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Phylogeny of Alariaceae, Laminariaceae, and Lessoniaceae (Phaeophyceae) Based on Plastid-Encoded RuBisCo Spacer and Nuclear-Encoded ITS Sequence Comparisons

Hwan Su Yoon,*,† Ju Yeon Lee,* Sung Min Boo,*,[1] and Debashish Bhattacharya†

*Department of Biology, Chungnam National University, Daejon 305-764, Korea and †Department of Biological Sciences, University of Iowa, 239 Biology Building, Iowa City, Iowa 52242-1324

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Concatenated sequences from the plastid-encoded RuBisCo spacer and nuclear-encoded rDNA ITS region of the Alariaceae, Laminariaceae, and Lessoniaceae as currently recognized were used to determine the phylogeny of kelps (Phaeophyceae). Our analyses indicate that all taxa in the Alariaceae, Laminariaceae, and Lessoniaceae form a monophyletic lineage (the Laminariales *sensu stricto*). The phylogenetic analyses show that the kelps form eight well-supported clades (represented by *Egregia, Laminaria, Hedophyllum, Macrocystis, Alaria, Agarum, Ecklonia,* and *Lessonia*) that conform to the tribes of the current morphological classification system of the "advanced" kelps. Our results suggest that the kelps should be classified into eight families rather than the three that are presently used. The interrelationships among the eight lineages were, however, unresolved in the phylogenetic analyses. In all trees, *Egregia* diverged first and is the sister to the other kelp taxa. Our phylogenetic analyses also indicate that *Kjellmaniella* and *Laminaria* do not form a monophyletic group. Taken together, the RuBisCo spacer and rDNA ITS prove useful for understanding the evolutionary history of the advanced kelps and provide a new framework for establishing the systematics of these commercially important brown algae. © 2001 Academic Press

Key Words: **Alariaceae; kelps; Laminariaceae; Lessoniaceae; Phaeophyceae; phylogeny; RuBisCo spacer; ITS.**

INTRODUCTION

Kelps (Laminariales, Phaeophyceae) are some of the best-known seaweeds with species like *Macrocystis pyrifera* reaching a maximum length of over 40 m (Bold and Wynne, 1985). Kelps create a marine "forest" that provides a habitat for a diversity of marine organisms. These brown algae are also commercially valuable foods, are a source for alginates, and are used in medicine (Lee, 1989). Despite their economic and ecological importance, kelp phylogeny remains unresolved (e.g., Saunders and Druehl, 1992; Druehl *et al.*, 1997; Boo *et al.*, 1999; Reviers and Rousseau, 1999).

The Laminariales has a heteromorphic, diplohaplontic life cycle, with an alternation of highly differentiated diploid sporophytes and microscopic haploid gametophytes (van den Hoek *et al.*, 1995). This order currently contains seven families: the Akkesiphycaceae, Pseudochordaceae, Chordaceae, Phyllariaceae, Alariaceae, Laminariaceae, and Lessoniaceae. The former four families are easily distinguished using morphological characters and are regarded as "primitive" kelps (Wynne and Loiseaux, 1976; Kawai and Kurogi, 1985; Henry and South, 1987; Maier, 1995; Boo *et al.*, 1999; Kawai and Sasaki, 2000). The Alariaceae, Laminariaceae, and Lessoniaceae share many characteristics such as the absence of an eyespot in the zoospore, and the presence of the hormone lamoxirene and of conductive tissues, which have been regarded as apomorphic features (Druehl and Saunders, 1992). These latter "advanced" families contain most kelp genera and species, and their interfamilial boundaries are unclear (Druehl and Saunders, 1992; Saunders and Druehl, 1992; Tan and Druehl, 1996; Yoon and Boo, 1999; Boo and Yoon, 2000).

The Alariaceae as currently defined is characterized by the restriction of the unilocular sporangia to sporophylls which arise in two rows in acropetal succession from the transition zone. This family is divided into three tribes (Setchell, 1893; Setchell and Gardner, 1925): the Alarieae which includes *Alaria* and *Pterygophora* and which has sporophylls on the stipe; the Eckloniеae which includes *Ecklonia*, *Eckloniopsis*, and *Eisenia* and which has sporangia borne on all blades arising from the transition zone; and the Egregieae which contains only *Egregia* with sporangia on both stipe and blade. The northwestern Pacific genus *Undaria*, although it has sporophylls as well as sporangia

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[1] To whom correspondence should be addressed. Fax: +82-42-822-9690. E-mail: smboo@hanbat.chungnam.ac.kr.

on blades, has also been placed in the Alarieae (Okamura, 1936; Kawashima, 1993).

The Laminariaceae, as currently recognized, is characterized by the presence of a distinct stipe, at least when young, paraphyses without hyaline appendages, and the absence of outgrowths or splitting of the transition zone. This family is divided into four tribes (Setchell, 1893; Setchell and Gardner, 1925): the Agarieae which includes *Agarum*, *Costaria*, and *Thalasiophyllum* and which has blades, either longitudinally ribbed or perforated, or both; the Cymathaereae which includes *Cymathaere* and *Pleurophycus* and which has blades with one or more longitudinal folds; the Hedophylleae which includes *Arthrothamnus*, *Hedophyllum*, and *Streptophyllopsis* and which has the false branches of pseudostipes formed from the thickened lower margins of the fronds; and the Laminarieae which includes *Kjellmaniella* and *Laminaria* and which shares a persistent stipe and a bullate (presence of dimpled depressions) or nonbullate (smooth thallus without depressions) frond.

The Lessoniaceae as currently described is the largest of all algae and is characterized by the splitting of the single blade into two, with the division extending through the transition zone. The family is divided into three tribes (Setchell, 1893; Setchell and Gardner, 1925): the Lessonieae which includes *Dictyoneurum*, *Dictyoneuropsis*, *Lessonia*, *Nereocystis*, and *Postelsia* and which has regularly dichotomous stipes but without specialized sporophylls; the Marcrocysteae which includes *Macrocystis* and *Pelagophycus* and which has unilateral splitting, thus producing a scorpioid sympodial stipe; and the Lessoniopsieae which contains only *Lessoniopsis* with specialized sporophylls and splitting of the basal blade. However, the division of the three described families and their subdivision into ten tribes is not clear from the existing classification. For example, Setchell and Gardner (1925) suggested that the Lessoniopsieae could be placed either in the Lessoniaceae because of its splitting blade or in the Alariaceae because of its specialized sporophylls.

Recent analyses of molecular sequence data (e.g., Saunders and Druehl, 1992; Druehl *et al.*, 1997; Yoon and Boo, 1999) are not congruent with the current classification system of the advanced kelps at the higher taxonomic categories both with regard to the phylogenetic relationships among families and tribes as well as the taxonomic associations of individual genera. However, because ITS sequences are too variable (Saunders and Druehl, 1993a,b; Druehl *et al.*, 1997) and the 18S rDNA gene too conserved (Saunders and Druehl, 1992; Tan and Druehl, 1996; Boo *et al.*, 1999), the phylogenetic relationships of the advanced kelps remain unresolved.

In order to reexamine the current classification system and the phylogenetic relationships of the taxa within the currently recognized Alariaceae, Laminariaceae, and Lessoniaceae, we used the complete sequence of the plastid-encoded RuBisCo spacer as well as the nuclear-encoded rDNA ITS region. The RuBisCo spacer and the ITS sequence data were analyzed both separately and in combination to infer kelp phylogeny. All currently recognized tribes and genera of advanced kelps except *Pleurophycus* and *Streptophyllopsis* of the Laminariaceae and *Dictyoneuropsis* of the Lessoniaceae were included in this study.

MATERIALS AND METHODS

Taxon Sampling

Forty-four species of kelps were collected from natural populations. The names, authorities, taxonomic positions, collection information, and GenBank accession numbers of these taxa are listed in Table 1. Voucher specimens have been deposited at the Chungnam National University Herbarium, Daejon, Korea. Outgroup taxa were chosen on the basis of published phylogenetic studies (van den Hoek *et al.*, 1995; Tan and Druehl, 1996; Peters, 1998; Boo *et al.*, 1999).

Plastid-Encoded RuBisCo Spacer Sequences

Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Approximately 20 mg of ground algal tissue was incubated in 500 μL of 2% CTAB buffer (with the addition of 2% β-mercaptoethanol) at 60°C for 45 min. Extractions with chloroform-isoamyl alcohol (CIA, 24:1 vol/vol) were performed repeatedly until complete removal of the interphase was achieved. Nucleic acids were precipitated with 1 mL of 100% cold ethanol (with 10 μL of 2 M sodium acetate) at -70°C for 30 min, pelleted by centrifugation (13,000 rpm/4°C/10 min), air dried, and dissolved in 200 μL distilled water (DW). The DNA was reprecipitated with the addition of 500 μL of 90% cold ethanol at -70°C for 30 min, washed with 70% ethanol twice, air dried, and then dissolved in 150 μL DW.

Polymerase chain reactions using genomic DNA were done for the complete RuBisCo spacer, including the partial 3′-terminus of *rbc*L and 5′-terminus of *rbc*S (RuBisCo spacer region) using the primer pair RS1/RS2 following the methods of Yoon and Boo (1999). PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's protocol. Sequencing templates were prepared using the two PCR primers and the BigDye Terminator Cycle Sequencing Kit [Applied Biosystems (ABI), Perkin-Elmer Cetus, Norwalk, CT]. Sequences of both strands were determined for all taxa using an ABI PRISM 377 DNA Sequencer (Foster City, CA). The electropherogram output for each sample was edited using the program SeqEd v. 1.0.3 (Applied Biosystems Inc., Foster City,

TABLE 1

List of Species Investigated in This Study

Taxa	Collection site and date	GenBank accession number	
		RuBisCo spacer	ITS region
Family Alariaceae			
Tribe Alarieae			
Alaria crassifolia Kjellman	Hakodate, Japan, Aug. 97	AF318957[a]	AF319001
A. esculenta (L.) Greville	Nova Scotia, Canada, July 97	AF318958[a]	AF319002
A. marginata Postels *et* Ruprecht	Bamfield, Canada, July 97	AF318959[c]	AF319003
A. praelonga Kjellman	Akkeshi, Japan, Aug. 97	AF318960[a]	AF319004
Pterygophora californica Ruprecht	Oregon, USA, July 98	AF318965	AF319005
Undaria peterseniana (Kjellm.) Okamura	Jeju, Korea, Jan. 97	AF318961[a]	AF319006
U. pinnatifida (Harv.) Suringar	Jeju, Korea, July 97	AF318966	AF319007
U. undarioides (Yendo) Okamura	Wakayama, Japan, Aug. 97	AF318962[a]	AF319008
Tribe Ecklonieae			
Ecklonia cava Kjellman	Chuksan, Korea, Feb. 99	AF318967	AF319009
E. stolonifera Okamura	Eupchon, Korea, Feb. 99	AF318968	AF319010
Eckloniopsis radicosa (Kjellm.) Okamura	Kochi, Japan, Jun. 98	AF318969	AF319011
Eisenia bicyclis (Kjellm.) Setchell	Ulreungdo, Korea, Oct. 97	AF318963[a]	AF319012
Tribe Egregieae			
Egregia menziesii (Turn.) Areschoug	Oregon. USA. July 98	AF318970	AF319013
Family Laminariaceae			
Tribe Laminarieae			
Laminaria diabolica Miyabe	Akkeshi, Japan, May 99	AF318975	AB022795-6[b]
L. digitata (Hudson) Lamouroux	Dover, Great Britain, Jan. 98	AF318971	AF319014
L. hyperborea (Gunnerus) Foslie	Roscoff, France, Apr. 00	AF318972	AF319015
L. japonica Areschoug	Jindo, Korea, Nov. 96	AF318976	AF319018
L. longipedalis Okamura	Akkeshi, Japan, May 99	AF318977	AB022797-8[b]
L. longissima Miyabe *in* Okamura	Akkeshi, Japan, May 99	AF318978	AB022801-2[b]
L. religiosa Miyabe *in* Okamura	Otaru, Japan, May 99	AF318979	AB022791-2[b]
L. saccharina (L.) Lamouroux	Oregon, USA, July 98	AF318980	AF319019
L. setchellii Silva	Oregon, USA, July 98	AF318973	AF319016
L. sinclairii (Harvey *ex* Hooker *et* Harvey) Farlow, Anderson and Eaton	Oregon, USA, July 98	AF318974	AF319017
Kjellmaniella crassifolia Miyabe	Gangreung, Korea, Dec. 96	AF318981	AF319020
K. gyrata (Kjellm.) Miyabe	Akkeshi, Japan, Aug. 97	AF318982	AF319021
Tribe Hedophylleae			
Arthrothamnus bifidus (Gmel.) Ruprecht *in* Middendorf	Akkeshi, Japan, May 99	AF318984	AF319023
Hedophyllum sessile (C. Ag.) Setchell	Oregon, USA, July 98	AF318985	AF319024
Tribe Agareae			
Agarum clathratum Dumortier	Akkeshi, Japan, Aug. 97	AF318986	AF319025
A. turneri Postels *et* Ruprecht	Kamchatka, Russia, July 98	AF318987	AF319026
Costaria costata (C. Ag.) Saunders	Shinnam, Korea, Jan. 97	AF318988	AF319027
Thalassiophyllum clathrus (Gmel.) Postels *et* Ruprecht	Kamchatka, Russia, July 98	AF318989	AF319028
Tribe Cymathaereae			
Cymathaere japonica Miyabe *et* Nagai *in* Nagai	Rausu, Japan, July 99	AF318983	AF319022
Family Lessoniaceae			
Tribe Lessonieae			
Dictyoneurum californicum Ruprecht	Carmel Bay, USA, Aug. 98	AF318990	AF319029
Lessonia flavicans Bory	Tierra del Fuego, Argentina, Nov. 98	AF318992	AF319031
L. trabeculata Villouta *et* Santelices	Ilo. Peru. Sep. 98	AF318991	AF319030
L. vadosa Searles	Chubut, Argentina. Dec. 98	AF318993	AF319032
Nereocystis luetkeana (Mer.) Postels *et* Ruprecht	Oregon, USA, July 98	AF318994	AF319033
Postelsia palmaeformis Ruprecht	Oregon, USA, July 98	AF318995	AF319034
Tribe Lessoniopseae			
Lessoniopsis littoralis (Tilden) Reinke	Oregon, USA, July 98	AF318996	AF319035
Tribe Macrocysteae			
Macrocystis integrifolia Bory	California, USA, Aug. 98	AF318997	AF319036
M. pyrifera (L.) C. Agardh	California, USA, Aug. 98	AF318998	AF319037
Macrocystis sp.	Chubut, Argentina, Dec. 98	AF318999	AF319038
Pelagophycus porra (Leman) Setchell	California, USA, Dec. 99	AF319000	AF319039
Outgroup			
Family Chordaceae			
Chorda filum (L.) Stackhouse	Taean, Korea, Aug. 96	AF318964[c]	Z98585-6[c]

[a] Yoon & Boo (1999).

[b] Yotsukura et al. (1999).

[c] Peters (1998).

CA). Sequences were initially aligned using CLUSTAL W version 1.6 (Thompson *et al.*, 1994) and the alignments were refined manually using SeqPup, a multisequence editing program (Gilbert, 1995) by first comparing sequences from species groups that are reported to be closely related on the basis of morphological evidence. Once these alignments were determined, more divergent groups of sequences were compared until all ingroup and outgroup taxa were aligned. Regions of ambiguous alignment (a total of 23 positions) were exclued for subsequent phylogenetic analyses.

The boundaries of the RuBisCo spacer and the flanking coding regions were determined by comparison to several published sequences obtained from a variety of brown algae (Assali *et al.*, 1990; Stache-Crain *et al.*, 1997; Lee *et al.*, 1999; Yoon and Boo, 1999). The RuBisCo spacer sequences were evaluated for several standard descriptive parameters, including size, percent G + C content, percent pairwise divergence, percent parsimony-informative sites, gamma shape parameter, and proportion of invariable sites using PAUP* v4.0b8 (Swofford, 2001).

Nuclear-Encoded rDNA ITS Sequences

The same genomic DNAs used for amplification of the RuBisCo spacer were used to amplify ITS regions. Reaction conditions and most of details of PCR and purification of the PCR products were identical to those used for the RuBisCo spacer. The complete ITS1, 5.8S gene, and ITS2, including partial 3′ end of SSU and 5′ end of LSU (ITS region), was amplified using the newly designed primer pairs LB1 (forward; 5′-CGCGAGTCATCAGCTCGCATT-3′) and LB2 (reverse; 5′-AGCTTCACTCGCCGTACTGG-3′) or JO6 (van Oppen *et al.*, 1995). The primers LB1 and BC2 were used for direct sequencing of the ITS1 region (Saunders and Druehl, 1992). The primers used to sequence the ITS2 region were LB2 or JO6 and YB1 (5′-TTGCAGAATCCAGTGAATCATC-3′). Previously published sequences were included in the alignment (Peters, 1998; Yotsukura *et al.*, 1999), of which a total of 770 bp was used to build trees. A number of hypervariable regions dispersed throughout the ITS (471 nt in total) could not be unambiguously aligned and were excluded from the phylogenetic analyses. The alignments of the RuBisCo spacer and the ITS sequences are available upon request from H. S. Y.

Phylogenetic Analyses

The program MODELTEST (V3.04, Posada and Crandall, 1998) was used to screen 56 different models of sequence evolution to identify which best fits the combined RuBisCo spacer and ITS data. The Akaike information criterion (Akaike, 1974) identified the general time-reversible (GTR) model (Rodríguez *et al*, 1990), incorporating estimates of the proportion of invariable sites (I) and alpha, which is the shape parameter of the gamma distribution (G, correction for rate variation across sites), as the best model for our data. The GTR + I + G model also showed the highest likelihood score of all 56 models tested with MODELTEST and was used thereafter in the neighbor-joining and ML analyses with the estimated I and G parameter values. For the heuristic ML analysis with the combined data, the tree was built stepwise with random sequence addition (ten replicates) and the resulting phylogeny was rearranged with tree bisection–reconnection (TBR). The bootstrap method (Felsenstein, 1985) was not implemented with the ML analysis due to the excessive computation time. We did, however, determine bootstrap support values for groups in the ML tree using the neighbor-joining method and distance matrices calculated with the GTR + I + G model (1500 replicates). Maximum parsimony (MP) analyses were done with combined or separate data sets using a heuristic search of 10 replicates with random stepwise addition and the TBR branch-swapping algorithm. Gaps were treated as missing data. Equally parsimonious trees were represented as strict consensus cladograms. Bootstrap MP analyses (1500 replicates) were done with the data sets. The kelp phylogenies were outgroup rooted with homologous sequences from *Chorda filum*.

RESULTS

Plastid-Encoded RuBisCo Spacer Sequence Data

Comparative descriptions and analyses of the sequences of 44 taxa including eight published sequences are based on 655 bp of the RuBisCo spacer region. The length of the RuBisCo spacer varied from 217 to 286 bp. The 3′-end of *rbc*L gene that was amplified was 240 bp in length, and that of the 5′-end of *rbc*S gene was 111 bp. The average G + C content across the entire data set was 30.3%: 18.2% for the RuBisCo spacer, 41.8% for the 3′-end of *rbc*L, and 34.8% for the 5′-end of *rbc*S. The RuBisCo spacer lengths and the general pattern of nucleotide composition in the sequences in this study are similar to those reported by Yoon and Boo (1999).

Alignment of the final matrix required the introduction of 22 small (1–7 bp) indels distributed throughout the RuBisCo spacer, with seven large (10–82 bp) indels. For example, five *Laminaria* species share a 55-bp indel, whereas three *Lessonia* species uniquely share a 51-bp indel. There were no differences between the sequences of *Ecklonia cava* and *E. stolonifera*, and between *Laminaria japonica* and *L. religiosa*. Of 632 bp which were used in phylogenetic analysis, 201 nucleotide positions were variable and 132 positions were parsimony informative; 91 in the RuBisCo spacer, 32 from the 3′-end of *rbc*L, and nine from the 5′-end of *rbc*S.

When sequences from all taxa used in this study were considered and gaps treated as missing data, the MP analysis recovered 212 shortest trees of 446 steps. A strict of the 212 trees showed a single kelp lineage comprising eight clades with weak (52% for *Agarum*) to strong (e.g., 100% for *Ecklonia*) bootstrap support for the monophyly of each of these clades.

Nuclear-Encoded rDNA ITS Sequence Data

Comparative descriptions and analyses of the sequences for 44 taxa including five published sequences are based on 1201 bp of the ITS region. The length of the ITS 1 varied from 224 to 346 bp, and that of the ITS2 from 239 to 385 bp. The coding 5.8S region was 160 bp in length. The 3′-end of the rDNA SSU gene that was determined was 159 bp, and the 5′-end of the LSU gene was 22 bp. Average G + C content across the entire set of sequences was 56.3%: 55.1% for ITS1 and 63.8% for ITS2. The ITS lengths and general pattern of nucleotide composition in the sequences obtained from our material are similar to those reported in Saunders and Druehl (1993a,b).

Alignment of the final data matrix required the introduction of numerous indels of 1–19 bp distributed throughout the ITS1 and the ITS2, with five large indels of over 20 bp. Furthermore, indels of length 80 bp or greater were introduced in the middle of the ITS1 region and the end of ITS2, respectively. As is the case between *Laminaria japonica* and *L. religiosa* (Yotsukura *et al.*, 1999), there were no differences between the sequences of *Macrocystis integrifolia* and *M. pyrifera*. Of 770 bp which were used in the analysis, 213 nt were variable, and 147 positions were parsimony informative: 84 nt in the ITS 1, 57 nt in the ITS 2, 3 nt in the 3′ end of the 18S, and 3 nt in the 5.8S.

The MP analysis recovered 105 shortest trees of 610 steps. A strict consensus of the 105 trees showed a single monophyletic kelp lineage, comprising eight clades with moderate (72% for *Laminaria*) to strong (100% for *Ecklonia*) bootstrap support for the monophyly of the clades. The composition of members in each clade was identical with those in the RuBisCo spacer analysis.

Combined RuBisCo Spacer + ITS Sequence Data

The model that best fits the combined sequence data was the general time-reversible model with among-site rate variation accommodated by assuming some proportion of sites to be invariable and rates at the remaining sites to follow the gamma distribution (GTR + I + G). The ML analyses using this model (Fig. 1) showed the advanced kelps to be monophyletic and to consist of eight well-supported clades (87–100% bootstrap support). *Egregia* was sister to the other advanced kelp genera, however, without bootstrap support (39%). Four *Laminaria* species (*L. digitata, L. hyperborea, L. setchellii,* and *L. sinclairii*) formed the well-resolved *Laminaria* clade (96%), whereas the other six *Laminaria* species, *Arthrothamnus, Cymathaere, Hedophyllum,* and *Kjellmaniella* formed another clade that includes *Hedophyllum* within the tribe Hedophylleae. The genus *Kjellmaniella* was not monophyletic. The *Macrocystis* clade comprised *Macrocystis, Nereocystis,* and *Postelsia* with strong bootstrap support (100%). *Alaria, Lessoniopsis, Pterygophora,* and *Undaria* formed a single clade with *Undaria* being sister to the remaining genera (98%). *Lessoniopsis* was more closely related to *Pterygophora* than to *Alaria*. *Agarum, Costaria, Dictyoneurum,* and *Thalassiophyllum* formed the strongly supported *Agarum* clade (87%). *Ecklonia, Eckloniopsis,* and *Eisenia* formed a robust grouping (*Ecklonia* clade, 100%), in which *Ecklonia cava* and *Eckloniopsis radicosa* shared a node. Three *Lessonia* species formed the monophyletic *Lessonia* clade with strong support (100%).

The combined RuBisCo spacer, ITS sequence data set contained 279 synapomorphic characters. The MP analysis of the combined data recovered four trees (length = 1083 steps; CI = 0.575; RI = 0.777). The strict consensus tree (Fig. 2) of the MP trees of the combined data set had a similar topology to the individual RuBisCo spacer and the ITS trees, but showed increased bootstrap support for the monophyly of each of the advanced kelp clades (see Fig. 2).

DISCUSSION

Congruence between RuBisCo Spacer and ITS Data Sets

This study is an initial estimate of phylogenetic relationships within the Alariaceae, Laminariaceae, and Lessoniaceae based on separate and combined analyses of RuBisCo spacer and ITS sequences. We sampled 22 of the 25 existing genera of advanced kelps. Our study shows that trees inferred from these data were generally in agreement with each other. As shown in Fig. 2, the values of bootstrap support increased in the tree inferred from the combined data set, and the number of the shortest trees decreased, consistent with an improved phylogenetic signal. All basal and terminal nodes were more strongly supported by combining the RuBisCo spacer and the ITS sequences. For example, the *Agarum* node, weakly supported in the RuBisCo spacer (52%, Fig. 2), shows strong support (94%, Fig. 2) in the tree constructed from the combined data set.

Phylogenetic Relationships at the Familial Level

The RuBisCo spacer and ITS data show that all the taxa in the Alariaceae, Laminariaceae, and Lessoniaceae are monophyletic, as found in previous analyses of 18S and ITS sequences (Saunders and Druehl, 1993a,b; Druehl *et al.*, 1997; Boo *et al.*, 1999, Boo and Yoon, 2000). The monophyly of these three families is

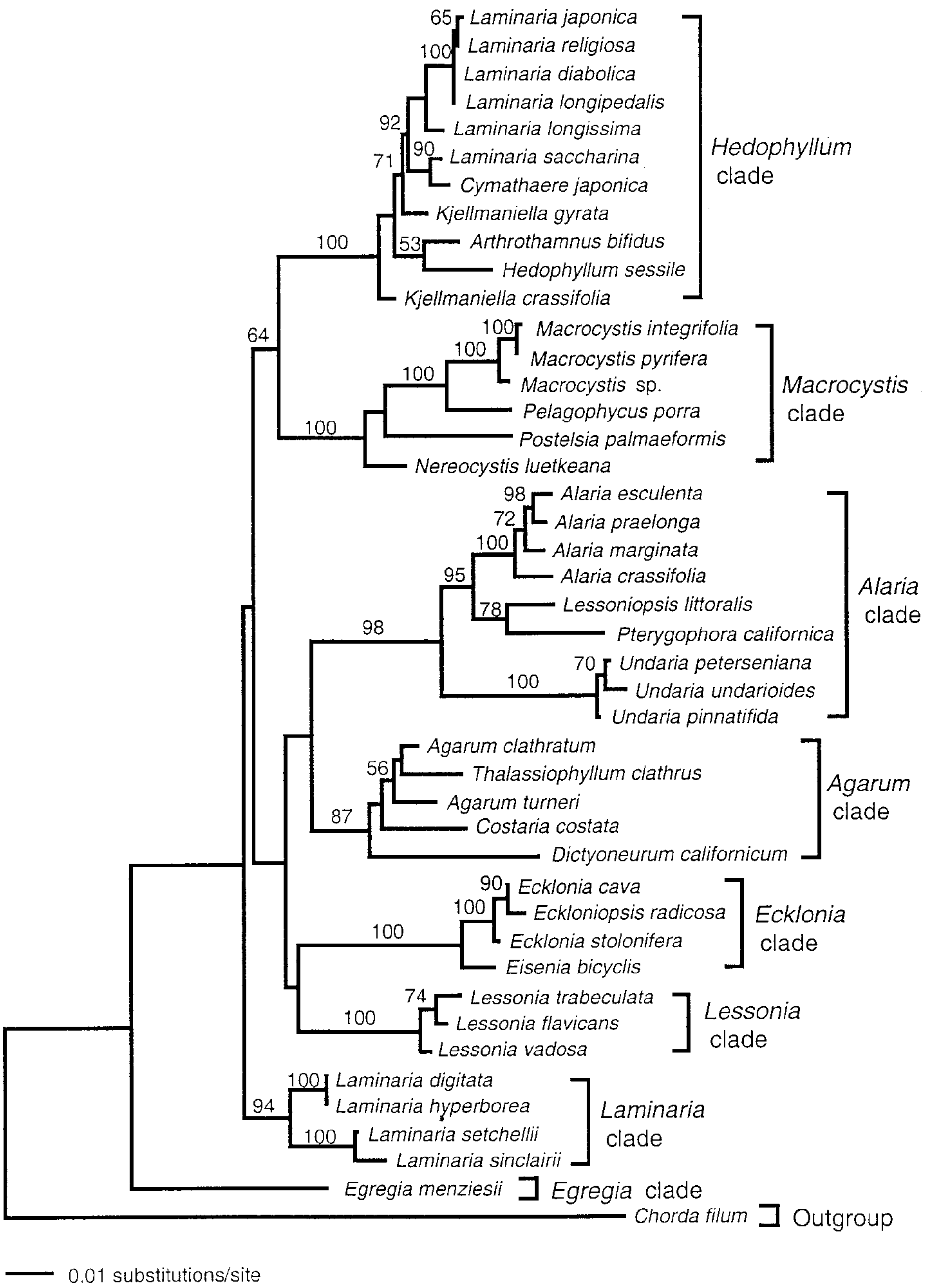

FIG. 1. Maximum-likelihood tree of kelps inferred from the concatenated RuBisCo spacer and ITS sequence data set for 44 taxa of the Alariaceae, Laminariaceae, and Lessoniaceae. This tree (-Ln likelihood = 7609.49) was inferred using the GTR + I + G model with different rates of substitution for each substitution class (A↔C = 1.1555, A↔G = 1.6482, A↔T = 0.5840, C↔G = 1.7225, C↔T = 2.8632, G↔T = 1), among-site rate variation accommodated by assuming a proportion of invariable sites (0.5135), and rates at the remaining sites following a gamma distribution (alpha = 0.5969). The numbers at the nodes are bootstrap support values (>50%) determined with the neighbor-joining method using distance matrices calculated with the GTR + I + G model.

strongly supported by synapomorphic characters such as the absence of a stigma in zoospores, dioecious and sexually dimorphic gametophytes, lamoxirene as the sexual pheromone, conducting systems in the sporophytes, and unicellular paraphyses (Kawai and Kurogi, 1985). We concur with Reviers and Rousseau (1999) that the three advanced kelp families should be considered as the Laminariales *sensu stricto,* exclusive of the primitive kelp families such as the Pseudochordaceae, Chordaceae, Phyllariaceae, and recently described Akkesiphycaceae (Kawai and Sasaki, 2000). Members of the primitive kelp families need to be analyzed with molecular markers to test this hypothesis (e.g., Kawai and Sasaki, 2000).

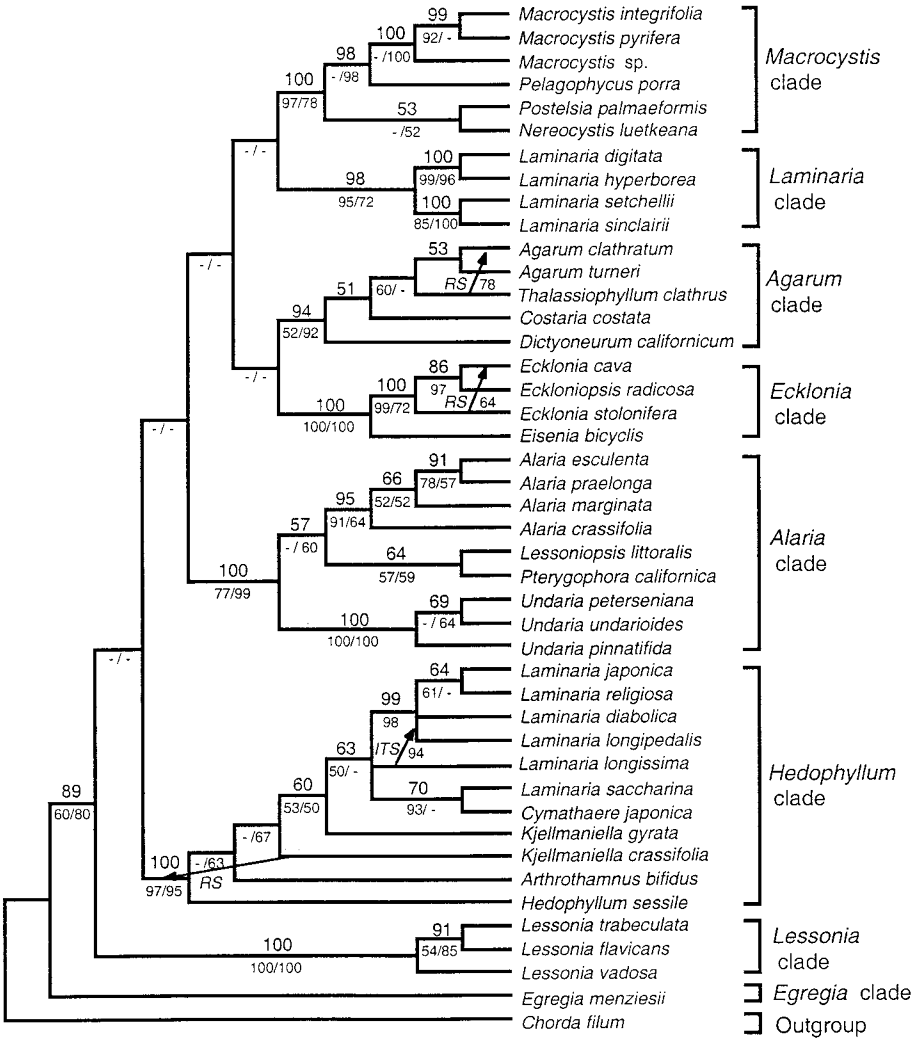

FIG. 2. Phylogenetic analysis of kelps. Strict consensus of the four equally parsimonious trees inferred from parsimony analysis of the combined RuBisCo spacer and ITS sequence data set for 44 taxa of the Alariaceae, Laminariaceae, and Lessoniaceae (length = 1083 steps; consistency index = 0.575; retention index = 0.777). The bootstrap values shown above the branches result from 1500 bootstrap resamplings of the combined data set, whereas the values shown below the branches are from bootstrap analysis of the RuBisCo spacer (left) and the ITS (right) data set only. Only bootstrap values >50% are shown. The arrows indiate rearrangement of taxa in ITS (*ITS*) and RuBisCo spacer (*RS*) bootstrap consensus trees relative to the phylogeny inferred from the combined data.

The major finding of our RuBisCo spacer and ITS sequence analyses is that the advanced kelp included in this study form eight distinct groups instead of the currently recognized three families. This indicates that the Alariaceae, based on the presence of specialized sporophylls arising from the transition zone, the Laminariaceae, based on sori being borne on simple blades, and the Lessoniaceae, based on branched blades arising from splitting between the blade and stipe, are not monophyletic. These results are consistent with previous analyses of the ITS (Saunders and Druehl, 1993b; Druehl *et al.*, 1997; Boo and Yoon 2000), 18S (Tan and Druehl, 1996; Boo *et al.*, 1999), and RuBisCo spacer (Yoon and Boo, 1999) sequences that show each of the three families not to be a natural taxon. The phylogenetic and taxonomic implications of these eight groups are discussed below.

Phylogenetic Relationships at the Tribal Level

Egregia is positioned basal as sister to the other seven lineages represented by *Laminaria, Hedophyllum, Macrocystis, Alaria, Agarum, Ecklonia,* and *Lessonia*. These eight clades are very similar to the tribes of the morphological classification system of the advanced kelps (Setchell, 1893; Setchell and Gardner, 1925; Bold and Wynne, 1985): the Egregieae, Laminarieae, Hedophyllieae, Lessonieae, Macrocysteae, Agareae, Ecklonieae, and Alarieae (Table 2). The exceptions are that the digeneric Cymathaereae is included in the

TABLE 2

A Comparison of the Currently Recognized Families to Phylogenetic Clades

Current classification system (e. g., Setchell and Gardner, 1925)	Phylogenetic clades identified in this study
Family Laminariaceae Bory	Not recognized
Tribe Agareae Kützing	*Agarum* clade
Agarum Dumortier, *Costaria* Greville *Thalassiophyllum* Postels *et* Ruprecht	*Agarum, Costaria, Dictyoneurum, Dictyoneuropsis, Thalassiophyllum*
Tribe Hedophylleae Setchell	*Hedophyllum* clade
Arthrothamnus Ruprecht, *Hedophyllum* Setchell, *Streptophyllopsis* Kajimura[a]	*Arthrothamnus, Cymathaere, Hedophyllum, Kjellamniella, Laminaria* in part
Tribe Cymathaereae Setchell *et* Gardner	Not recognized
Cymathaere J. Agardh, *Pleurophycus* Setchell *et* Saunders[a]	
Tribe Laminarieae Bory	*Laminaria* clade
Laminaria Lamouroux, *Kjellmaniella* Myabe *in* Okamura	*Laminaria* in part
Family Alariaceae Setchell *et* Gardner	Not recognized
Tribe Alarieae Setchell	*Alaria* clade
Alaria Greville, *Pterygophora* Ruprecht, Undaria Suringar	*Alaria, Pterygophora, Undaria, Lessoniopsis*
Tribe Ecklonieae Setchell	*Ecklonia* clade
Ecklonia Hornemann, *Eckloniopsis* Okamura, *Eisenia* Areschoug	*Ecklonia, Eckloniopsis, Eisenia*
Tribe Egregieae Setchell	*Egregia* clade
Egregia Areschoug	*Egregia*
Family Lessonaceae Setchell *et* Gardner	Not recognized
Tribe Lessonieae Setchell	*Lessonia* clade
Lessonia Bory, *Nereocystis* Postels *et* Ruprecht, *Postelsia* Ruprecht, *Dictyoneurum* Ruprecht, *Dictyoneuropsis* Smith[a]	*Lessonia*
Tribe Macrocysteae Kützing	*Macrocystis* clade
Macrocystis C. Agardh, *Pelagophycus* Areschoug	*Macrocystis, Nereocystis, Pelagophycus, Postelsia*
Tribe Lessoniopsieae Setchell	Not recognized
Lessoniopsis Reinke	

[a] Taxa not included in the present analysis.

Hedophyllum lineage and the monotypic Lessoniopsieae is in the *Alaria* lineage.

The basal position of *Egregia* is supported by the presence of irregular branching with numerous lateral blades, small pneumatocysts, and the outgrowth of sporophylls on both stipe and blade in this taxon (Setchell and Gardner, 1925). These characters are absent in other members of the advanced kelps. The *Egregia* clade conforms to the tribe Egregieae (Setchell, 1893; Setchell and Gardner, 1925).

The *Laminaria* clade, comprising nonbullate species of *Laminaria*; *L. digitata* (the type of the genus *Laminaria*), *L. hyperborea*, *L. sinclairii*, and *L. setchellii*, is strongly supported (94% in ML, 98% in MP) by the combined RuBisCo spacer and the ITS data set. This *Laminaria* group is similar to the tribe Laminarieae (Setchell and Gardner, 1925), except that the bullate *Laminaria* and *Kjellmaniella* are excluded from the group (Table 2). Other nonbullate members, *Laminaria longipes* and *L. yezoensis* from Kamchatka, are also included in this clade (Yoon and Boo, unpublished data). The clear separation of these nonbullate *Laminaria* from the bullate species, as seen in Figs. 1 and 2, leads us to conclude paraphyly of the genus *Laminaria*, which some authors (Druehl, 1968; Kain, 1979; Kawashima, 1993) have considered to be a distinct taxonomic group. Therefore, we suggest that *Laminaria sensu stricto* should be established based on the presence of nonbullate fronds. This suggestion is different from the proposal of Yotsukura *et al.* (1999) that the bullation of fronds may be diagnostic only at the species level. Furthermore, our molecular analyses do not support the subdivision of *Laminaria* into the four subgenera (Petrov, 1974); *Laminaria* Lamouroux, *Rhizomaria* Petrov, *Solearia* Petrov, and *Renfrewia* (Griggs) Schmitz. Instead, our data are consistent with the geographical distribution patterns of the *Laminaria* species. For instance, *L. digitata* and *L. hyperborea* from the Atlantic are closely related to each other, as are the Pacific *L. sinclairii* and *L. setchellii*. These results indicate that the phylogenetic relationships between kelp species and their geographic distributions are intimately linked. We suggest that some ancestor of *Laminaria* dispersed from the North Pacific via the Bering Strait and the Arctic Ocean into the North Atlantic and likely diverged into the taxa presently found there (see Estes and Steinberg, 1988). However, broader sampling of taxa from the northern Atlantic (e.g., *L. ochroleuca,* and *L. solidungula*) and from the Southern Hemisphere (e.g., *L. abyssalis, L. brasiliensis,* and *L. pallida*) is required to test this hypothesis of kelp speciation.

The *Hedophyllum* clade comprise *Cymathaere, Arthrothamnus, Hedophyllum, Kjellmaniella* and six bullate *Laminaria* species and is very strongly supported by our RuBisCo spacer and the ITS sequence analyses. This *Hedophyllum* group differs substantially from the tribe Hedophylleae (Setchell and Gardner, 1925) in its constituent genera (Table 2). The monophyly of *Laminaria japonica, L. diabolica, L. religiosa, L. longissima,* and *L. longipedalis* indicates close relationship among taxa which have bullations found only in the young thallus (Kawashima, 1993), as in multivariate analyses of morphological characters (Druehl *et al.,* 1988). Sexual compatibility is reported among *L. religiosa, L. japonica,* and *L. ochotensis,* between *L. japonica* and *L. diabolica* (Yabu, 1964), among *L. japonica, L. ochotensis,* and *L. longissima,* and between female *L. diobolica* and male *L. longissima* (Migita, 1985a). Chromosome numbers are the same ($n = 32$) in *L. japonica, L. ochotensis,* and *L. religiosa* (Yabu and Yasui, 1991). Our sequence analyses, taken together with the morphological data, results of hybridization experiments, and chromosome numbers, lead us to conclude that *L. diabolica, L. japonica, L. longissima, L. ochotensis,* and *L. religiosa* represent a single biological species, as suggested by Yotsukura *et al.,* (1999). This hypothesis agrees with Hasegawa (1959), Yabu (1964), and Petrov (1972) who recognized all these five species as infraspecific taxa of *L. japonica sensu lato. Laminaria saccharina* is more closely related to *Cymathaere* than to *L. japonica s. l.* Our RuBisCo spacer and ITS data suggest that the genus *Kjellmaniella* may not be monophyletic because of the close relationship of *K. gyrata* to the bullate *Laminaria* species. Taxonomic and nomenclatural changes of the *Kjellamniella* species should, therefore, be considered. Our results are consistent with those of Yotsukura *et al.* (1999) who used ITS data to show that *K. gyrata* was included in the *L. saccharina* clade. However, *K. crassifolia* was not included in their study.

The *Macrocystis* clade is comprised of *Macrocystis, Nereocystis, Pelagophycus,* and *Postelsia*. This group is characterized by the splitting of the transition zone and the air-bladder structure as a pneumatocyst or hollow stipe and is similar to the tribe Macrocystieae (Setchell and Gardner, 1925) with the absence of *Nereocystis* and *Postelsia* (Table 2). Although the close relationships among the genera of the *Macrocystis* group are reported in published cpDNA data (Fain *et al.,* 1988) and in hybridization experiments (Neushul, 1971; Sanbonsuga and Neushul, 1978), our analysis of the RuBisCo spacer and the ITS sequences shows the four genera to be evolutionarily distinct. *Pelagophycus* appears to be closely related to *Macrocystis,* both having unilateral splitting and sympodial feature of the stipe (Setchell and Gardner, 1925). *Nereocystis* and *Postelsia* are closely related (see Fig. 1) in this analysis, as in previous reports (Setchell and Gardner, 1925; Saunders and Druehl, 1993b). Of the known four species of *Macrocystis*, the three taxa included in our study are monophyletic, a result supported by their similar morphology (see Setchell and Gardner, 1925; North, 1994). There are large length differences (26 bp in the ITS sequences and 6 bp in the RuBisCo spacer sequences) between Californian *M. integrifolia* and Argentine *Macrocystis* sp., which supports a relatively distant relationship between these two geographically isolated species. However, the sequences of the two morphologically and ecologically distinct *M. integrifolia* and *M. pyrifera* species from California are the same in the ITS region or differ by one substitution in the RuBisCo spacer. Our results corroborate the hypothesis by Druehl and Saunders (1992) who proposed that *M. integrifolia* and *M. pyrifera* were separated prior to bipolarization, with the northern *M. integrifolia* recently acquiring the *M. pyrifera* genome through introgression. The alternative is that these two species diverged subsequent to the bipolarization of *M. integrifolia* (Druehl and Saunders, 1992).

The *Alaria* clade, comprised of *Alaria, Lessoniopsis, Pterygophora,* and *Undaria,* conforms to the tribe Alarieae (Setchell and Gardner, 1925), but includes *Lessoniopsis* that belongs to the tribe Lessoniopsieae in the morphotaxonomy (Setchell and Gardner, 1925; Okamura, 1936). The monophyly of these genera has been reported in cpDNA RFLP (Fain *et al.,* 1988), ITS (Saunders and Druehl, 1993b; Druehl *et al.,* 1997), and RuBisCo spacer (Yoon and Boo, 1999) sequence analyses. The *Alaria* group is characterized by sporophylls on the stipe and midrib, or thickening of the blade (Widdowson, 1971). *Undaria* is positioned basal as sister to the other representatives as suggested by Druehl *et al.* (1997) and Yoon and Boo (1999).

The *Agarum* clade is comprised of *Agarum, Costaria, Dictyoneurum,* and *Thalassiophyllum* in our analyses and includes *Dictyoneuropsis* in trees inferred from ITS-1 data (Saunders and Druehl, 1993b). The *Agarum* group is similar to the tribe Agarieae (Setchell and Gardner, 1925), except that it includes *Dictyoneurum,* which was considered part of the Lessonieae, and is characterized by having longitudinal rib or rib-like reticulation and perforation of the blade (Table 2). Our RuBisCo spacer and ITS data show that *Agarum clathratum* (= *A. cribrosum,* Silva, 1991) and *A. turneri* are distinct taxa and not conspecific as suggested by Silva (1991). This result strongly supports the separation of both species by Klochkova and Berezovskaja (1997) and Klochkova (1998), who distinguished *A. turneri* as having a broad (2–3 cm) midrib, whereas *A. clathratum* as having a narrow midrib (1.5 cm). If it is being nested in the *Agarum* clade (Fig. 1), the genus *Thalassiophyllum* may be combined with *Agarum*.

The *Ecklonia* clade, comprised of *Ecklonia*, *Eckloniopsis*, and *Eisenia*, conforms to the tribe Ecklonieae (Setchell and Gardner, 1925), as shown previously with RuBisCo spacer data (Yoon and Boo, 1999). The *Ecklonia* group is characterized by the origin of the secondary blades from the transition zone, sori forming on the blade, and the presence of a solid stipe. Our RuBisCo spacer and ITS data analyses clearly show *Ecklonia*, *Eckloniopsis*, and *Eisenia* to form a distinct clade, only distantly related to both the *Alaria* and *Laminaria* clades. The phylogenetic independency of the *Ecklonia* group forces us to stop the long debate of whether all of the three genera should be positioned in the Alariaceae (Setchell and Gardner, 1925; Papenfuss, 1951; Bolton and Anderson, 1994) because of the production of secondary blades or, in the Laminariaceae, because of the position of sporangial sori on blades (Okamura, 1936; Kawashima, 1993). Notably, the members of the *Ecklonia* group usually occur in warm waters (Bolton and Anderson, 1994), while members of the *Alaria* and *Laminaria* groups mostly occur in cold (see Bold and Wynne, 1985). This distributional difference causes us to speculate that some ancestor of kelps in warm waters likely diverged to the present members of the *Ecklonia* group. The very close relationships within the *Ecklonia* group indicate that they likely had a recent common ancestor. Our RuBisCo spacer and ITS data also indicate a possible paraphyly of the genus *Ecklonia*, but cannot also identify the exact position of *Eckloniopsis* in relation to *Ecklonia* representatives. The morphological separation of *Ecklonia* from *Eisenia* is questioned (Yendo, 1902). The intergeneric hybridizations are reported between the two genera (Migita, 1984) and between *Ecklonia* and *Eckloniopsis* (Migita, 1985b). In our trees, *Eckloniopsis radicosa* is nested within the clade formed by other representatives. This topology provides a clue that *Eckloniopsis* may be combined with *Ecklonia*. The questions of whether *Ecklonia* is monophyletic or is synonymized with *Eckloniopsis* need additional examples such as *E. maxima*, a type of *Ecklonia*.

The *Lessonia* clade, comprised of only *Lessonia* species, differs from the tribe Lessonieae (Setchell, 1893; Setchell and Gardner, 1925; Papenfuss, 1951) in that *Dictyoneurum*, *Nereocystis*, and *Postelsia* are excluded (Table 2). The monophyly of three *Lessonia* species included in this study is clearly resolved in the trees but have a confused taxonomy because of extreme phenotypic variation (Edding *et al.*, 1994). Except for *L. laminarioides* in the Okhotsk Sea, *Lessonia* species are most abundant in the Southern Hemisphere, particularly along the South Pacific of Peru and Chile, and in the circumpolar islands (Searles, 1978; Edding *et al.*, 1994). We speculate, therefore, that the *Lessonia* species likely originated and diversified in the southern Pacific. However, because the *Lessonia* clade is sister to the *Ecklonia* clade in the ML tree and is nested in other kelps in the MP tree, more details remain unclear. According to previous studies, *Lessonia* probably migrated to the Southern Hemisphere probably during the Miocene cooling, based on distribution of kelps in the North Pacific (Lüning, 1990; Chin *et al.*, 1991), or its ancestor likely diversified in the South Pacific after its entrance via a cold-water corridor along the coast of the Americas before the introduction of *Macrosystis* and *Eisenia* to the Southern Hemisphere (Saunders and Druehl, 1993b).

Taxonomic Implications

Setchell and Gardner (1925) classified the laminarialean genera (except *Chorda*) into the Alariaceae comprising three tribes, the Laminariaceae comprising four tribes, and the Lessoniaceae comprising three tribes (Scenario 1). Alternative systems, proposed by these authors, are either to retain all the genera in one family divided into numerous tribes (Scenario 2), or to divide the genera among numerous families (Scenario 3). The current classification system (e.g., Bold and Wynne, 1985) stems from Scenario 1 of Setchell and Gardner (1925), who suggested that the most useful markers for distinguishing among families are frond characteristics such as its place of origin (e.g., the transition zone) and the relationship of fronds to reproductive tissues.

The eight clades shown in our analyses of the RuBisCo spacer and the ITS data strongly support either Scenarios 2 (Fig. 3B) or 3 (Fig. 3C) rather than Scenario 1 (Fig. 3A) of Setchell and Gardner (1925). The fact that the eight groups are very morphologically diverse appears to favor Scenario 3. We, therefore, suggest that each clade in our analyses should be considered at the family level within the order Laminariales *s.s.* and the familial names should be defined based on formerly recognized tribes. For example, the Agariaceae would become the *Agarum* clade, Alariaceae the *Alaria* clade, Eckloniaceae the *Ecklonia* clade, Egregiaceae for *Egregia* only, Hedophylleaceae the *Hedophyllum* clade, Laminariaceae the *Laminaria* clade, Lessoniaceae the *Lessonia* clade, and Macrocysteaceae the *Macrocystis* clade. The alternative is that all the advanced kelps could be placed in one large family and then classified into eight tribes (Scenario 2). A suitable name for this grouping would be the Laminariaceae Bory (1827) instead of the Alariaceae Setchell and Gardner (1925), and the Lessoniaceae Setchell and Gardner (1925).

CONCLUSIONS

In summary, phylogenetic analyses of the RuBisCo spacer and ITS sequences are useful for understanding relationships of advanced kelps and provide a frame-

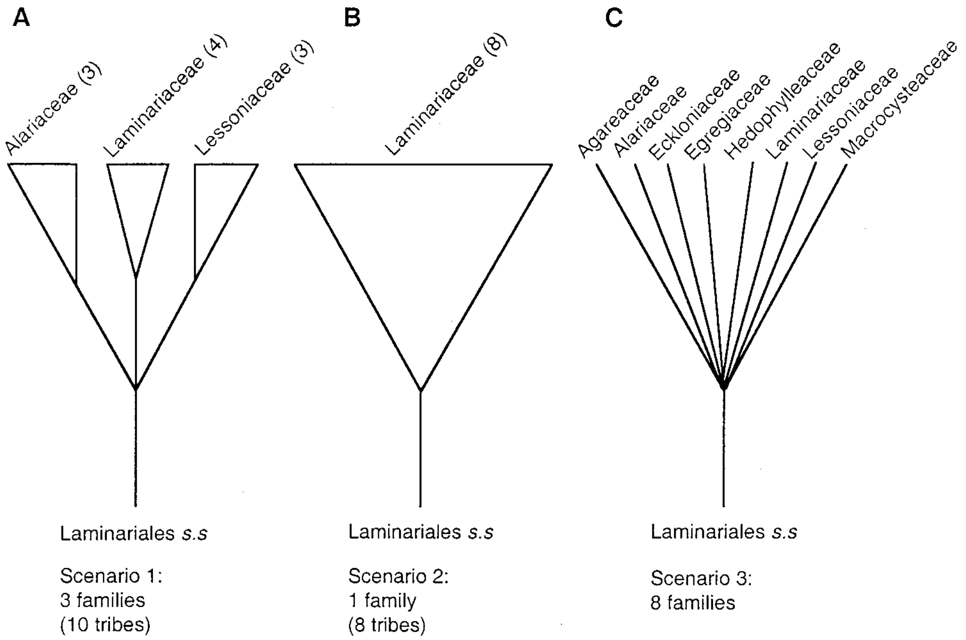

FIG. 3. Three possible scenarios for the classification of the Laminariales *s.s.* (A) The currently recognized three families and 10 tribes system based on the mode of tissue differentiation at the transition zone. (B) The single family and eight tribes system based on limited molecular data. (C) The eight families system based on our molecular data.

work for revising the systematics of this group. All genera currently assigned to the Alariaceae, Laminariaceae, and Lessoniaceae included in this study share a monophyletic origin, suggesting that these taxa may best be classified as a single order. Another important conclusion is the finding of eight strongly supported terminal clades, represented by *Egregia*, *Laminaria*, *Hedophyllum*, *Macrocystis*, *Alaria*, *Agarum*, *Ecklonia*, and *Lessonia*. These eight clades conform to the tribes of the current morphological classification system, with the exception of the two tribes, Cymathaereae and Lessoniopseae, which are subsumed in the *Hedophyllum* and *Alaria* clades, respectively. However, these eight clades differ in detail from the morphologically recognized tribes (see Table 2). For example, the *Macrocystis* clade including *Macrocystis*, *Nereocystis*, *Pelagophycus*, and *Postelsia* differs from the tribe Macrocysteae (comprised of *Macrocystis* and *Pelgophycus*), and the *Hedophyllum* clade including *Arthrothamnus*, *Hedophyllum*, *Kjellmaniella*, and bullate *Laminaria* differs from the tribe Hedophylleae (*Arthrothamnus*, *Hedophyllum*, and *Streptophyllopsis*). Morphological diversity of the advanced kelps suggests that each of these eight groups should be considered at the family rank. A final observation is that some kelp genera are paraphyletic in the RuBisCo spacer and the ITS trees. For example, *Kjellamaniella* from the northwest Pacific comprise two lineages; *K. gyrata* is closely related to the bullate members of *Laminaria* and *Kjemaniella crassifolia* is positioned basal in the *Hedophyllum* clade. *Laminaria* is also positioned in two separate lineages; one comprises *L. digitata*, the type of *Laminaria* having nonbullate blades, and the other comprises members with bullate blades.

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