CHARACTERISTICS AND UTILITY OF PLASTID-ENCODED 16S rRNA GENE SEQUENCE DATA IN PHYLOGENETIC STUDIES OF RED ALGAE

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Abstract: A set of oligonucleotide primers was designed for amplifying and sequencing the plastid-encoded 16S rRNA gene from red algae. Gene sequences (16S rRNA) were generated from 28 species representing 11 red algal orders. Alignment of sequences from a wide taxonomic sampling required only a small number of gapped sites and revealed distinct variable and conserved regions in the gene. Likewise, a more narrow taxonomic sampling of 15 sequences from Gelidiales species was analyzed and compared with complementary sequence data sets for the nuclear-encoded 18S rRNA and 28S rRNA genes and plastid-encoded *rbc*L. Alignment of the Gelidiales 16S rRNA gene sequences also required only a small number of gapped sites and the resulting data set contained both highly conserved and variable regions. The amount of variation in 16S rRNA gene sequences was similar to that found in the 28S rRNA gene, and intermediate between the level of variation in *rbc*L and the 18S rRNA gene when complementary data sets were compared. Phylogenetic analyses of 16S rRNA gene sequences should be a useful tool for researchers studying the evolution, systematics and taxonomy of red algae.

Key Words: cp 16S rRNA; Gelidiales; phylogenetic analyses; red algae.

INTRODUCTION

Studies of evolution, systematics, and taxonomy of organisms have been greatly enhanced by the phylogenetic analysis of DNA sequence data. A large disparity exist in the number and types of DNA loci available for study in particular groups of organisms. Red algal researchers for example, do not have as extensive a "tool box" of loci for phylogenetic studies as do vascular plant researchers. A quick perusal of any volume of *Systematic Botany* will reveal the truth in this statement, i.e., volume 28(2) 2003 contains vascular plant studies analyzing sequence data from 11 different loci including ITS (Zomlefer et al., 2003; Gengler–Nowak, 2003), *rbc*L (Lamb Fry and Kron, 2003; Evans et al., 2003) and a variety of chloroplast-encoded intron and spacer regions (Ebihara et al., 2003; Santiago–Valentin and Olmstead, 2003). Molecular systematic studies in red algae in contrast, have predominately utilized only two loci, the gene for the nuclear-encoded 18S rRNA and plastid-encoded *rbc*L.

Bird et al. (1992) published one of the first red algal phylogenetic studies analyzing 18S rRNA gene sequence data. The potential of 18S data to elucidate relationships of red algae was further substantiated by Ragan et al. (1994) and many subsequent studies, a large number published by Saunders and collaborators (Saunders and Kraft, 1994; Saunders and

Bailey, 1997; Choi et al., 2002), have utilized this gene. Application of *rbc*L sequence data in phylogenetic studies of red algae was established with a series of papers in 1994 (Freshwater and Rueness, 1994; Freshwater et al., 1994; Hommersand et al., 1994). The use of other loci in phylogenetic studies of red algae has been limited, although works have been published utilizing 28S rRNA gene and ITS sequences (van Oppen et al., 1995; Chopin et al., 1996; Freshwater et al., 1999; Hughey et al., 2001; Harper and Saunders, 2002; Kamiya et al., 2003), RuBisCO spacer sequences (Destombe and Douglas, 1991; Goff et al., 1994; Müller et al., 1998), and more recently the mitochondrial *cox*2-*cox*3 intergenic spacer (Zuccarello et al., 1999; Chiasson et al., 2003).

We present the sequences of oligonucleotide primers that may be used to amplify and sequence the plastid-encoded 16S rRNA gene (16S) from many red algae. Comparisons of complementary sequence data sets of plastid-encoded 16S rRNA and *rbc*L, and nuclear-encoded 18S rRNA (18S) and 28S rRNA (28S) genes from members of the red algal order Gelidiales are made to demonstrate the characteristics and potential utility of 16S sequences in studies of red algae.

METHODS

Total genomic DNA was extracted from analyzed species following the methods of Freshwater and Rueness (1994) or Hughey et al. (2001). All taxa included in this study and GenBank accession numbers for the sequences generated from these taxa are listed in Table 1. Sequences of the oligonucleotide primers used to initially amplify and sequence the 16S gene were based on conserved loci in an alignment of three red algal 16S sequences: *Antithamnion sp.* (GenBank Accession #X54299, Maid and Zetsche, 1991), *Palmaria palmata* (GenBank Accession # Z18289, submitted by R.K. Singh), and *Chondrus crispus* (GeneBank Accession # Z29521, LeBlanc et al., 1995). The 16S gene was amplified and sequenced in two overlapping fragments using primer pairs F16S–A: 5'-GGA GAG TTT GAT CCT GGC TC–3'; R16S-C: 5'-CGT GCA ACA TCT AGT ATC–3', and F16S–B: 5'-CCA GTG TAG CGG TGA AAT GC–3'; R16S-E: 5'-GGA GGT GAT CCA GCC GCA CC–3'. Three additional primers were designed and sometimes used: R16S-A.2: 5'–TCC TCT CAA ACC AGC TAC–3' and F16S–A.3: 5'-GCA ATG GGC GAA AGC TGA CG–3', which are ca. 300 and 340 base pairs 3' of the F16S-A priming site, and R16S-D: 5'–CAT TGT AGC ACG TGT GTA GC–3', which is ca. 300 base pairs 5' of the R16S–E priming site.

Amplification reactions consisted of the basic reaction mixture and thermocycling protocol outlined in Freshwater et al. (2000). Sequencing reactions used Big Dye sequencing chemistry (Applied Biosystems, Foster City, CA, USA) and were run on either an ABI 377 or ABI 3100 genetic analyzer (DNA Analysis Core Facility, Center for Marine Science). Sequence reaction results were compiled and edited using Sequencher (Gene Codes Corp., Ann Arbor, MI). Sequences were aligned using MacClade (v. 4.0, Maddison and Maddison, 2000) or interactive CLUSTAL X (Thompson et al., 1997) running on the late North Carolina Super Computer. Sequence characteristics were determined and analyses performed using MacClade and PAUP* v. 4.0 (Swofford, 2002).

RESULTS AND DISCUSSION

The plastid–encoded 16S rRNA gene was amplified and sequenced from red algae representing 11 different orders. These sequences were aligned to generate two separate data sets. The first data set consisted of the three previously available sequences from GenBank and 15 newly generated sequences including at least one species from 11

Table 1.   Species and GenBank accession numbers for sequences derived from the taxa studied.

Species	18S	rbcL	28S	16S
Gelidiales				
Capreolia implexa	U60344	L22456	AF039545	AY706943
Gelidiella acerosa	U60342	L22457	AF039551	AY706939
Gelidium americanum	U60347	L22459	AF039536	AY706953
Gelidium caulacantheum	U60343	U00103	AF039544	AY706944
Gelidium crinale	U60355	U00981	AF039543	AY706947
Gelidium floridanum	U60351	U00107	AF039537	AY706952
Gelidium latifolium	U60350	U00112	AF039540	AY706950
Gelidium pristoides	U60353	U01044	AF039541	AY706948
Gelidium serrulatum	U60340	U01042	AF039538	AY706951
Gelidium sesquipedale	U60354	L22071	AF039539	AY706949
Pterocladia lucida	U60349	U01048	AF039550	AY706940
Pterocladiella capillacea	U60346	U01896	AF039549	AY706941
Pterocladiella melanoidea	U60341	U01046	AF039548	AY706942
Ptilophora leliaertii	U60345	U16834	AF039547	AY706945
Ptilophora subcostata	U60348	U16835	AF039546	AY706946
Ceramiales/Ceramiaceae				
Aglaothamnion halliae	—	—	—	AY731508
Ceramium diaphanum	—	—	—	AY731509
Polysiphonia harveyi	—	—	—	AY731510
Ahnfeltiales				
Ahnfeltia plicata	—	—	—	AY731515
Batrachospermales				
Paralemanea annulata	—	—	—	AY731517
Bonnemaisoniales				
Bonnemaisonia asparagoides	—	—	—	AY731516
Compsopogonales				
Compsopogon coeruleus	—	—	—	AY731520
Corallinales				
Corallina officinalis	—	—	—	AY731519
Gigartinales				
Chondrus crispus	—	—	—	AY731511
Hypnea sp.	—	—	—	AY731512
Gracilariales				
Gracilariopsis sjoestedtii	—	—	—	AY731514
Palmariales				
Palmaria palmata	—	—	—	AY731518
Plocamiales				
Plocamium cartilagineum	—	—	—	AY731513

different orders, and was used to determine the complexity of insertion–deletion mutations (indels) and sequence variation over a wide taxonomic sampling. The second data set consisted of an alignment of 15 Gelidiales sequences, and was used to characterize 16S data within a restricted taxonomic sampling.

The designed primers were found to work well with the majority of red algal species for which 16S amplifications were attempted. Less 'universal' primers may be found to work better in some red algal groups. The wide taxonomic sampling alignment was truncated because the 5′ and 3′ ends of some sequences were incomplete so that all sequences were the same length. This data set included 1,261 sites, of which 300 (23.8%) were variable. A histogram of the number of inferred mutational changes per non-overlapping 20 site segments of this alignment revealed distinct variable and conserved regions across the
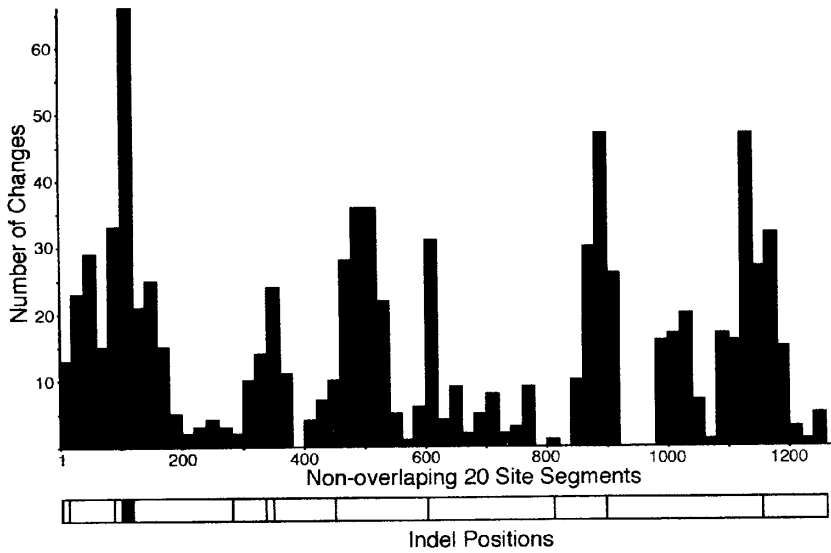
FIG. 1. Number of changes per non-overlapping 20 site segments of an alignment of 16S rRNA gene sequences from 18 species representing 11 different red algal orders. The positions of insertion–deletion mutations (vertical lines) are shown below the histogram of changes.

length of the gene (Fig. 1). Insertion–deletion mutations were small in this alignment, and never involved more than two successive gapped sites. Both the small number and size of indels in the 16S gene facilitates the alignment of sequence data sets and reduces the number of sites that may have to be excluded from phylogenetic analyses because the homology of sites among the aligned sequences is uncertain (Oliveira et al., 1995).

The distribution of variable sites in the Gelidiales alignment also revealed distinct variable and conserved regions within the 16S gene (Fig. 2). Indels again involved no more than two successive gapped sites and were mostly positioned in variable regions at the beginning and end of the gene. Sites within the Gelidiales alignment were characterized as being in loop or stem sections by comparing these sequences with the 16S rRNA secondary structure inferred for *Chondrus crispus* Stackhouse (LeBlanc et al., 1995). The highly conserved and variable regions of the alignment were not strongly associated with either stems or loops (Fig. 2).

The Gelidiales 16S alignment was also compared to complementary 18S, 28S, and *rbc*L data sets. The 18S, 28S, and 16S alignments required the inclusion of only 9, 15, and 11 gapped sites respectively. No gaps of more than two contiguous sites occurred in the 18S and 16S alignments, while the 28S alignment contained no gaps of more than three contiguous sites. Characteristics of the four sequence data sets are presented in Table 2. Prior comparisons of 18S, 28S, and *rbc*L data sets from Gelidiales species revealed that 18S sequences were the most conserved and *rbc*L sequences the most variable (Freshwater and Bailey, 1998). The 16S data set was found to have a similar level of variation as that found in the 28S data set. The 181 variable and 103 parsimony informative sites within the 16S data set were on a percentage basis comparable to 28S data (13.31% vs. 12.51% and 7.57% vs. 7.25% respectively). The percentage of variable and parsimony informative sites in the 18S data set were only 3.98% and 2.02%, while these values were 33.26% and 22.79% for the *rbc*L data set.
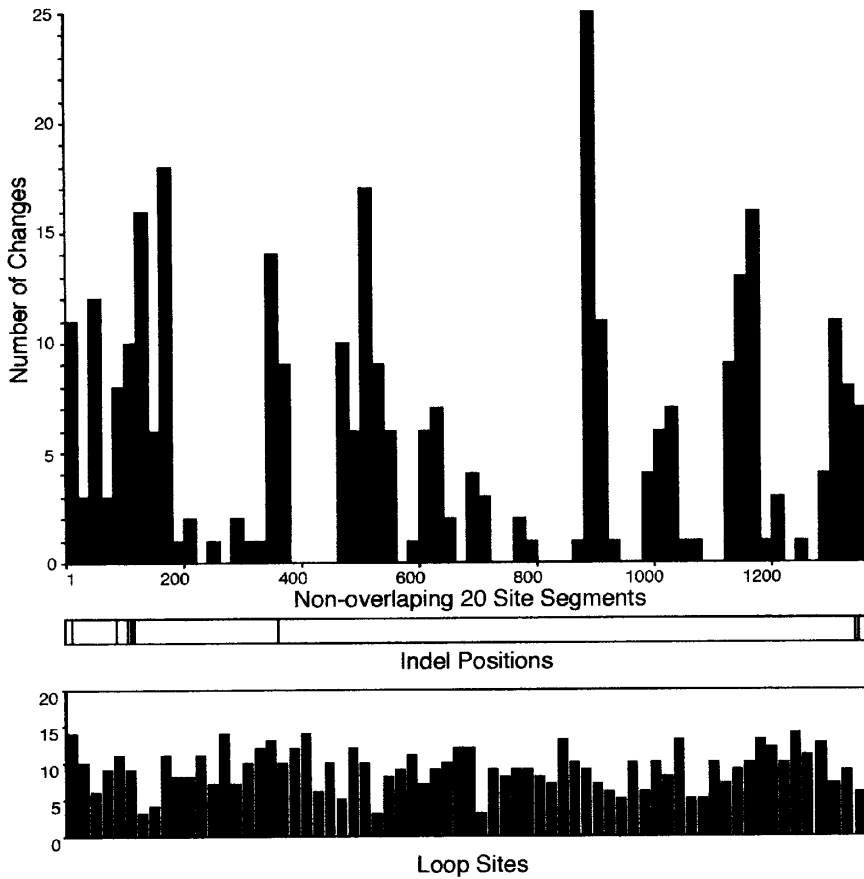
FIG. 2. Number of changes per non-overlapping 20 site segments of an alignment of 16S rRNA gene sequences from 15 Gelidiales species. The positions of insertion-deletion mutations (vertical lines) and the number of sites per 20 site segment in loops of an inferred secondary structure are shown below the histogram of changes.

Plastid genomes are generally AT rich (Howe et al., 2003) and this is reflected in the 62% AT bias seen in the rbcL data set. Although the 16S rRNA gene is located in the plastid genome, the Gelidiales 16S data set shows no AT bias, and its composition of 50.8% AT is similar to that of the nuclear encoded 18S (50.0%) and 28S (48%) data sets. The AT bias present in the rbcL data set is in large part a reflection of the 81% AT nucleotide composition of synonymous third codon positions. The structural constraints for a functional 16S rRNA molecule may preclude any strong nucleotide composition bias in this gene in contrast to other noncoding loci or synonymous sites in the plastid genome.

All four data sets have transition-transversion ratios (Ti:Tv) greater than two. The Ti:Tv of the 28S and rbcL data sets were similar (2.20 and 2.12 respectively), but the Ti:Tv of the 18S (3.33) and 16S (5.21) data sets were much higher. The distribution of transitions between purine-purine and pyrimidine-pyrimidine mutations was also variable in the four data sets. The 18S and 16S data sets were similar with 47.5% and 53.5% purine-purine mutations respectively, while the 28S and rbcL data sets had 60.8% and 38.5% purine-purine mutations. The high proportion of pyrimidine-pyrimidine mutations in the rbcL data set is probably because of the small number of C's present in these sequences.

Table 2.　Characteristics of DNA sequence data sets for four genes from 15 Gelidiales species.

Gene	# of Sites	Sites w/Gaps	Sites	Parsimony Informative	Mean Base Use (%)				Transitions (Ti)			Transversions (Tv)	Ti: Tv
					A	C	G	T	Pur-Pur	Pyr-Pyr	Total		
18S no gaps	1,581	9	63	32	24.4	20.7	29.3	25.6	19	21	40	12	3.33
18S w/ gaps	"	"	68	33	"	"	"	"	"	"	"	"	"
28S no gaps	1,159	15	145	84	24.2	21.3	30.7	23.8	59	38	97	44	2.20
28S w/ gaps	"	"	155	93	"	"	"	"	"	"	"	"	"
rbcL	1,413	na	470	322	30.8	16.7	21.4	31.2	186	297	483	228	2.12
16S no gaps	1,360	11	181	103	27.5	20.9	28.3	23.3	92	80	172	33	5.21
16S w/ gaps	"	"	189	110	"	"	"	"	"	"	"	"	"

The number of variable and parsimony informative sites within the four data sets is reflected in the number, position, and bootstrap support for branches resolved in parsimony analyses (Fig. 3). The 18S parsimony tree is poorly resolved with a basal polytomy of the four major Gelidiales lineages (for a discussion of these lineages see Bailey and Freshwater, 1997) and a larger polytomy that includes species of *Gelidium*, *Capreolia*, and *Ptilophora*. The rbcL parsimony tree is fully resolved except for a polytomy of the *Pterocladiella*, *Ptilophora*, and *Gelidium+Capreolia* lineages, but the position of the *Capreolia+G. caulacantheum* lineage has only weak bootstrap support. The rbcL parsimony analysis is the only one that fully resolves the relationships among the included *Gelidium* species. The 28S and 16S parsimony analyses resulted in an intermediate level of resolution.

CONCLUSIONS

The plastid-encoded 16S rRNA gene has been amplified and sequenced from a taxonomically diverse group of red algae using the presented primer sequences. Over 1,300 bp of sequence data can be generated in both directions for most taxa with only four sequencing reactions. The 16S sequences are generally easy to align and require only a small number of gapped sites. This may reduce or eliminate the need to exclude portions of the aligned sequence data sets from analyses because of site homology uncertainty. Gaps may also be included as characters in data sets where the homology of gapped sites is not in question, adding to the overall information content of the sequences.

The amount of variation among Gelidiales 16S sequences is intermediate to that found in rbcL and 18S sequences. Consequently, 16S analyses do not resolve all the species relationships resolved in rbcL analyses but more than are resolved by 18S analyses. The amount of variation found at different loci may vary depending upon the evolutionary lineage of red algae being examined. Preliminary data are required to make specific assumptions as to which DNA loci will contain the proper signal for phylogenetic analyses addressing a particular question. Thus, it is important that researchers have a "tool box" of loci from which to choose. Nucleotide sequences of the plastid-encoded 16S rRNA gene promise to be a useful addition to the tools available for red algal researchers.

A. 18S

- P. leliaertii
- P. subcostata
- C. implexa
- G. caulacantheum
- G. crinale
- G. pristoides
- G. sesquipedale
- G. latifolium
- G. serrulatum
- G. floridanum
- G. americanum
- P. capillacea
- P. melanoidea
- P. lucida
- G. acerosa

(node values: 99, 89, 100)

B. 28S

- G. sesquipedale
- G. latifolium
- G. serrulatum
- G. floridanum
- G. americanum
- G. pristoides
- G. crinale
- C. implexa
- G. caulacantheum
- P. leliaertii
- P. subcostata
- P. capillacea
- P. melanoidea
- P. lucida
- G. acerosa

(node values: 95, 77, 100, 99, 93, 83, 100)

C. rbcL

- G. serrulatum
- G. americanum
- G. floridanum
- G. sesquipedale
- G. latifolium
- G. pristoides
- G. crinale
- C. implexa
- G. caulacantheum
- P. leliaertii
- P. subcostata
- P. capillacea
- P. melanoidea
- P. lucida
- G. acerosa

(node values: 52, 100, 93, 62, 99, 93, 68, 91, 100, 100, 100)

D. 16S

- G. sesquipedale
- G. latifolium
- G. serrulatum
- G. floridanum
- G. americanum
- G. pristoides
- G. crinale
- C. implexa
- G. caulacantheum
- P. leliaertii
- P. subcostata
- P. capillacea
- P. melanoidea
- P. lucida
- G. acerosa

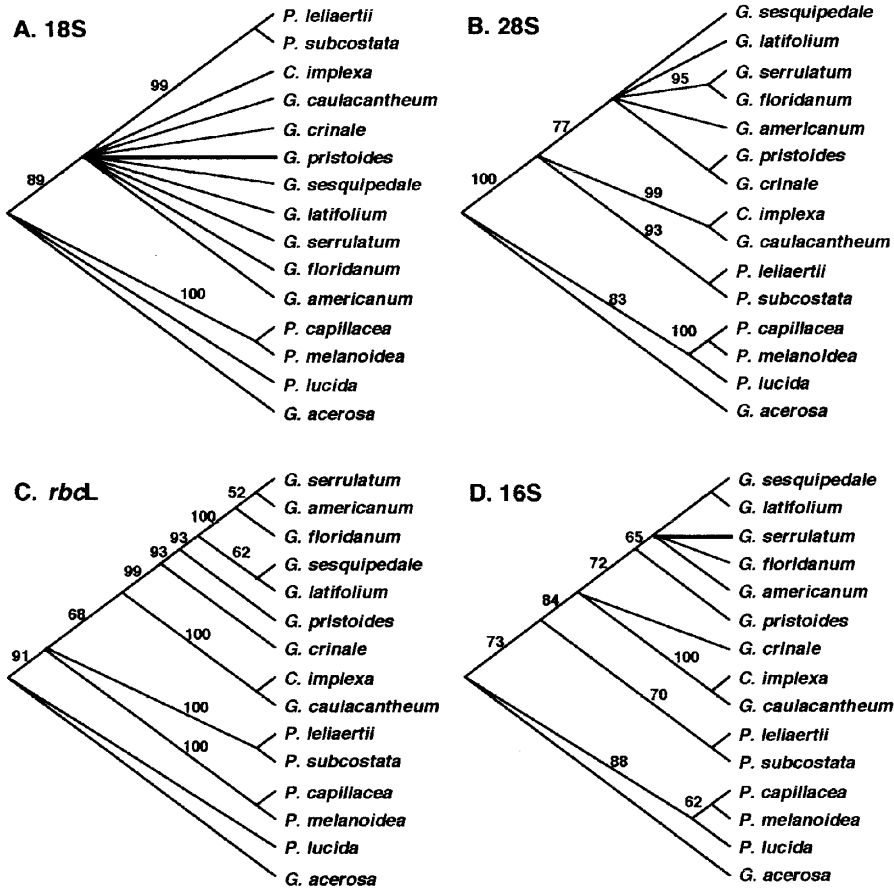*(node values: 65, 72, 84, 73, 100, 70, 88, 62)*

FIG. 3. Results of maximum parsimony analyses using the branch and bound algorithm of four complementary sequence data sets from 15 Gelidiales species. (A) Strict consensus of 25 minimal 18S trees of 81 steps [Consistency Index (CI) = 0.755; Retention Index (RI) = 0.821]. (B) Strict consensus of five minimal 28S trees of 217 steps (CI = 0.669; RI = 0.750). (C) Strict consensus of two minimal *rbc*L trees of 1032 steps (CI = 0.519; RI = 0.538). (D) Strict consensus of three minimal 16S trees of 322 steps (CI = 0.534; RI = 0.611). Gaps were treated as missing data in all analyses. Calculations of tree lengths (steps) were made including all characters, and calculations of CI were made including only parsimony informative characters. Bootstrap proportion values resulting from 1,000 replications of branch and bound searches are shown for branches when >50.

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