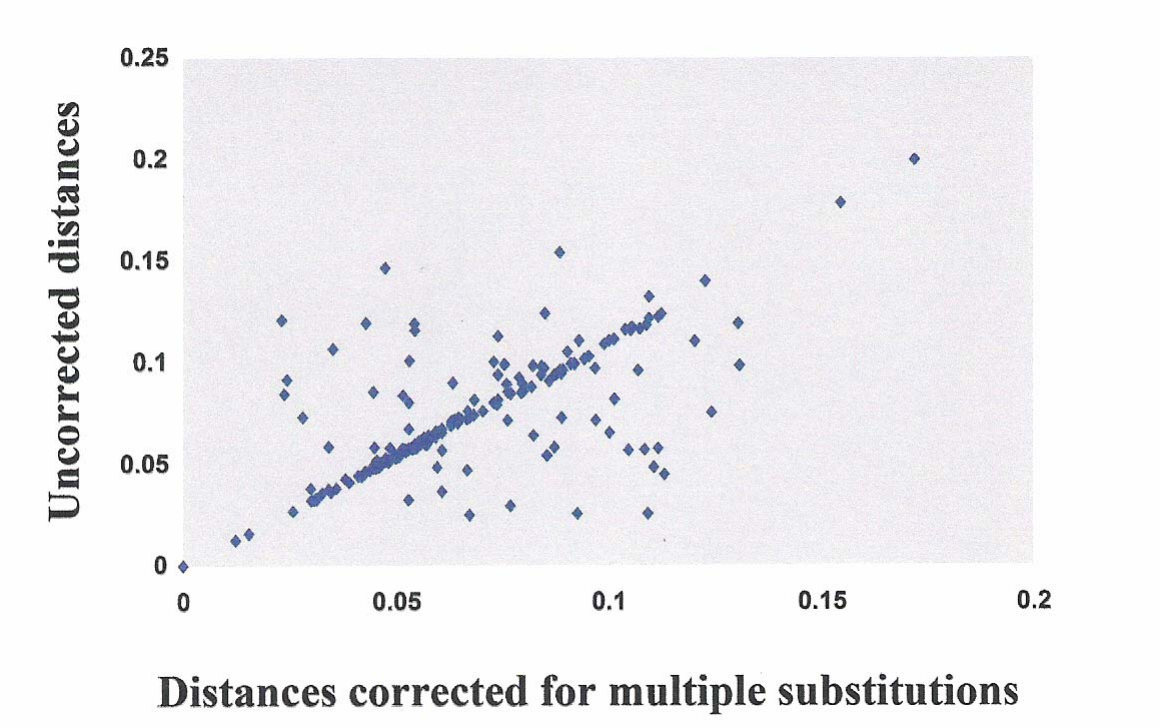

Figure 1. Graph showing mutational saturation at the third codon position.

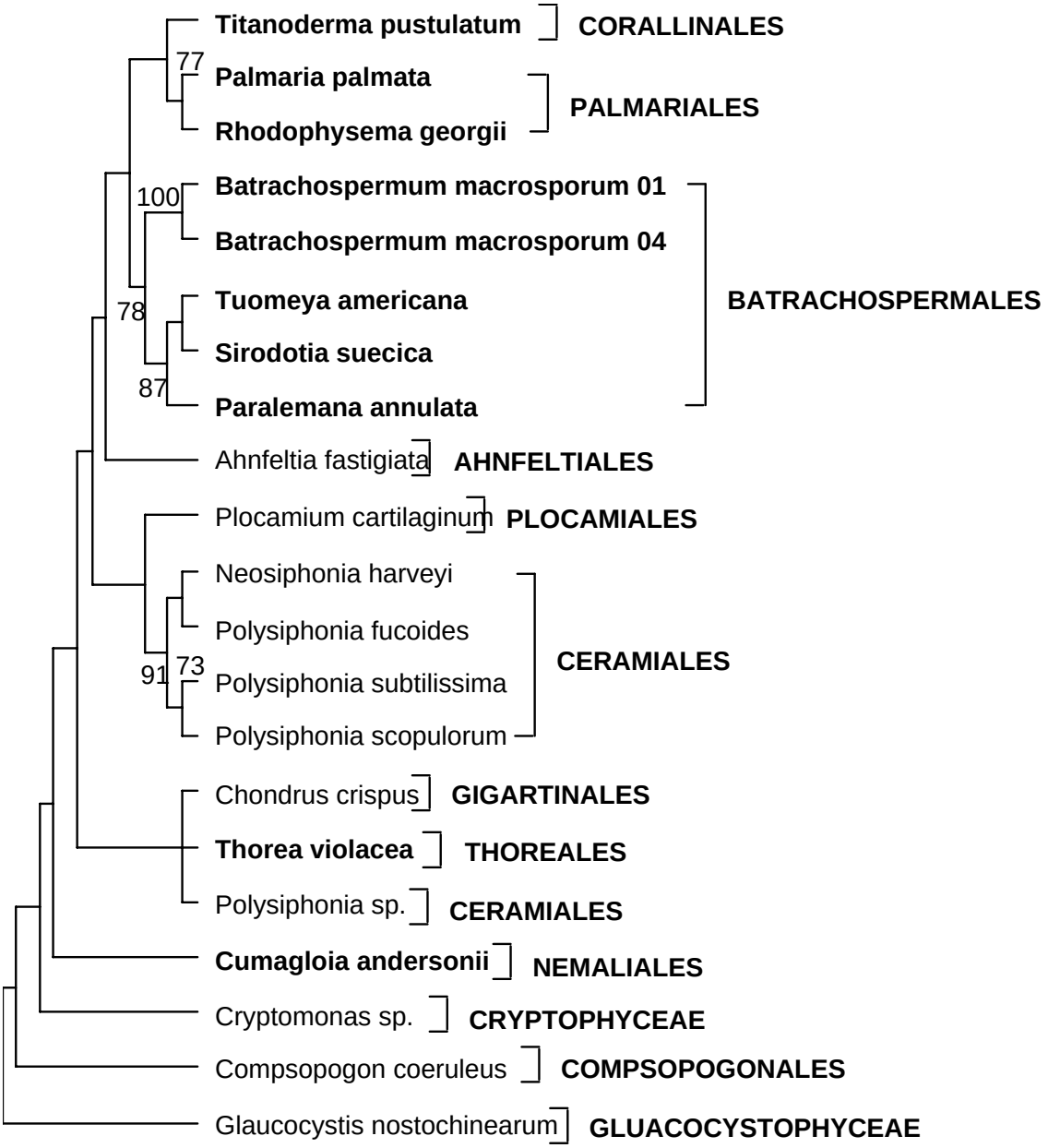

Figure 2. Phylogenetic relationships inferred among 18 ingroup taxa (red algal species) based upon parsimony analysis of first and second codon positions of the plastid-encoded *psbA* gene. Bootstrap values over 70 are indicated on the tree. Species in the "two cap-layer clade" are shown in bold.

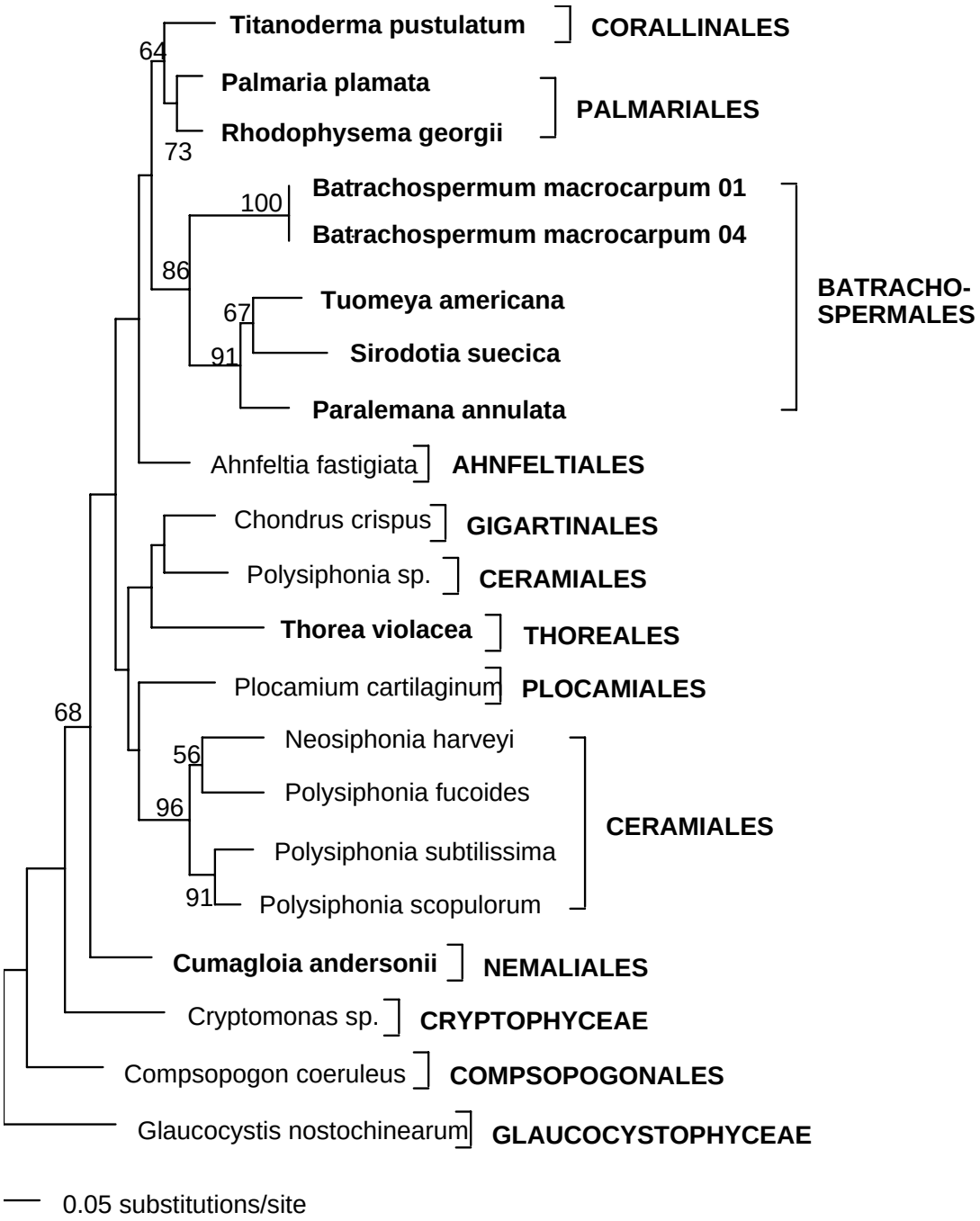

Figure 3. Phylogenetic relationships inferred among 18 ingroup taxa (red algal species) based upon maximum likelihood analysis of first and second codon positions of the plastid-encoded *psbA* gene. Bootstrap values above 50 are indicated on the tree. Species in the "two cap-layer clade" are shown in bold.

Polysiphonia sp. outside the 'core' ceramialean clade that includes other *Polysiphonia* species investigated as well as *Neosiphonia harveyi*. Finally, my trees imply that the *psb*A sequence

determined for *Compsopogon coeruleus* (Compsopogonales) is highly divergent from the other red algal taxa examined in this study. In the parsimony and ML trees *C. coeruleus* is positioned between outgroup species representing the classes Cryptophyceae and Glaucocystophyceae (Figs. 2, 3).

DISCUSSION

I have developed primers that have been successfully used to amplify the *psbA* gene in a phylogenetically diverse array of red algal species. This protein-encoding gene should provide useful data for red algal systematics studies, within limitations. The limits of resolution for this gene - specifically in red algae - are discussed below.

In both the parsimony and maximum likelihood analyses, the Corallinales, Palmariales and Batrachospermales, all members of the "two cap-layer clade," are shown to be closely related to one another. However, representatives of the Thoreales (*T. violacea*) and Nemaliales (*C. andersonii*), also members of the two cap-layer clade are resolved as unrelated to one another and to other two cap-layer clade orders. Thus, the hypothesis that *psb*A sequences are capable of resolving deep-level phylogenetic divergences among red algal taxa is rejected.

Both trees also show the single member of the Compsopogonales resolving between the two outgroup taxa. This may be due to the fact that this order is among the oldest orders of the red algae. In fact, Saunders & Hommersand (2004) have suggested this order be placed in its own subphylum and my data provide some support for their taxonomic scheme.

Polysiphonia sp. is not placed with congeners in the Ceramiales in our analyses. This result, coupled with the information above, suggests that the rate of nucleotide substitution in

*psb*A genes of red algae may significantly vary within species and among lineages. The combination of short and long branch lengths evident in the ML phylogram support such a conclusion, but in this pilot study the uneven sampling of red algal taxa might (alone, or in combination with taxon-specific substitution rate variation) help explain the observed branch length variation. In any event, more taxa within different lineages need to be examined to provide a meaningful (useful) examination of *psb*A variation.

As mentioned previously, my results indicate that translated *psbA* amino acids are too conserved for resolving relationships among representatives of the orders examined in this study. Not surprisingly, my results, and preliminary observations by others, also indicate that *psbA* amino acid sequences are too conserved among species within families to be of phylogenetic utility at that level of investigation (Yang and Boo, 2004; Yang et al., 2004; Seo et al., 2003). That amino acid substitutions are rare, even among apparently distantly related species, is not unexpected given the critical role that the D1 protein plays in photosynthesis.

Multi-gene analyses are rapidly becoming commonplace in systematics studies, even among eukaryotic algal taxa that have heretofore received little attention because their genomes are so poorly known. In this study, I present a new plastid-encoded marker for use in studies of red algal evolution and taxonomy. Although it is most likely that DNA sequences for the *psb*A alone will be most useful for conducting intraspecific studies, DNA or amino acid sequences could conceivably prove useful for examining questions at other levels in combination with data from other loci.

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