The application of sequences of the ribosomal cistron to the systematics and classification of the florideophyte red algae (Florideophyceae, Rhodophyta)

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Abstract: This review discusses the impact that molecular studies of the genic and spacer regions of the ribosomal cistron have had on the systematics and classification of the florideophyte red algae. Each ribosomal cistron consists of a non-transcribed spacer region (NTS), an external transcribed spacer region (ETS), the small-subunit (SSU) rRNA gene, internal transcribed spacer (ITS 1) region 1, the 5.8S rRNA gene, internal transcribed spacer (ITS 2) region 2, and the large-subunit (LSU) rRNA gene. To date, molecular investigations on the Rhodophyta that utilize the ribosomal cistron have been based largely on the SSU and, to a lesser extent, the ITS regions. We then outline the methods and procedures we use to obtain sequence data from the various regions of the ribosomal cistron. A discussion of recent advances in florideophyte systematics within the four florideophyte lineages outlined by Saunders & Kraft (1997) is presented. We then close the review with an introduction to, and discussion of the potential utility of sequences of the LSU for resolving relationships within the Rhodophyta.

Résumé : Cette revue discute de l'impact des études moléculaires des régions géniques et des espaceurs du cistron ribosomal sur la systématique et la classification des algues rouges Florideophytes. Chaque cistron ribosomal est constitué par un espaceur non trancrit (NTS), une région espaceur externe transcrite (ETS), le gène de la petite sous-unité (SSU) de l'ARN ribosomal, la région 1 de l'espaceur interne transcrit (ITS 1), le gène 5.8S de l'ARNr, la région de l'espaceur interne transcrit (ITS 2) et le gène de la grande sous-unité (LSU) de l'ARNr. A ce jour, la plupart des études moléculaires sur les Rhodophytes ont utilisé les séquences de la SSU et, dans une moindre mesure, les régions des ITS. Nous détaillons les méthodes et les protocoles que nous avons utilisés pour obtenir les séquences des différentes régions du cistron ribosomal. Une discussion des avancées récentes sur la systématique des algues rouges au sein des quatre lignées de Floridéophytes, déjà abordée par Saunders & Kraft (1997), est présentée. Nous achevons cette revue par une introduction et une discussion de l'utilisation potentielle des séquences de la LSU pour résoudre des parentés évolutives chez les Rhodophytes.

Keywords : Florideophyceae; internal transcribed spacer regions; large-subunit rRNA; molecular systematics; phylogeny; red algae; ribosomal cistron; small-subunit rRNA.

Introduction

The phylum Rhodophyta (red algae) is a morphologically diverse and distinct assemblage of eukaryotes with as many as 6000 species in upwards of 700 genera (Woelkerling, 1990). Red algae are characterized as eukaryotic, nonflagellate, photosynthetic organisms that contain phycobilin pigments organized into phycobilisomes (Gabrielson & Garbary, 1986). Although the phylum as a whole is well delimited (Garbary & Gabrielson, 1990),

relationships with other groups remain uncertain and the systematics and classification of the included members have been rather unstable (Saunders & Kraft, 1997). Traditionally, the phylum has been considered to encompass two lineages, variously referred to as either two classes in their own right (Bangiophyceae and Florideophyceae) or as two subclasses (Bangiophycidae and Florideophycidae) within a single class (Rhodophyceae).

The utility of molecular data in reconstructing phylogenies has yielded favourable results in many taxonomic groups (Leffers et al., 1987; Woese, 1987; Cedergren et al., 1988) and the Rhodophyta have been no exception (Saunders & Kraft, 1997). Since their inception as phylogenetic tools, a number of gene systems have been used to investigate red algal phylogeny (see Saunders & Kraft, 1997). We present here a review of the impact that analyses of the genic and spacer regions of the ribosomal cistron have had on the systematics and classification of the Florideophyceae, focusing on the work of the second author over the past decade and future directions that our research is taking.

An introduction to ribosomes and ribosomal RNA

Ribosomal RNA (rRNA) is by far the most abundant and most stable form of RNA in most cells (Snustad et al., 1997). It represents about 70-80% of the total cellular RNA and is the major component of ribosomes - the organelles that serve as the site of protein synthesis (Alberts et al., 1994). Ribosomes consist of two unequal subunits, each containing one or more specific rRNAs and a variety of ribosomal proteins. Ribosome subunits are usually identified according to their sedimentation coefficients, or S values (Table 1). The prokaryotic ribosome, for example, is a 70S particle whose larger 50S subunit includes one 23S and one 5S rRNA molecule. The smaller subunit is 30S and contains one 16S rRNA molecule. The eukaryotic ribosome, on the other hand, is an 80S particle. The larger 60S subunit contains one each of a 25S-28S, 5.8S, and 5S rRNA molecule. The small (40S) subunit in eukaryotes, as is the case in prokaryotes, possesses a single rRNA molecule - 18S rRNA (Alberts et al., 1994). These different-sized subunits act in concert to facilitate translational processes within the cell.

Of the four species of eukaryotic rRNA, the three larger ones (18S, 5.8S, and 25S-28S) are coded for by contiguous nuclear genes located in a ribosomal cistron, 100s to 1000s of the latter organized in a tandem array (Fig. 1). Conversely, the 5S rRNA molecule is usually encoded by DNA located elsewhere in the genome (Snustad et al. 1997). Recently, however, Kawai et al. (1995) found evidence for a linked 5S rRNA gene in the brown alga *Scytosiphon lomentaria* (Lyngbye) Link. The first such instance reported for multicellular photosynthetic eukaryotes. Each ribosomal cistron consists of a non-transcribed spacer region (NTS) and an external transcribed spacer region (ETS) [together referred to as the intergenic spacer (IGS)], as well as the small-subunit (SSU) rRNA gene, internal transcribed spacer region 1 (ITS 1), the 5.8S rRNA gene, internal transcribed spacer region 2 (ITS 2), and the large-subunit (LSU) rRNA gene (Fig. 1) (Appels & Honeycutt, 1986). In most cells the three larger rRNAs are transcribed as a single precursor transcript about 8000-13000 base pairs (bp) in length, depending on the organism. The three rRNA molecules processed during maturation of this transcript are approximately 1400-2000 (18S rRNA), 100-200 (5.8S rRNA), and 2500-5000 bp (25S-28S rRNA) in length (Appels & Honeycutt, 1986, Alberts et al., 1994). Consequently, only about 55% of the primary transcript contributes to the final products. The remaining 45% consists of the transcribed spacer regions that are excised

Table 1. Properties of prokaryotic and eukaryotic ribosomes[a].
Tableau 1. Propriétés des ribosomes procaryotiques et eucaryotiques[a].

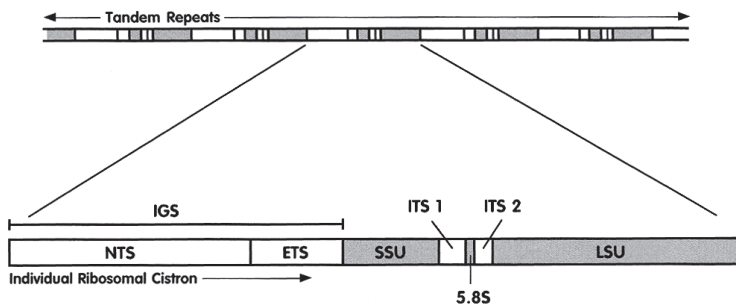| Source	Ribosome Size	Subunit	Subunit Size	Subunit rRNA Size	Approx. Length of rRNA (bp)
Prokaryotic Cells	70S	Large	50S	23S	2900
				5S	125
		Small	30S	16S	1540
Eukaryotic Cells	80S	Large	60S	25-28S	5000
				5.8S	160
				5S	125
		Small	40S	18S	2000

[a] Alberts et al., 1994, Snustad et al., 1997.

Figure 1. Composition of one of the tandemly repeated ribosomal cistrons. The coding units (shaded) within the cistron (small-subunit (SSU), 5.8S, and large-subunit (LSU)) are separated by two internal transcribed spacer regions (ITS 1 and ITS 2). Coding regions between two adjacent repeat units are separated by the intergenic spacer (IGS), which consists of a nontranscribed spacer (NTS) and an external transcribed spacer (ETS).

Figure 1. Composition de l'un des cistrons ribosomaux répétés en tandem. Les régions codantes (ombrées) au sein du cistron (petite sous-unité (SSU), 5.8S, et grande sous-unité (LSU)) sont séparées par deux espaceurs internes transcrits (ITS 1 et ITS 2). Les régions codantes entre deux régions répétées uniques sont séparées par un espaceur intergénique (IGS), qui consiste en un espaceur non transcrit (NTS) et un espaceur transcrit (ETS).

during the various processing steps and eventually degraded in the nucleus (Snustad et al., 1997).

Tandem repeats, concerted evolution, and molecular systematics

The hundreds to thousands of ribosomal cistrons in a eukaryotic genome are referred to as ribosomal DNA (rDNA) and are clustered in the nuclear organizing region(s) of one or more chromosomes in the genome. During the course of evolution each cistron is subject to independent mutational events and, therefore, it should be expected that the nucleotide sequences of the repeat units will diverge in primary sequence composition relative to one another. This, however, is not what happens in ribosomal DNA evolution. Rather, the numerous copies appear to evolve together. The solution to this paradox is found in a homogenizing process termed concerted evolution (Zimmer et al., 1980; Li, 1997). Concerted evolution is effected by gene conversion and unequal crossing-over (Dover, 1982; other agents have been advanced, see Callan, 1967; Britten & Kohne, 1968; Buorgiorno-Nardelli et al., 1972; Dover, 1987; Walsh, 1987) and has the net result of maintaining homogeneity of ribosomal cistrons within a population. This homogenisation is essential for organismal survival (Li, 1997) and contributes to the utility of these genes for systematics (Sogin et al., 1986).

Despite the overall homogenization between cistrons themselves, different regions of the ribosomal cistron are under varying degrees of functional constraint and hence evolve at different rates. The IGS and the two ITS regions

(Fig. 1) have little functional constraint and evolve most rapidly, thus accumulating mutations at a much greater rate than the rRNA genes. Coding regions of the cistron, on the other hand, tend to be highly conserved because their transcripts are directly involved in the formation of ribosomes - organelles requiring the correct 'fit' of many protein and RNA components to function properly - and hence mutations are more likely to be deleterious and therefore subject to purifying selection. Of these regions, the small-subunit gene is the more conserved whereas the large-subunit gene possesses the greatest variability (Appels & Honeycutt, 1986).

Pre-molecular florideophyte systematics

Schmitz (1892) formulated one of the first comprehensive classifications of florideophyte red algae, emphasizing female reproductive anatomy and post- fertilization development. In his work, four orders of red algae were recognized: Cryptonemiales, Gigartinales, Nemaliales (as Nemalionales), and Rhodymeniales. Oltmanns (1904-1905) modified this system slightly by segregating the Ceramiales from the Rhodymeniales.

In 1923, Kylin extended this emphasis on female reproductive anatomy and laid the basis for what was to become the predominant classification of the Florideophyceae. Kylin (1923) recognized a total of six orders, adding two more to those delineated by Schmitz (1892) and Oltmanns (1904-1905). The Gelidiales was described as a segregate of the Nemaliales (auxiliary cells functioning as nutritive cells and not initiating gonimoblast formation) while a new character - whether the auxiliary cell is borne in a special (accessory) filament or is non-accessory - was introduced by Kylin (1925) and used to distinguish the order Cryptonemiales from the Gigartinales (procarpic) and Nemastomatales (non procarpic; this order, subsequently merged with the Gigartinales, Kylin, 1932). Though Kylin would refine this classification system over time, these basic ordinal delineations would be maintained (Kylin, 1932, 1956).

Feldmann & Feldmann (1942) were the first to attempt to alter substantially Kylin's (1932) system of red algal classification. They demonstrated that the nemalialean family Bonnemaisoniaceae displayed a heteromorphic, diplobiontic life history. As Kylin's Nemaliales was characterized by the presence of a haplobiontic life history, the Bonnemaisoniaceae was raised to ordinal rank. Feldmann (1953) further dismantled the Nemaliales by elevating the family Acrochaetiaceae to ordinal rank because of the morphological simplicity and absence of a

carpogonial branch for the included species. Despite the recognized heterogeneity of the Nemaliales, there was no consensus among red algal systematists during the 1960s and 1970s regarding which of the segregate orders should be recognized. Dixon (1961), Abbott (1962), and Papenfuss (1966) argued against recognizing the Acrochaetiales, while the Bonnemaisoniales and Gelidiales received relative degrees of acceptance (Papenfuss, 1966; Chihara & Yoshizaki, 1972).

The (essentially) Kylinian classification (Kylin, 1956) began to face the challenge of new interpretations of characters as well as the advent of new ultrastructural features during the late 1970s. The first major departure was in the Rhodymeniales, an order that had been regarded as homogeneous. Guiry (1978) elevated the family Palmariaceae to ordinal rank based on the presence of a stalk cell within each tetrasporangium and the absence of female gametophytes and carposporophytes. In addition, Garbary (1978) renewed arguments for recognition of the Acrochaetiales based on its speculative position as the most primitive florideophyte lineage.

An important contribution to red algal systematics was the report by Pueschel & Cole (1982) that the differences in the morphology and number of cap layers that overlay red algal pit plugs are useful characters in distinguishing among higher taxa of rhodophytes. Their findings provided another line of evidence supporting the segregation of the Gelidiaceae and Bonnemaisoniaceae from the Nemaliales, and the Palmariaceae from the Rhodymeniales. In addition, Pueschel & Cole (1982) described two new orders: Batrachospermales for the freshwater families of the Nemaliales; and, Hildenbrandiales for the cryptonemialean family Hildenbrandiaceae. Pueschel & Cole (1982) provided further evidence for elevating the cryptonemialean family Corallinaceae to ordinal status, a proposal formalized by Silva & Johansen (1986).

In 1989 Maggs & Pueschel demonstrated that the genus *Ahnfeltia* (previously housed in the Gigartinales), long recognized as reproductively unusual, displayed a number of unique features among the Florideophycidae. Based on observations of ultrastructural and reproductive anatomy, they established the order Ahnfeltiales. In that same year, the family Gracilariaceae was segregated from Kylin's Gigartinales following detailed cytological and anatomical observations on *Gracilaria verrucosa* (Hudson) Papenfuss (Fredericq & Hommersand, 1989).

Considering the upheaval that these new ultrastructural and reproductive data have had on red algal systematics, it was inevitable that molecular data would have an equally profound impact.

Acquisition and analysis of molecular data from the ribosomal cistron

Regardless of the final region of the ribosomal cistron desired for molecular systematics (SSU, ITS, or LSU), the methods we use generally follow a common procedure with modifications as noted below. Algal samples are processed and genomic DNA isolated following the procedure of Saunders (1993). Regions of the ribosomal cistron are PCR-amplified as overlapping fragments using oligonucleotide primers complementary to coding and noncoding portions of the gene/spacer of interest (Fig. 2). Overlap of the PCR products is a useful strategy because it confirms the sameness of the independently amplified fragments. We use the *Taq* DNA polymerase PCR kit (Promega) to amplify the fragments, but most amplification systems should work equally as well. The reaction profiles for each gene system currently studied by us are given in Fig. 3. Each profile involves an initial DNA denaturation phase; 38 cycles of denaturation, primer annealing, and primer extension; an extended phase of primer extension; and, a final quiescent phase.

Normally, the SSU is amplified as two overlapping fragments, E' and F (ca. 1000 bp each), using PCR primers G01-G14 and G04-G07, respectively (Saunders & Kraft, 1994; 1996). If one or both of these fragments fails to amplify, the smaller fragments L and M (corresponding to fragment E') and N and O (corresponding to fragment F) are amplified (ca. 450-600 bp each) using primers G01-G10, G02-G14, G04-G13, and G06-G07, respectively (Fig. 2A). In many cases, especially for members of the Ceramiales, G05 is preferred to G10 (fragment L). Similarly, primers H1 and H2 replace G01 (E' and L fragments) and HR replaces G10 and G05 (fragment L) for members of the Hildenbrandiales (Saunders & Bailey, 1999).

We have developed PCR primers that consistently amplify three overlapping fragments of the florideophyte LSU, representing approximately 85% of this gene. These fragments are labeled X, Y and Z (Fig. 2B). The X fragment is amplified using primers T01-T13 (ca. 1100 bp), the Y fragment using primers T04-T08 (ca. 800 bp), and the Z fragment using primers T05-T15 (ca. 700 bp).

As with the system for the SSU, there are two approaches to amplifying fragments of the ITS regions. The ITS may be amplified as a single fragment, labelled fragment I, or as two overlapping fragments, labelled II and III (Fig. 2C). Primers ITSP1-G4 are used to amplify fragment I (ca. 700 bp), primers ITSP1-ITSR1 for fragment II, and primers P5-G4 for fragment III (ca. 400 bp each).

The Wizard (TM) PCR Preps DNA purification system (Promega, Madison, WI) is used to agarose gel purify PCR products. Once cleaned by this method, DNA is sequenced with the dRhodamine Terminator Cycle Sequencing Kit
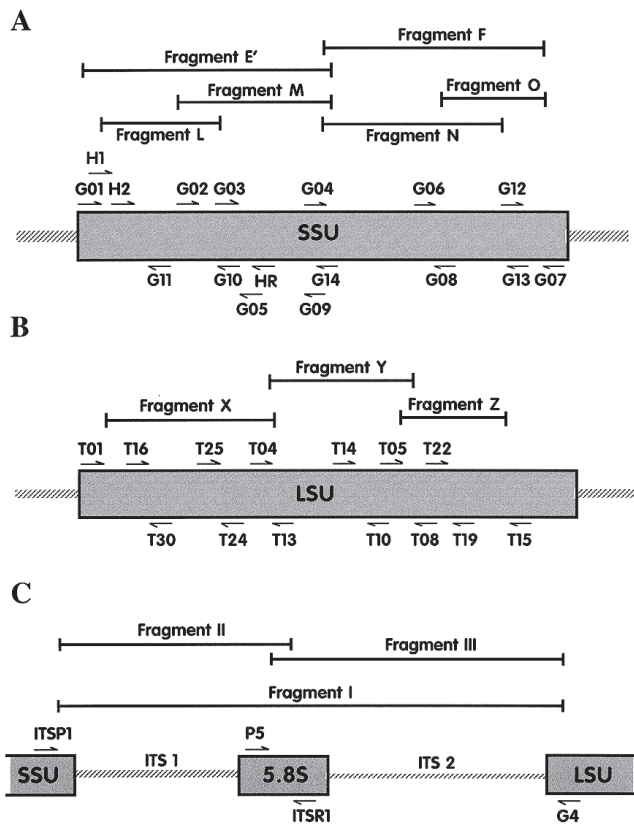
A

B

C

Figure 2. Schematic representations of cistronic regions investigated. Approximate position of primers utilized for PCR amplification and sequencing are indicated. A) The SSU is usually amplified as two or four overlapping fragments: E' (G01-G14) and F (G04-G07), and L (G01-G10), M (G02-G14), N (G04-G13), and O (G06-G07). B) Three overlapping fragments are amplified for the LSU: X (T01-T13), Y (T04-T08), and Z (T05-T15). C) The ITS regions may be amplified as a single fragment - I (ITSP1-G4), or as two overlapping fragments, II (ITSP1-ITSR1) and III (P5-G4). Note: primer ITSP1 is not the kelp primer P1 of Saunders & Druehl (1993). Nucleotide sequences for all primers are listed in Table 2.

Figure 2. Représentations schématiques des régions cistroniques étudiées. La position approximative des amorces utilisées pour l'amplification par PCR et le séquençage est indiquée. A) La SSU est habituellement amplifiée en deux ou quatre fragments chevauchants : E' (G01-G14) et F (G04-G07), et L (G01-G10), M (G02-G14), N (G04-G13), et O (G06-G07). B) Trois fragments chevauchants de la LSU sont amplifiés: X (T01-T13), Y (T04-T08), et Z (T05-T15). C) Les régions ITS sont amplifiées en un fragment unique - I (ITSP1-G4), ou en deux fragments chevauchants, II (ITSP1-ITSR1) et III (P5-G4). Note : l'amorce ITSP1 n'est pas l'amorce P1 pour les Laminariales de Saunders & Druehl (1993). Les séquences nucléotidiques de toutes les amorces sont listées dans le Tableau 2.

[Applied Biosystems (ABI), division of Perkin Elmer Cetus]. The reaction profile for all sequencing primers is given in Fig. 3E. When sequencing PCR-amplified

Table 2. Nucleotide sequences for PCR primers and sequencing primers listed in Fig. 2.

Tableau 2. Séquences nucléotidiques des amorces de PCR et de séquençage cités sur la figure 2.

Coding Region	Primer	Primer Sequence	Strand Complement
SSU	G01	CACCTGGTTGATCCTGCCAG	Coding
	G02	GGACTCCGGAGAGGGAGCCTG	Coding
	G03	GTCTGGTGCCAGCAGCCGCGG	Coding
	G04	CAGAGGTGAAATTCTTGGAT	Coding
	G05	TGGTATACGCTTACAGAGCTG	Noncoding
	G06	GTTGGTGGTGCATGGCCGTTC	Coding
	G07	AGCTTGATCCTTCTGCAGGTTCACCTAC	Noncoding
	G08	GAACGGCCATGCACCACCACC	Noncoding
	G09	ATCCAAGAATTTCACCTCTG	Noncoding
	G10	CCGCGGCAGCTGGCACCAGAC	Noncoding
	G11	TCTCTCAGGCTCCCTCTCCGG	Noncoding
	G12	GAACGAGGAATACCTTGTAAG	Coding
	G13	GCTTACAAGGTATTCCTCGTTC	Noncoding
	G14	CTTGGCAGACGCTTTCGCAG	Noncoding
	H1	GTCTTGTCTCAAGGACTAAG	Coding
	H2	CATGCAAGTGCCAGTATGAG	Coding
	HR	CCAAGTAGAAGAGCCGATGGC	Noncoding
LSU	T01	TAAGCATATCAGTAAGCGGAG	Coding
	T04	GCAGGACGGTGGCCATGGAAGT	Coding
	T05	GCAACGGGCAAAGGGAATCCG	Coding
	T08[a]	CAGAGCACTGGGCAGAAATCAC	Noncoding
	T10[a]	CACCTTGGAGACCTGCTGCGG	Noncoding
	T13	GCAGGTGAGTTGTTACACTC	Noncoding
	T14[a]	CGTCCGGCTCGCCTTCGACGG	Coding
	T15	TGATAGGAAGAGCCGACATCGA	Noncoding
	T16	GAGACCGATAGCGAAACAAGTAC	Coding
	T19	TTATCCTACACCTCTCAAGTAC	Noncoding
	T22	GGAAAGAAGACCCTGTTGAGCTT	Coding
	T24	GCACTAATCATTCGCTTTACC	Noncoding
	T25	GAAAGATGGTGAACTATGCC	Coding
	T30	TGTTAGACTCCTTGGTCCGTG	Noncoding
ITS	G4	CTTTTCCTCCGCTTATTGATATG	Noncoding
	ITSP1	GGAAGGAGAAGTCGTAACAAGG	Coding
	ITSR1	TTCAAAGATTCGATGATTCAC	Noncoding
	P5	GCATCGATGAAGAACGCAG	Coding

[a] LSU primers based on sequences kindly provided by Drs C. Bailey and W. Freshwater.

fragments, a number of internal oligonucleotide primers are used in addition to a fragment's respective PCR primers (Fig. 2). For the SSU, the number of internal primers needed depends on both the number of fragments amplified and the number of nucleotides acquired per sequencing reaction. For sequencing reactions that read up to 600 bp, internal primers G02 and G10 are used for fragment E', and primers G06 and G13 are used for fragment F, whereas no internal primers are generally required for fragments L, M, N, and O. Conversely, for sequencing reactions yielding less than 600 bp (ca. 400 bp), internal primers are required for fragments E' (G02, G03, G10, and G11), F (G06, G08, G12, and G13), L (G11), M (G03), N (G08), and O (G12)
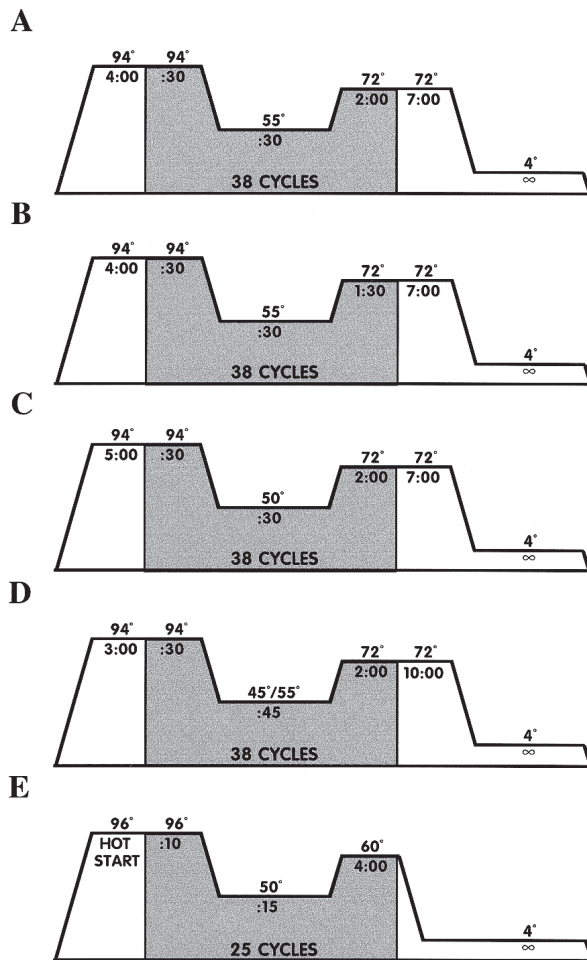
A

B

C

D

E

Figure 3. Schematic of the PCR profiles for amplifying and sequencing regions of the ribosomal cistron. A) PCR profile for amplifying fragments E' and F of the SSU. B) PCR profile for amplifying fragments L, M, N, and O of the SSU. C) PCR profile for amplifying fragments X, Y, and Z of the LSU. D) PCR profile for amplifying fragments I, II, and III of the ITS1/5.8S/ITS2 region. A primer annealing temperature of 45°C is used for fragments I and II, whereas 55°C is used for fragment III. E) Sequencing reaction profile for all ribosomal cistron primers. Temperatures listed are in Celsius and times are in minutes and seconds (above and below lines, respectively). The shaded area of each diagram includes those times and temperatures that are cycled during the reaction.

Figure 3. Schémas des conditions de PCR pour amplifier les régions séquencées du cistron ribosomal. A) Conditions de PCR pour amplifier les fragments E' et F de la SSU. B) Conditions de PCR pour amplifier les fragments L, M, N, et O de la SSU. C) Conditions de PCR pour amplifier les fragments X, Y, et Z de la LSU. D) Conditions de PCR pour amplifier les fragments I, II, et III de la région ITS1/5.8S/ITS2. Une température d'hybridation de 45°C est utilisée pour les fragments I et II, tandis q'une température de 55°C est utilisée pour le fragment III. E) Conditions des réactions de séquence pour les amorces du cistron ribosomal. Les températures sont exprimées en degré Celsius et le temps en minutes et secondes (au dessus et en dessous des lignes, respectivement). L'aire ombrée de chaque diagramme représente la répétition des cycles de temps et de température durant la réaction.

(Fig. 2A). To acquire sequence data for the LSU, fragment X requires primers T16, T24, T25, and T30, fragment Y requires T14 and T10, and fragment Z requires T19 and T22, in addition to the respective PCR primers (Fig. 2B). For the long ITS fragment (Fragment I, Fig. 2C) the internal primers P5 and ITSR1 are necessary.

Sequence data are compared and edited using the SeqEd DNA sequence editor (ABI) software package. This allows sequences from both strands to be compared and the overlapping fragments combined to derive the corresponding gene sequences. For phylogenetic analyses, homologous sequences from a number of taxa are aligned relative to one another with the assistance of a multiple alignment program (e.g. SeqPup: A Biosequence Editor and Analysis Program; Gilbert, 1995). This multiple alignment then becomes a data set for input into phylogenetic analysis programs.

Three analyses are currently in routine use for deriving phylogenies from molecular data: distance, parsimony, and maximum-likelihood. Our approach for distance analyses involves converting aligned sequences into a distance matrix using the DNADIST program of PHYLIP (Felsenstein, 1995). We then construct phylogenetic trees using the neighbor-joining algorithm (Saitou & Nei, 1987) of PHYLIP. Parsimony analyses are completed using the program PAUP (Phylogenetic Analysis Using Parsimony; Swofford, 1991). Bootstrap resampling (generally 1000 replicates) is used for distance and parsimony methods to estimate the robustness of internal branches (Felsenstein, 1985). For maximum likelihood analyses we use the fastDNAml program for the Power Macintosh (V. 1.0.8; Olsen et al., 1994). This program is preferred as maximum likelihood analyses are computationally demanding and time-consuming.

Sequences of the small-subunit ribosomal DNA

Nucleotide sequences of the small-subunit ribosomal DNAs (18S rDNAs) are one of the most common molecular markers used in phylogenetic reconstruction (Woese, 1987; Bhattacharya, 1997). The larger size of the SSU (ca. 1800 base pairs (bp) in red algae) when compared to other systems such as the 5.8S gene, its mosaic of conserved and variable regions (Medlin et al., 1988), and ease of isolation using PCR methodology, have all prompted the suggestion that this gene provides the best system for phylogenetic investigations at a variety of taxonomic levels (Sogin et al., 1986). Within the Rhodophyta, SSU-based phylogenies have been successfully employed to distinguish between species, families, and orders within various red algal lineages (Bird et al., 1990; 1992; Ragan et al., 1994; Saunders & Kraft, 1994; 1996; 1997; Lluisma & Ragan, 1995; Millar et al., 1996; Saunders et al., 1996).

Ragan et al. (1994) published the first extensive SSU-based molecular phylogeny for the Rhodophyta that included 52 representatives from 15 red algal orders. The class Bangiophyceae (as Bangiophycidae) was found to be paraphyletic, encompassing species with relatively large sequence divergence. The other class, the Florideophyceae (as Florideophycidae), was resolved as a monophyletic clade emerging from among the bangiophytes. The monophyly of the Florideophyceae and the paraphyly of the Bangiophyceae was reinforced in the analyses of Saunders & Bailey (1997; 1999).

Saunders and Bailey (1997) analysed an expanded data set of florideophyte red algae and recognized four distinct lineages within this class (Fig. 4). Saunders & Kraft (1997) reviewed the taxonomic history of these lineages and implications of these results for the phylogeny and classification of the florideophyte red algae.

Lineage 1 - Hildenbrandiales
As Ragan et al., (1994) illustrated earlier (although with comparatively weaker support), the order Hildenbrandiales represented the most basal florideophyte lineage in the phylogenies of Saunders & Bailey (1997; 1999). Further, the position of the enigmatic genus *Apophlaea* (represented by *Apophlaea lyallii* Hooker f. et Harvey) as a member of the Hildenbrandiales was confirmed (Fig. 5). Interestingly though, the sequence generated by Saunders & Bailey (1999) for *Hildenbrandia rubra* (Sommerfelt) Meneghini
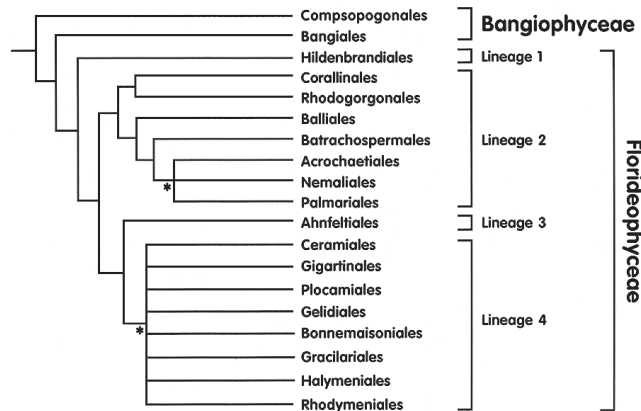

Figure 4. Schematic of SSU-based phylogenies outlined in Saunders & Bailey (1997) and Saunders & Kraft (1997), indicating the four lineages of florideophytes. Asterisks denote nodes which have traditionally received low bootstrap support and where phylogenetic relationships are presently unresolved.

Figure 4. chématisation des phylogénies basées sur les SSU à partir de Saunders & Bailey (1997) et Saunders & Kraft (1997), montrant les quatre lignées de Florideophytes. Les astérisques indiquent les nœuds qui correspondent à de faibles valeurs de "bootstrap" et où les relations phylogénétiques sont encore non élucidées.
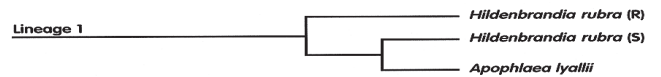

Figure 5. Florideophyte lineage 1, the Hildenbrandiales. Note the apparent paraphyly of the genus *Hildenbrandia*. R = sequence published in Ragan et al. (1994), S = sequence published in Saunders & Bailey (1999).

Figure 5. Lignée 1 des Florideophytes, Hildenbrandiales. Noter l'apparente paraphylie du genre *Hildenbrandia*. R = séquence publiée par Ragan et al. (1994), S = séquence publiée par Saunders & Bailey (1999).

did not group with a published sequence for that species (Ragan et al., 1994), a conundrum yet to be solved.

Lineage 2 - Two cap-layer group
In their 1997 study, Saunders & Bailey used SSU-based phylogenies to test Pueschel's (1994) hypotheses of pit-plug evolution. The results of the molecular data were consistent with four aspects of these hypotheses: 'naked' plugs represent the ancestral type (similar to those found in the Bangiophyceae), outer cap layers are homologous for all species that possess these structures, domed outer caps are ancestral to plate-like outer caps, and cap membranes are a derived feature within the two-cap layer lineage (Fig. 6). Therefore, all orders with two-layered pit plugs share a common ancestor (Acrochaetiales, Balbianiales (Sheath & Müller, 1999), Balliales (Choi et al., 2000), Batrachospermales, Corallinales, Nemaliales, Palmariales, and Rhodogorgonales.

Saunders & Bailey (1997) were the first to illustrate the close relationship between the recently described Rhodogorgonales (Fredericq & Norris, 1995) and the Corallinales. Further, this grouping was resolved as sister to a clade containing the Acrochaetiales, Batrachospermales, Nemaliales, and Palmariales.

Previously, Saunders et al. (1995) demonstrated a close relationship between the Acrochaetiales and Palmariales, and the position of the Nemaliales as sister to this assemblage (Fig. 6). In their study, *Rhodothamniella floridula* (Dillwyn) J. Feldmann, previously considered a member of the Acrochaetiales, was found to represent an early lineage within the Palmariales, and the earlier tentative placement of the Rhodophysemataceae within the Palmariales (Saunders & McLachlan, 1989; 1991) was confirmed. The position of the Acrochaetiales as a recently derived lineage within the phylogenies of Saunders & Bailey (1997) was also resolved by Ragan et al. (1994) and clearly refutes the hypothesis that this order is the most primitive florideophyte lineage (Garbary, 1978; Gabrielson et al., 1985; Gabrielson & Garbary, 1986; 1987; Garbary & Gabrielson, 1987).

More recently, Harper & Saunders (1998) performed an expanded study of the Acrochaetiales and found an amazing diversity within this morphologically homogenous order. Two clearly divergent groups of acrochaetioid algae were resolved (Fig. 6), but the relationships between these groups and the closely related Palmariales and Nemaliales remained equivocal. Harper & Saunders (1998) also illustrated that the enigmatic *Camontagnea oxyclada* (Montagne) Pujals grouped within the Rhodothamniellaceae of the Palmariales (Fig. 6), and was not allied to either the Ceramiales (lineage 4 herein) or Acrochaetiales, as had been previously suggested (Wollaston, 1968; Pujals, 1981; Womersley, 1994).
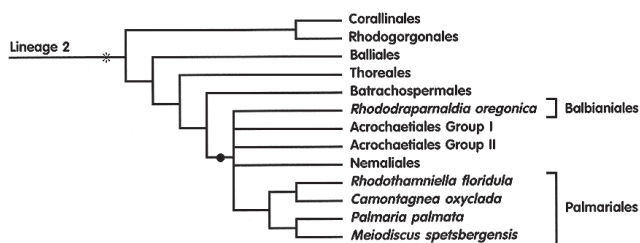

Figure 6. Florideophyte lineage 2, the 'two cap-layer group'. Asterisk denotes the hypothesized evolution of the domed outer cap while the dot represents the hypothesized evolution of the plate-like outer cap from a dome (Saunders & Bailey, 1997).
Figure 6. Lignée 2 des Floridéophytes, 'groupe à bouchon synaptique à deux membranes'. L'astérisque indique l'évolution du bouchon externe en forme de dôme alors que le point représente l'évolution hypothétique du bouchon externe en forme de plaque (Saunders & Bailey, 1997).

Using combined SSU and *rbc*L data, Vis et al. (1998) examined the systematics of the Batrachospermales. The family Thoreaceae was found to be only distantly related to other members of the Batrachospermales, a circumstance that Harper & Saunders (1998) confirmed, leading them to suggest that this family should receive ordinal recognition.

During the past year, three new red algal orders have been proposed to accommodate the diversity of taxa within lineage 2 (Fig. 6). Sheath & Müller (1999) recommended an order, Balbianiales, for two species of freshwater red algae, *Balbiania investiens* (Lenormand) Sirodot and *Rhododraparnaldia oregonica* Sheath, Whittick & Cole, both previously considered members of the Acrochaetiales (Swale & Belcher, 1963; Vis et al. 1998; respectively). In a more dramatic move, Choi et al. (2000) demonstrated that *Ballia callitricha* (C. Agardh) Kützing, long considered a representative of the Ceramiales (lineage 4 herein), occupied a basal position to the Acrochaetiales, Batrachospermales, Nemaliales, and Palmariales clade, and have proposed the order Balliales. Lastly, Sheath et al. (2000) have formally proposed the order Thoreales to accommodate this distinct clade.

Lineage 3 - Ahnfeltiales
The single genus *Ahnfeltia* is the only member within lineage 3 (Fig. 7). Maggs & Pueschel (1989) recognized that this genus was morphologically and reproductively unique in the Rhodophyta and proposed ordinal status. The position of the Ahnfeltiales was essentially unresolved in the phylogenies of Ragan et al. (1994), whereas Saunders & Bailey (1997) found this lineage to be weakly allied with lineage 2. Recently, however, Saunders & Bailey (1999) uncovered increased support for the Ahnfeltiales as sister to lineage 4 (Fig. 7).
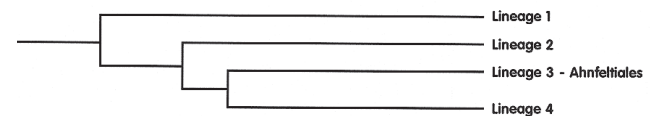

Figure 7. Florideophyte lineage 3, the Ahnfeltiales. In Saunders & Bailey (1999), lineage 3 grouped with lineage 4 in 90% (distance) and 53% (parsimony) of bootstrap replicates.
Figure 7. Lignée 3 des Floridéophytes, Ahnfeltiales. Dans Saunders & Bailey (1999), la lignée 3 est groupée avec la lignée 4 dans 90 % (distance) et 53 % (parsimonie) des réplicats de bootstrap.

Lineage 4 - Membrane-only group
This lineage contains the multitude of florideophyte orders (Fig. 8) characterized by pit plugs which have only an associated membrane (the Gelidiales is an exception in possessing an apparently independently derived single cap layer; Saunders & Bailey, 1997). SSU phylogenies for the included orders (Saunders & Kraft, 1994; 1996; Saunders & Bailey, 1997) have provided little resolution (Fig. 4) in this region of the tree, and relationships among these taxa remain largely equivocal.

In light of the poor to absent support for monophyly of the Ceramiales in previous works (Rice et al., 1991; Ragan et al., 1994; Saunders & Kraft, 1994; 1996), Saunders et al. (1996) performed an expanded study of the Ceramiaceae. They noted that the inclusion of a published sequence for *Ceramium nodulosum* (Lightfoot) Ducluzeau in their multiple alignment was causing reduced bootstrap support for a monophyletic Ceramiales. This sequence has, nevertheless, been recently confirmed (Choi & Saunders, unpublished) and is a bona fide SSU sequence for this species. Thus, its detrimental impact on phylogenetic resolution is not the result of incorrect data. A noteworthy discrepancy has been found between a published sequence by Ragan et al. (1994) for the genus *Griffithsia* and a sequence determined by Saunders et al. (1996) for another species of this genus (Fig. 8). Ragan et al. (1994) found their representative of *Griffithsia* (*G. globulifera* Harvey) to be strongly allied to the Gelidiales, whereas Saunders et al.
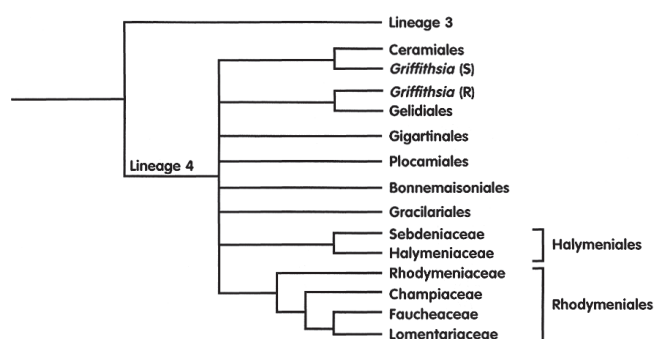
Figure 8. Florideophyte lineage 4, the 'membrane-only group'. Note the differing positions of the sequences for the genus *Griffithsia*. R = sequence published in Ragan et al. (1994), S = sequence published in Saunders et al. (1996).

Figure 8. Lignée 4 des Floridéophytes, le groupe à bouchon synaptique à membrane unique. Noter les différentes positions des séquences pour le genre *Griffithsi*a. R = séquence publiée par Ragan et al. (1994), S = séquence publiée par Saunders et al. (1996).

(1996) resolved *Griffithsia monilis* Harvey as a member of the Ceramiales. It was concluded that the published sequence for the former was not correct (Saunders et al., 1996).

In the first of a series of publications dealing with the Gigartinales and Rhodymeniales, Saunders & Kraft (1994) examined the relationships between the gigartinalean families Plocamiaceae, Sphaerococcaceae, Phacelocarpaceae, and Nizymeniaceae. Comparatively large nucleotide differences and a suite of unique anatomical and morphological characters led to their proposal of a new rhodophyte order, the Plocamiales (Fig. 8). Recently, this proposal has received support from published *rbc*L phylogenies (Fredericq et al., 1996).

Subsequently, Saunders & Kraft (1996) examined representatives of the Kylinian orders Cryptonemiales and Gigartinales, using molecular data to test the validity of the merger of these two orders (Kraft & Robins, 1985). With only two exceptions - the families Halymeniaceae and Sebdeniaceae - the phylogenies of Saunders & Kraft (1996) supported a monophyletic origin for a combined Gigartinales and Cryptonemiales. Thus, they proposed resurrection of the Cryptonemiales as a new order, the Halymeniales (Fig. 8), to accommodate the Halymeniaceae and Sebdeniaceae. Ironically, these two families were exemplars of the previous concepts for the Cryptonemiales and Gigartinales.

Until recently, relatively few molecular studies have focused on the Rhodymeniales. Millar et al. (1996) used a combination of morphological and molecular evidence to describe a new genus within the Rhodymeniaceae,

Cephalocystis. An expanded study of the Rhodymeniales has recently been performed by Saunders et al. (1999), with a complete revision of the systematics and classification of the order, including the description of two new genera (*Irvinea* and *Sparlingia*), support for a previously contentious genus (*Cryptarachne*) as distinct from the genus *Chrysemenia*, and the description of a new family (Faucheaceae) (Fig. 8).

Sequences of the internal transcribed spacer regions

In contrast to the conserved sequences of the coding regions of the rRNA genes, the two ITS regions are highly variable. Although the ITS1 and ITS2 regions are included in the initial ribosomal transcript (Fig. 1), they are excised after transcription and not used in the final functioning of ribosomes. Hence, the ITS1 and ITS2 regions experience less evolutionary constraint (purifying selection) and accumulate many more mutations than the rRNA coding regions. The ITS1 and ITS2 regions are less variable than the IGS, and are valuable for phylogenetic comparisons at the intrafamilial and intrageneric levels (Appels & Dorvak, 1982).

Within the Rhodophyta, ITS regions have been useful in determining phylogenetic relationships at lower taxonomic levels and for population studies (Goff et al., 1994; Chiovitti et al., 1995; Coleman & Mai, 1997; Müller et al., 1997). Present research in our laboratory involves examining the relationships among species of the gigartinalean family Dumontiaceae using a combination of SSU and ITS data (Tai et al. 2001). Although this family has been the subject of many studies (e.g. Bert, 1965; Lindstrom & Scagel, 1987; Lindstrom, 1988), the phylogenetic relationships between the genera of the Dumontiaceae, and among this family and the other families of the Gigartinales, have not been fully resolved.

Sequences of the large-subunit ribosomal DNA

Sequences of this region of the ribosomal cistron display a common, largely conserved core that in eukaryotes is interspersed by 12 divergent, rapidly-evolving domains (D1-D12; Fig. 9) (Hassouna et al., 1984; Michot et al., 1984; Michot & Bachellerie, 1987; Lenaers et al., 1989). The conservative core (>2000 bp) is constrained by purifying selection, and thus is suitable for phylogenetic evaluation among distant taxa. LSU core data have been used to infer evolutionary relationships among archaebacteria (Leffers et al., 1987; Gouy & Li, 1989a), eukaryotic kingdoms (Cedergren et al., 1988; Gouy & Li, 1989b; Lenaers et al., 1989), helminths (Qu et al., 1986; Gill
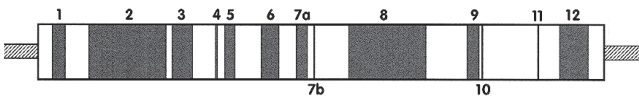
Figure 9. A schematic of the LSU, showing the relative sizes and positions of the twelve divergent domains (shaded gray and labeled 1-12). This diagram is based on the multiple alignment of Lenaers et al. (1989). Scale bar = 250 bp.
Figure 9. Schéma de la LSU, montrant les tailles et positions relatives de 12 domaines divergents (ombrage gris et numérotation de 1 à 12). Ce diagramme est basé sur un alignement multiple publié par Lenaers et al. (1989). Barre d'échelle = 250 bp.

Figure 10. Simplified distance trees (Neighbour-joining) for selected orders of florideophyte red algae. A) Phylogenetic tree based on SSU data. B) Phylogenetic tree based on LSU data. Numbers at nodes indicate percentage of times individual nodes were supported in 1000 bootstrap replicates.
Figure 10. Arbres de distance simplifiés (Neighbour-joining) pour certains ordres de Floridéophytes. A) Arbre phylogénétique basé sur la SSU. B) Arbre phylogénétique basé sur la LSU. Les numéros au niveau des nœuds indique les pourcentages de validation de ces nœuds sur 1000 réplicats.

et al., 1988), protists (Baroin et al., 1988; Preparata et al., 1989), and higher plants (Kuzoff et al., 1998). LSU divergent domains, on the other hand, have the potential to be useful for phylogenetic and taxonomic analyses of closely related species (Baroin et al., 1988; Qu et al., 1988; Lenaers et al., 1991).

Sequences of the LSU have been used only sparingly in algal systematics, and even less so with respect to the Rhodophyta. In the only published results to our knowledge, Freshwater & Bailey (1998) and Freshwater et al. (1999) obtained partial LSU sequence for members of the Gelidiales, and used this data in a multi-gene comparison for gelidealean phylogeny.

Our present research focus is on the LSU and its application in resolving phylogenetic relationships within the Rhodophyta. Previous SSU-based phylogenies on the Rhodophyta have revealed a number of unresolved nodes (Saunders & Bailey, 1997; Saunders & Kraft, 1997; Fig. 4), and these equivocal regions are our targets of primary interest. Sequence data from the LSU possess both the conservation of the SSU (>2000 bp of conservative core) and ITS-level variation (>1500 bp of divergent domains) all within one gene system. We postulate the LSU will provide the resolution that the SSU could not.

We designed PCR and sequencing primers by aligning LSU sequences of *Chlorella* (Chlorophyta) and *Prorocentrum* (Dinophyta). We reasoned that regions conserved between these two very divergent groups would most likely be conserved within the Rhodophyta. At present, we have obtained LSU sequence data for seven species of red algae spanning six florideophyte orders: Acrochaetiales, Ceramiales, Gigartinales, Palmariales, Rhodogorgonales, and Rhodymeniales. Partial data for the Gelidiales generated by Freshwater & Bailey (1998) were also used in our preliminary multiple alignment. Fig. 10 compares distance analyses of complimentary SSU and LSU data. In terms of tree topology, the resulting phylogenies were identical. However, bootstrap values on the LSU tree were comparable or considerably higher than respective values on the SSU tree. These early results are encouraging and as
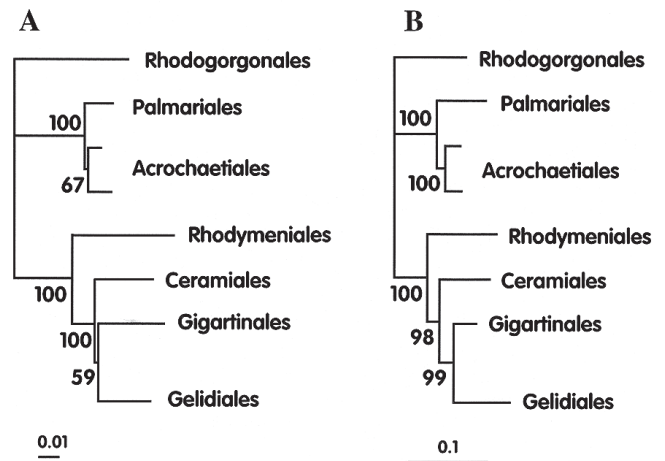
more LSU data for red algae are determined, the full potential of the LSU in resolving red algal relationships will be realized.

Conclusion

Sequences derived from the ribosomal cistron of red algae have had a profound impact on the classification and systematics of this Division. By far, data generated for the SSU rDNA have had the largest impression at higher taxonomic levels. In the last four years alone, molecular data have been responsible for the proposal of four florideophyte orders (Plocamiales - Saunders & Kraft, 1994; Halymeniales - Saunders & Kraft, 1996; Balliales - Choi et al., 2000; and Balbianiales - Sheath & Müller, 1999), as well as supporting earlier ordinal proposals (Pueschel & Cole, 1982) based on pit plug ultrastructure. Data from the ITS regions have not been utilized to the same extent as the SSU in red algal systematics, but considering that the extreme variability of ITS data has restricted their use to the level of genus and/or species, this is not surprising. In our opinion, sequences of the LSU hold the most promise for the future. In possessing a number of divergent domains interspersed among a conservative core, these data offer the promise of increased resolution at equivocal nodes and applicability across a broader taxonomic spectrum.

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