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A MULTI-GENE MOLECULAR INVESTIGATION OF THE KELP (LAMINARIALES, PHAEOPHYCEAE) SUPPORTS SUBSTANTIAL TAXONOMIC RE-ORGANIZATION[1]

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Every year numerous ecological, biochemical, and physiological studies are performed using members of the order Laminariales. Despite the fact that kelp are some of the most intensely studied macroalgae in the world, there is significant debate over the classification within and among the three "derived" families, the Alariaceae, Laminariaceae, and Lessoniaceae (ALL). Molecular phylogenies published for the ALL families have generated hypotheses strongly at odds with the current morphological taxonomy; however, conflicting phylogenetic hypotheses and consistently low levels of support realized in all of these studies have resulted in conservative approaches to taxonomic revisions. In order to resolve relationships within this group we have sequenced over 6000 bp from regions in the nuclear, chloroplast, and mitochondrial genomes and included 42 taxa in Bayesian, neighbor-joining, and parsimony analyses. The result is the first comprehensive and well-supported molecular phylogeny for the ALL complex of the Laminariales. We maintain the three recognized families (Alariaceae, Laminariaceae, and Lessoniaceae), but with vastly different compositions, as well as propose the Costariaceae fam. nov. for *Agarum*, *Costaria*, *Dictyoneurum*, and *Thalassiophyllum*, the only genera in the Laminariales with flattened, occasionally terete, stipes and either a perforate or reticulate blade. In addition, our data strongly support a split of the genus *Laminaria*. We resurrect the genus *Saccharina* Stackhouse for the *Laminaria* clade that does not contain *L. digitata* (Hudson) J.V. Lamouroux, the type of the genus.

Key index words: **Costariaceae; Laminariales; long branch attraction; nested analyses; phylogenetics; *Saccharina***

The order Laminariales Migula, commonly called kelp, includes the largest algae in the world, reaching up to 50 m in length (Van den Hoek et al. 1995). Kelp are ubiquitous in coastal waters of cold-temperate regions from the Arctic to the Antarctic, and their size and biomass establishes a unique and essential habitat for hundreds of species (Steneck et al. 2002). They are used as a food source in Asia and Europe, and are also economically important for their extracts (Chapman 1970, Ohno and Crithchley 1998), which are used in consumer and medical products.

There are approximately 30 genera within the Laminariales, many of which are monotypic, whereas a few contain a large number of species [*ca*. 45 species in *Laminaria* (Kain 1971), 12 species in *Alaria* (Widdowson 1971, Kraan et al. 2001)]. Nearly 100 species of kelp are currently recognized across the order, with the majority of them occurring in the North Pacific, where 40 species are currently recognized from the coast of North America (Druehl 1970), and *ca*. 41 from Asia (Yoshida 1998, Kawai and Sasaki 2000, Kawai et al. 2000). The North Atlantic contains only a fraction of the diversity seen in the Pacific, with only eight kelp species recognized (South and Tittley 1986, Kawai and Sasaki 2000). However, these species span a taxonomic range equal to that among the 40 species on the West Coast of North America. The Southern Hemisphere kelp species are limited to *Eisenia galapagensis* (near the equator), two species of *Laminaria*, three species of *Macrocystis*, the introduced species *Undaria pinnatifida* (also introduced in Europe, but originally from East Asia), and the largely Southern Hemisphere genera *Ecklonia* and *Lessonia* (Womersley 1987, Adams 1994, Stegenga et al. 1997).

While morphology is highly variable among members of the Laminariales, kelp are typically connected

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to the substrate by a holdfast of branching haptera, rhizoids, or a disc. A stipe is borne centrally from the holdfast and has at its upper end an intercalary meristem at the "transition zone" between the stipe and the terminal blade. Extension of both the stipe and the blade occur by growth in this transition zone, with growth in girth produced by a superficial meristem, the meristoderm (Bold and Wynne 1985). Kelp are the only seaweed with specialized cells (trumpet hyphae) for the transport of nutrients, stored mainly as laminaran and mannitol (Parker 1965, Lüning 1990). Another defining feature of the Laminariales is their heteromorphic, diplohaplontic life cycle, which alternates between a microscopic haploid gametophyte and a macroscopic parenchymatous diploid sporophyte. Whereas many kelp are annuals, some, such as *Laminaria hyperborea*, reportedly live up to 20 years (Van den Hoek et al. 1995).

Kelps are well studied seaweeds; the ISI Web of Knowledge lists 376 papers in just a 3 year period (January 2003–December 2005) with "kelp" in the key words. Despite the conspicuous nature of kelp, and considerable attention they receive from both biochemical and ecological researchers, little consideration had been given to their systematics until recently. Several molecular studies over the past 20 years have called into question the widely accepted classification system for the Laminariales (Fain et al. 1988, Saunders and Druehl 1993b, Druehl et al. 1997, Yoon and Boo 1999, Kawai and Sasaki 2000, Sasaki et al. 2001, Yoon et al. 2001). In the past 5 years publications have changed the three "ancestral" families that were traditionally recognized, viz., the Chordaceae, Phyllariaceae, and Pseudochordaceae. The Phyllariaceae was moved out of the Laminariales based on its close molecular affinity to the Tilopteridales (Kawai and Sasaki 2000). The monotypic genus *Halosiphon* was, at the same time, elevated from the Chordaceae to familial status (Halosiphonaceae) and moved out of the Laminariales, but left *incertae sedis* at the ordinal level. The monotypic family Akkesiphycaceae was then added to the Laminariales (Kawai and Sasaki 2000). Subsequently, the Phyllariaceae and Halosiphonaceae were placed in the Tilopteridales based on both nuclear and chloroplast DNA sequence data (Sasaki et al. 2001). Thus, the "ancestral" clade in the Laminariales continues to have three families, but with substantially different composition.

The three "derived," or "ALL" families (Saunders and Druehl 1993b), including the Alariaceae, Laminariaceae, and Lessoniaceae, have diverse morphologies (Fig. 1), but are differentiated from other taxa in the Laminariales by the presence of mucilaginous organs in the sporophyte, lack of an eyespot in meiospores, and unique flagellation of the sperm (Kawai and Sasaki 2000). Further indicating a close association, only species in the ALL families produce the sexual pheromone Lamoxirene (Müller et al. 1985).

The ALL families have remained virtually unchanged because their classification on the basis of gross morphology of the sporophyte by Setchell and Gardner (1925). The Alariaceae was defined by the presence of sporophylls (special blades for reproductive sori). The Laminariaceae have simple, single blades, whereas representatives of the Lessoniaceae display splitting at the transition zone (between the stipe and the blades). Some genera, however, do not fit neatly into the morphological scheme. In fact, Setchell and Gardner (1925) stated that the genus *Lessoniopsis* (Fig. 1b), which has both sporophylls and splitting, could be placed either in the Alariaceae or the Lessoniaceae and the decision to place this genus in the latter family was based on its habit being more similar to *Lessonia* than *Alaria*. Despite uncertainty as to the usefulness of the characters that define the ALL families, these taxa had remained largely unchallenged at the familial level until the application of molecular techniques to kelp systematics.

Fain et al. (1988) were the first to apply molecular tools to kelp systematics. They initiated a restriction fragment length polymorphism (RFLP) investigation of the chloroplast genome for five species [*Alaria marginata* (Al), *Laminaria saccharina* (La), *Lessoniopsis littoralis* (Le), *Nereocystis luetkeana* (Le) and *Macrocystis integrifolia* (Le); Al = Alariaceae, La = Laminariaceae, Le = Lessoniaceae] of the Laminariales, of which three were members of the Lessoniaceae. Rather than grouping together, members of the Lessoniaceae were polyphyletic—*Nereocystis* (Le) consistently grouped with *Laminaria* (La), whereas *Alaria* (Al) was variously resolved with *Macrocystis* (Le), or *Lessoniopsis* (Le).

Subsequent to the chloroplast studies of Fain et al. (1988), a number of publications directed at resolving kelp systematics have focused on the nuclear ribosomal cistron using either the small subunit rDNA (SSU) (Saunders and Druehl 1992, Boo et al. 1999) or the internal transcribed spacer regions (ITS) (Saunders and Druehl 1993b, Druehl et al. 1997, Yoon et al. 2001). Phylogenetic trees generated from the first ITS region (ITS1) (Saunders and Druehl 1993b) added further evidence that the Lessoniaceae was polyphyletic, and also indicated a similar situation for a second family, the Alariaceae. Three groups were resolved by their data: Group 1 for *Alaria* (Al), *Lessoniopsis* (Le), and *Pterygophora* (Al); Group 2 for *Costaria* (La) and *Dictyoneurum* (Le); and Group 3 for *Egregia* (Al), *Eisenia* (Al), *Lessonia* (Le), *Macrocystis* (Le), *Nereocystis* (Le), and *Postelsia* (Le). Only one member of the Laminariaceae (*Costaria*) was included in their study and, therefore, no conclusion could be framed regarding this family. Druehl et al. (1997) added representatives of the Laminariaceae (*Hedophyllum* and *Laminaria*) to the ITS1 data set and discovered that this family was also polyphyletic.

The RUBISCO spacer (*rbc*Sp) region was used in Yoon and Boo's (1999) study of the Alariaceae, which included 14 species, representing seven genera. Although they disregarded earlier work, which established that the traditional Alariaceae was an artificial

FIG. 1. Morphological variation in the Laminariales: (a) *Alaria marginata* (Al), (b) *Lessoniopsis littoralis* (Le), (c) *Pleurophycus gardneri* (La), (d) *Agarum clathratum* (La), (e) *Costaria costata* (La), (f) *Pterygophora californica* (Al), (g) *Egregia menziesii* (Al), (h) *Eisensia arborea* (Al), (i) *Macrocystis integrifolia* (Le), (j) *Postelsia palmaeformis* (Le), (k) *Laminaria sinclarii* (La), (l) *Cymathaere triplicata* (La); Al = Alariaceae, La = Laminariaceae, Le = Lessoniaceae. Photographs (a), (c), (e)–(i), (k), and (l) by Colin Bates, photograph (b) and (j) by Saunders and (d) by Lane and Saunders.

grouping (Saunders and Druehl 1993b, Druehl et al. 1997) and thus analyzed an unnatural assemblage, they introduced data from a genomic region that had not been previously used for kelp phylogeny. In 2001, the ITS and *rbc*Sp sequences of 44 kelp taxa were combined to produce a phylogeny that was well supported at the level of tribe, but that had virtually no bootstrap support at the familial level (Yoon et al. 2001). The Yoon et al. (2001) study supported earlier conclusions regarding the inappropriateness of the morphological classification system and they proposed three possible classification scenarios without making formal modifications. Additionally, they were the first to show the paraphyly of the genus *Laminaria* using molecular data, but did so without making formal changes in nomenclature. The ITS/*rbc*Sp combined data set yielded a tree with a topology at odds with those found earlier using ITS1 data alone (Saunders and Druehl 1993b, Druehl et al. 1997). The trees produced by Yoon et al. (2001) recovered Groups 1 and 2 from Druehl et al. (1997) as monophyletic, but Group 3 was polyphyletic, and, most notably, *Egregia menziesii* was positioned as sister to all remaining ingroup taxa.

Despite all of the previous efforts, there remains considerable uncertainty concerning the phylogenetics of kelp genera, and the number and composition of families that should be recognized among the ALL taxa. Neither the ITS nor *rbc*Sp data have provided robust resolution at the deeper nodes. Although the *rbc*Sp and ITS data have indicated that the traditional families recognized within the ALL clade are unnatural groupings, better resolution is required to understand fully the evolutionary relationships of these taxa.

We set out to resolve kelp phylogeny and distinguish between the two published hypotheses (based on molecular data) for the evolution of the Laminariales by bringing more data, with appropriate levels of variation, to bear on the question. The ITS and large subunit (LSU) rDNA from the nuclear genome, the RUBISCO operon [including the large subunit (*rbc*L), spacer, and small subunit (*rbc*S)] from the chloroplast genome and the NADH dehydrogenase subunit 6 (*nad*6) gene from the mitochondrial genome were selected because these genes have shown phylogenetic utility in other groups with similar levels of divergence and provide a large number of nucleotides. Sequences from these regions were used alone, and in combination, in a series of nested analyses to maximize the resolving power of our data at all levels of taxonomy within and among the ALL families of the Laminariales. The final result is the most thorough examination of ALL systematics published to date, which allows for formal modifications to the system of classification for these taxa.

MATERIALS AND METHODS

DNA extraction and sequencing. Samples of the species listed in Table 1 were dried on silica gel in the field and returned to the lab where they were immersed in liquid nitrogen and ground to a fine powder using a chilled mortar and pestle. Because of the high levels of polysaccharides, tannins, and phenolics present in most kelp tissue, DNA extractions kits generally fail to recover DNA adequate for PCR. Therefore, the following procedure was developed as a cost-effective and rapid DNA extraction for kelp.

Steps involving organelle extraction were performed on ice, with stirring, until the material was transferred to the DNA extraction buffer. Organelles were isolated from the ground material using a modification of Fain et al.'s (1988) procedure as follows: ground tissue (80–100 μg) was slowly added to 4 mL of Buffer A [1.65 M Sorbitol, 50 mM MES pH 6.1, 10 mM EDTA, 2% (w/v) PVP-40, 0.1% (w/v) BSA and 5 mM β-mercaptoethanol] while stirring for 2–3 min. The mixture was then filtered through Miracloth (Calbiochem®, La Jolla, CA, USA) with two layers of cheesecloth on either side. The resulting filtrate was centrifuged for 2 min at 3000*g* in an IEC MicroMax centrifuge. Buffer A was poured off and the pellet was resuspended in 1 mL of Buffer B (Buffer A without PVP) and centrifuged again for 2 min at 3000*g*; this step was repeated up to four times until the pellet was compact. After the last centrifugation, all of the buffer was poured off and the pellet resuspended in 300 μL of the DNA extraction buffer (Saunders and Kraft 1995), 30 μL of 10% Tween-20 and 3 μL of Proteinase K (20 mg mL^{-1}) (Fisher Scientific Ltd., Ottawa, ON, Canada). All subsequent centrifugations occurred at 15,000*g* in a microcentrifuge. After a 1 h incubation at room temperature, samples were placed on ice for 20 min, then centrifuged for 10 min. The aqueous phase was transferred to 1.5 mL microcentrifuge tubes containing 250 μL of a phenol, chloroform and isoamyl alcohol (25:24:1 v/v/v) mixture, and was thoroughly mixed for 5 min, then centrifuged for 5 min. The aqueous phase was transferred to new 1.5 mL microcentrifuge tubes containing 250 μL chloroform and isoamyl alcohol (24:1 v/v), and mixed for 2 min, then centrifuged for 2 min. After this final centrifugation, the aqueous layer was transferred to new microcentrifuge tubes containing 1 mL of Wizard® DNA Clean-Up System resin (Promega, Madison, WI, USA), and the manufacturer's protocol was followed.

Purified DNA was PCR amplified in 50 μL volumes using 50 ng of DNA in a 2400 GeneAmp PCR System (Perkin Elmer, Boston, MA, USA) or Icycler (Bio-Rad Laboratories, Hercules, CA, USA), using the Takara Ex-Taq DNA polymerase kit (PanVera, Madison, WI, USA). With the exception of two modifications (KT14—5′ CGTCCGCGTGCGCTCTCGACGG 3′ instead of T33 and KT05—5′ GAGCGGACAAGGGGAATCCG 3′ instead of T05), PCR primers for the LSU were used from Harper and Saunders (2001). The ITS primers from Tai et al. (2001) were used with modifications to P5 (KP5—5′ ACAACGATGAAGAACGCAG 3′), R1 (KIR1—5′ TTCAAAGTTTTGATGATT 3′) and G4 (KG4—5′ CTTTTCCTCCGCTTAGTTATATG 3′) to achieve greater specificity. Thermal profiles for all nuclear PCR reactions were identical to those described previously (Harper and Saunders, 2001; Fig. 3). RUBISCO (Fig. 2a) and nad6 (Fig. 2b) PCR and sequencing primers were developed based on available brown algal sequences (Daugbjerg and Andersen 1997, Yoon et al. 2001, Oudot-Le Secq et al. 2002). Because the *rbc*L and *rbc*S are adjacent in the chloroplast genome of brown algae, the RUBISCO operon was amplified in two overlapping sections (Fig. 2a). The thermal profile for PCR amplification of both the RUBISCO and mitochondrial genes included: an initial denaturation cycle of 94.0° C for 4 min, followed by 38 cycles of 94.0° C for 1 min, 50.0° C for 1 min, and 72.0° C for 2 min. A final annealing step occurred at 72.0° C for 7 min followed by storage at 4.0° C until the samples were processed.

After 2 μL of the PCR product was used to check for a successful reaction, the remaining product was cleaned as in Saunders (1993). The DNA sequencing was performed with the

TABLE 1. Collection locations or reference and GenBank numbers for species used in this study.

Classification	Location	LSU	ITS	*rbc* operon	*rbc*Sp only	nad6
Chordaceae						
Chorda filum (L) Stackhouse	Blacks Harbour, NB, Canada	**AY851505**	**AY857872**	**AY851533**	----	**AY857903**
Alariaceae						
Alaria marginata Post. and Rupr.	Seal Rock, OR, USA	**AY851525**	AF362997	**AY851537**	----	**AY857907**
Alaria fistulosa Post. and Rupr.	Seldovia Point, AL, USA	**AY851527**	**AY857878**	**AY851536**	----	**AY857908**
Ecklonia cava Kjell.	Yoon et al. (2001)	----	----	----	AF318967	----
Ecklonia stolonifera Okamura	Yoon et al. (2001)	----	----	----	AF318968	----
Ecklonia radiata (C. Ag) J. Ag	Pt. Lonsdale, Vic, Australia	**AY851512**	----	**AY851552**	----	**AY857919**
Ecklonia radiata	Whitfords Reef, WA, Australia	----	**AY857898***	----	----	----
Eckloniopsis radicosa (Kjell.) Okamura	Yoon et al. (2001)	----	----	----	AF318969	----
Egregia menziesii (Turn.) Aresch.	Boiler Bay, OR, USA	**AY851506**	**AY857897***	**AY851551**	----	**AY857917**
Eisenia arborea Aresch.	Kelp Point, Bamfield, BC, Canada	**AY851511**	**AY857899***	**AY851550**	----	**AY857918**
Eisenia bicyclis (Kjell.) Setch.	Yoon and Boo (1999)	----	----	----	AF318963	----
Pterygophora californica Rupr.	Cape Beale, Bamfield, BC, Canada	**AY851523**	**AY857875***	**AY851539**	----	**AY857910**
Undaria pinnatifida (Harv.) Suringar	l'Etang de Thau, France	**AY851528**	**AY857873**	**AY851535**	----	**AY857912**
Laminariaceae						
Agarum clathratum Dumortier	Grand Mannan Is., NB, Canada	**AY851521**	**AY857880**	**AY851542**	----	**AY857905**
Arthrothamnus bifidus (Gemelin) Rupr.	Yoon et al. (2001)	----	AF319023	----	AF318984	----
Costaria costata (C. Ag.) Saunders	Whiffen Spit, Sooke, BC, Canada	**AY851522**	**AY857879***	**AY851541**	**AY851541**	**AY857904**
Cymathere japonica Miyabe and Nagai	Yoon et al. (2001)	----	AF319022	----	----	----
Cymathaere triplicata Post. and Rupr.	Whiffen Spit, Sooke, BC, Canada	**AY851519**	**AY857884**	**AY851562**	----	**AY857932**
Hedophyllum sessile (C. Ag) Setch.	Cape Beale, Bamfield, BC, Canada	**AY851513**	**AY857896**	**AY851553**	----	**AY857928**
Kjellmaniella crassifolia Miyabe	Yoon et al. (2001)	----	**AF319020**	----	----	----
Kjellmaniella gyrata (Kjell.) Miyabe	L. Druehl culture	**AY851526**	----	**AY851560**	----	**AY857925**
Kjellmaniella gyrata	Yoon et al. (2001)	----	AF319021	----	----	----
Laminaria angustata Kjell.	Muroran, Hokkaido, Japan	----	**AY857891**	----	----	----
Laminaria angustata	L. Druehl culture	**AY851515**	----	**AY851554**	----	**AY857927**
Laminaria dentigera Kjell.	Dutch Harbor, AL, USA	----	**AY857895**	----	----	----
Laminaria digitata Kjell.	Green Pt., Lepreau, NB, Canada	**AY851517**	**AY857886**	**AY851559**	----	**AY857921**
Laminaria ephemera Setch.	Botanical Beach, Port Renfrew, BC, Canada	----	**AY857887**	**AY851557**	----	**AY857924**
Laminaria farlowii Setch.	Carmel Bay, CA, USA	----	**AY857888**	----	----	----
Laminaria groenlandica Rosenvinge	Whiffen Spit, Sooke, BC, Canada	----	**AY857894**	----	----	----
Laminaria hyperborea (Gunnerus) Foslie	Erting et al. (2003)	----	AY441773	----	----	----
Laminaria japonica Aresch.	Muroran, Hokaido, Japan	----	**AY857892**	----	----	----
Laminaria saccharina (L.) Lamouroux	Green Pt., Lepreau, NB, Canada	**AY851514**	**AY857893**	**AY851561**	----	**AY857926**
Laminaria setchellii P. C. Silva	Cape Beale, Bamfield, BC, Canada	----	**AY857890**	----	----	----
Laminaria sinclairii (Harv.) Farlow, Anderson and Eaton	Mud Cove, Bamfield, BC, Canada	**AY851516**	**AY857889**	**AY851558**	----	**AY857920**
Laminaria solidungula J. Ag.	Pond Inlet, Baffin Is. Nunavut, Canada	----	----	**AY851556**	----	----
Laminaria yezoensis Miyabe	L. Druehl culture	**AY851518**	----	**AY851555**	----	**AY857922**
Pleurophycus gardneri Setch. and Saunders	Pachena Beach, Bamfield, BC, Canada	**AY851529**	**AY857876**	**AY851534**	----	**AY857911**
Lessoniaceae						
Dictyoneurum californicum Rupr.	Agassiz Beach, CA, USA	**AY851520**	**AY857881**	**AY851540**	----	----
Lessonia corrugata Lucas	Gov. Is. Reserve, Tas., Australia	**AY851532**	**AY857902**	**AY851545**	----	**AY857930**
Lessonia flavicans Bory	Rookery Bay, Falkland Islands	**AY851531**	**AY857900**	**AY851543**	----	**AY857931**
Lessonia nigrescens Bory	Las Cruces, Chile	**AY851530**	**AY857901***	**AY851544**	----	**AY857929**
Lessonia trabeculata Villouta and Santelices	Yoon et al. (2001)	----	----	----	AF318991	----
Lessonia vadosa Searles	Yoon et al. (2001)	----	----	----	AF318993	----
Lessoniopsis littoralis (Farlow and Setch.) Reinke	Frank Island, Uculet, BC, Canada	**AY851524**	**AY857874**	**AY851538**	----	**AY857909**
Macrocystis integrifolia Bory	Cape Beale, Bamfield, BC, Canada	**AY851507**	**AY857882***	**AY851546**	----	**AY857915**
Nereocystis luetkeana (Mertens) Post. and Rupr.	Cape Beale, Bamfield, BC, Canada	**AY851509**	**AY857883***	**AY851548**	----	**AY857914**
Pelagophycus porra (Leman) Setch.	Yoon et al. (2001)	----	AF319039	----	----	----
Pelagophycus porra	San Diego, CA, USA	**AY851508**	----	**AY851547**	----	**AY857916**
Postelsia palmaeformis Rupr.	Saunders and Druehl (1993a)	**AY851510**	AF362998	**AY851549**	----	**AY857913**

Data reported on the same line are from the same isolate. Entries in bold were completed in this study, while (----) indicates no data. Sequences denoted with an (*) include ITS1 data from Saunders and Druehl (1993b), and these data are submitted to GenBank.

LSU, large subunit; ITS, internal transcribed spacer regions.

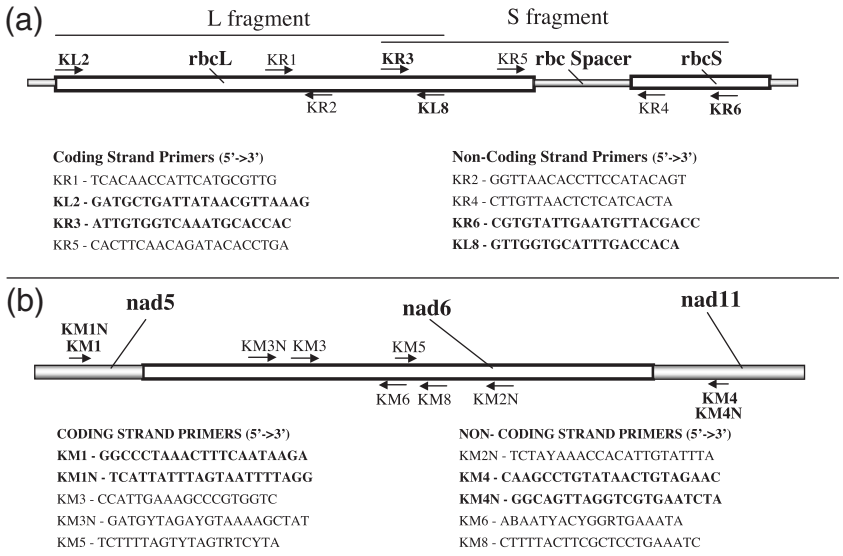

FIG. 2. Primer design for the chloroplast RUBISCO operon (a) and the nad6 region (b) of the mitochondrion. All primers were used for sequencing; bold indicates primers used for PCR as well. The RUBISCO Operon was amplified in two overlapping fragments while the nad6 region was amplified as a single product. Owing to the variability of the nad6 region, more than one external primer had to be designed, as well as a number of internal primers to acquire product and sequence data from all taxa.

PE Applied Biosystems (Foster City, CA, USA) Big Dye (V 3.0) sequencing kit according to the manufacturer's protocol. Samples were analyzed using a PE Applied Biosystems 3100 automated sequencer. Complementary and overlapping sequences were edited and aligned using SeqEd (PE Applied Biosystems).

Alignments. Alignments were prepared by eye with the computer program SeqPup (Gilbert 1995) and raw elements are available upon request whereas edited alignments can be downloaded from GenBank. Forty-two species from the ALL families were included in various analyses, with *Chorda filum* as the outgroup (Table 1). *Chorda filum* was chosen as the outgroup because *Chorda* shares unique morphological features with the ALL families (Kawai and Sasaki 2000) and has been resolved as sister to the ALL complex in molecular investigations (Sasaki et al. 2001). Seven different alignments, five single region and two concatenated, were used to investigate evolutionary relationships among the ALL families: (1) Nuclear alignment, consisting of the ITS and LSU data for 3429 bp from 28 taxa; (2) RUBISCO alignment, incorporating 2069 bp of the RUBISCO operon, including the *rbc*L, *rbc*Sp, and all but *ca*. 100 bp of the 3′ end of the *rbc*S for 30 taxa; (3) Mitochondrial alignment, including 131 bp of nad5, followed by the entire 936 bp of nad6, and 46 bp of nad11 for a total of 1113 bp from 30 taxa; (4) Combined Total, consisting of 5873 bp from 28 taxa, including all the previous sequences except 1st and 3rd codon positions from the mitochondrial alignment; (5) Combined Ingroup, excluding the outgroup and comprised of 6192 bp and 27 taxa, including all data except the 3rd codon positions from the mitochondrial alignment; (6) Group 3-ITS alignment, with 14 taxa and 590 bp, including portions of the ITS1, 5.8S and ITS2; and (7) Group 4-*rbc*Sp (and flanking regions) alignment, with 642 bp, including 12 taxa.

Saturation and data congruency. Data saturation was evaluated by comparing uncorrected divergence values (p) against corrected values (Daugbjerg and Andersen 1997, Draisma et al. 2001). Coding regions were partitioned by codon position, whereas data from each spacer region (ITS, *rbc*Sp) and the LSU were each analyzed as a partition. The DNA substitution model for each partition was calculated individually using Modeltest (Posada and Crandall 1998). The relationship between the uncorrected P values and those of the models used to correct for multiple substitutions is an indicator of saturation—the larger the discrepancy between the corrected and uncorrected values for a partition, the greater the saturation (observed as a plateau in uncorrected values when plotted against corrected values; cf. Daugbjerg and Andersen 1997).

Congruence of the three organellar genome regions in our combined data sets was tested using the Incongruence Length Differential (ILD) test, implemented as the Partition Homogeneity Test in PAUP* v4.0b10 (Swofford 2002). Invariant sites were removed from the data set as suggested by Cunningham (1997) because of the large discrepancy in the size of the partitions. One thousand runs, with 10 replicates of random sequence addition for each, were performed using the heuristic search option. The ILD test was performed in pairwise comparisons of the three different regions, both with and without the outgroup.

The Shimodaira–Hasegawa test (Shimodaria and Hasegawa 1999) (SH test) and the Templeton test (Templeton 1983) were used in PAUP* to test for significant difference between the conflicting backbone topologies of Druehl et al. (1997), which we term the "Type I" topology, and Yoon et al. (2001), termed the "Type II" topology. Using our Combined Total alignment, a tree file defining a Type I backbone topology only (i.e. not enforcing any constraints within the lineages themselves), was constructed by hand in PAUP* and used as a constraint tree for likelihood analyses. The resulting likelihood tree was then compared against the best tree (Type II topology) for the Combined Total alignment.

Phylogenetic analyses. Gaps in the alignment were treated as missing data in unweighted parsimony analyses performed in PAUP*, and 50 heuristic search replicates were used with TBR branch swapping. Bootstrap values (Felsenstein 1985) were calculated with 1000 replicates using 10 random additions under the heuristic search method. Model parameters used in neighbor-joining (NJ) and minimum evolution (ME) analyses, performed in PAUP*, were estimated with Modeltest. These two distance methods produced no supported differences in topology, so NJ was used in bootstrap analyses to reduce computing time. One thousand NJ bootstrap replicates were performed.

Bayesian analyses were used as a proxy for maximum likelihood topologies because of computing time the flexibility associated with unlinking data partitions, and were completed with MrBayes v 3.0b4 (Huelsenbeck and Ronquist 2003) using the GTR + I + G model with parameters estimated during the

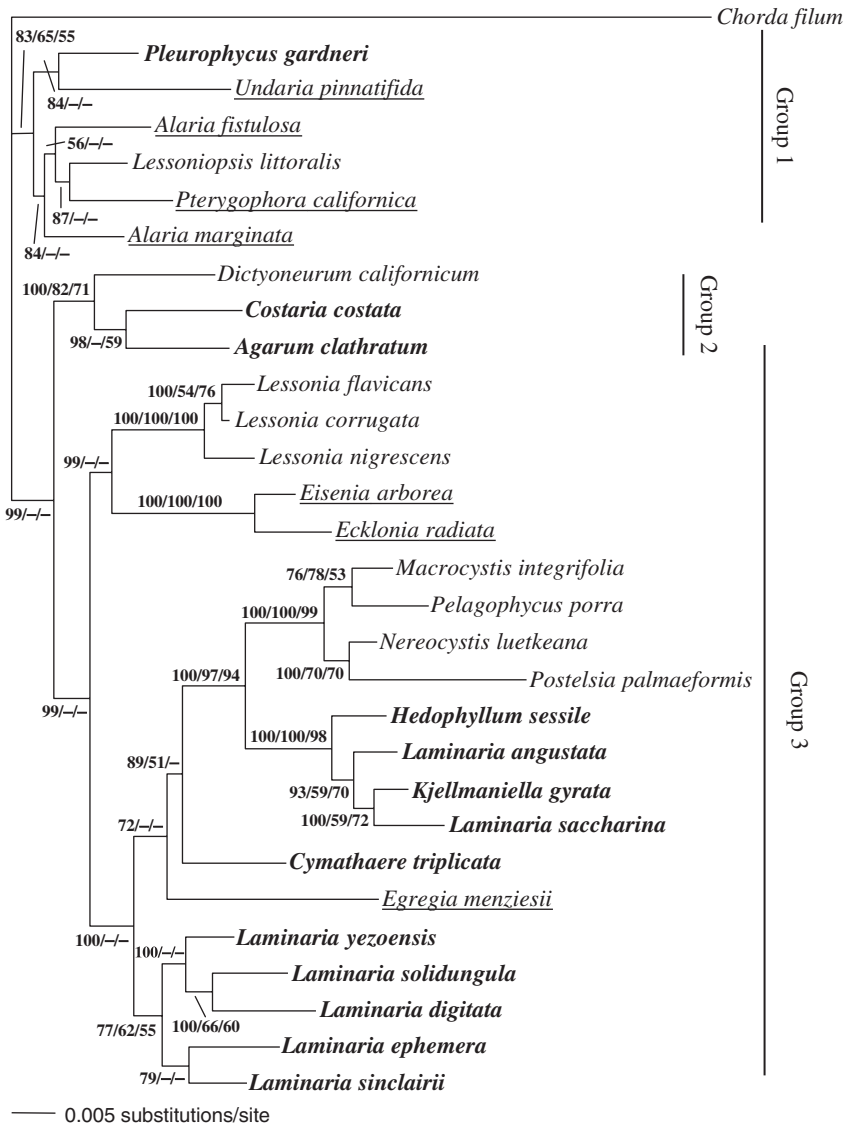

FIG. 3. Bayesian consensus tree for the RUBISCO alignment. The backbone relationships resolved in this tree is an example of a Type I topology. When this topology is recovered, all three groups are monophyletic and stable in their composition. Underlined taxa are those traditionally ascribed to the Alariaceae, taxa in bold are those placed in the Laminariaceae, and taxa in plain text formerly belonged to the Lessoniaceae (except *Chorda filum*). Groups 1–3 correspond to clades resolved in an earlier publication by Saunders & Druehl (1993b). Support values are presented as Bayesian posterior probabilities, neighbor-joining bootstrap and parsimony bootstrap, respectively, and "-" indicates <50% support in a particular analysis.

analyses. Alignments were partitioned by gene and codon position in Bayesian analyses, with Ti/Tv ratio, substitution rate of the GTR model, nonsynonymous/synonymous rate ratio, gamma shape parameter, and the proportion on invariant sites, all "unlinked" between partitions. Where indicated, the covarion model option was implemented in MrBayes. Each Bayesian data set was independently analyzed three times, using 1, 1, and 4 million generations, respectively, to ensure stability in tree topology. The default parameters were used for temperature and swapping, and trees were sampled every 100 generations. Examination of the $-\ln$ likelihood ($-\ln L$) scores indicated that stability was reached in the first 200,000 generations. In order to ensure stability, the first 400,000 generations were discarded as the "burn-in" phase and the remaining trees were used to compute the consensus tree.

RESULTS

Saturation. Only mitochondrial partitions showed evidence of saturation. The third codon partition produced values indicating more than one substitution per site when compared across all of the taxa in our trees using the model generated by Modeltest. In addition, the mitochondrial first codon partition showed the characteristic "plateau" of values described by Graybeal (1994) (e.g. Daugbjerg and Andersen 1997, Fig. 1b) for data too variable for the question being asked. Both the first and third codon partitions were thus removed from combined analyses including the outgroup (Combined Total alignment). When *Chorda* was removed from the data set, however, only the mitochondrial third codon partition continued to show signs of saturation, and was the only partition removed from our Combined Ingroup alignment analyses. In both cases, analyses were conducted with these partitions included to investigate the effect of these data on the tree topology and their inclusion produced a consistent loss of resolution in our analyses.

RUBISCO alignment. The RUBISCO data set had 220 parsimony informative sites and Modeltest identified the TVM + I + G model as the best fit. The base

frequency had an A–T bias (A = 0.3113, C = 0.1533, G = 0.1872, T = 0.3482), which became more apparent when the codon positions were partitioned.

Data from the third codon position of the *rbc*L showed a marked difference from the other two codon positions. Third positions from ALL taxa had a maximum divergence of 40% when compared with the outgroup, over 10× the level of divergence in the first position, and 25× the second position. In addition, the bias toward A–T in the *rbc*L was predominantly in the third position (72% A–T compared with the second position 58% A–T). The *rbc*S was slightly more variable than the *rbc*L, but with less discrepancy between the different codon positions (divergence levels for the third codon postion were <2× the first position and <6× the second position). However, the A–T bias of the third codon position was higher in the *rbc*S (77%) than the *rbc*L.

Bayesian analysis ($-\ln L$ of 8413.07730) produced the Type I (cf. Druehl et al. 1997—Group 1 sister to Groups 2 and 3, all groups monophyletic) topology (Fig. 3) and resolved Group 1 as sister to the other ingroup lineages with a posterior probability of 99. Parsimony analysis recovered 16 trees with a length of 895, a consistency index (CI) of 0.564 and a retention index (RI) of 0.581. Parsimony analysis also supported a Type I topology, but the backbone of the tree received no bootstrap support (Fig. 3). NJ analysis recovered a tree with a Type II topology (Group 3 forming a paraphyletic grade leading to an association between Groups 1 and 2) with *Lessonia* as sister to the remaining ingroup taxa (tree not shown). However, there was only support among relationships within clades, not along the backbone.

Phylogenetic trees produced by Bayesian and parsimony analyses for the RUBISCO alignment were remarkably similar in topology to the likelihood tree from the short and variable ITS1 data set of Druehl et al. (1997, Fig. 3c). However, a better representation of taxa in our data resolved relationships not previously found with the ITS1 data. Two distinct *Laminaria*-containing clades were resolved in our RUBISCO trees (Fig. 3), indicating that this genus is polyphyletic.

Nuclear alignment. All of the LSU data in the nuclear alignment were produced during this study, but the ITS sequences came from a variety of sources (Table 1), including Saunders and Druehl (1993a). The first 1027 bp of the alignment consisted of the ITS/5.8S region and the remaining 2890 bp were LSU data. For the purposes of analyses, 491 bp of ambiguously aligned data were removed from the ITS region of the data set. The model chosen by Modeltest was the TrN + I + G with fairly equal base frequencies (A = 0.2341, C = 0.2468, G = 0.2989, T = 0.2191).

The LSU and ITS data, not surprisingly, contrasted sharply in their level of variation. Even after removing all but the most conservative regions, the ITS data had high divergence values (up to 31% between ALL taxa) when the model chosen by Modeltest was used. On the other hand, the LSU data were less than 3% divergent among the ALL taxa.

In contrast to our RUBISCO data, all analyses of the Nuclear alignment produced the Type II topology. Bayesian analysis produced a Type II topology tree with a $-\ln L$ score of 9392.85883 (Fig. 4) and resolved *Egregia* as sister to the remaining ALL taxa with a posterior probability score of only 78 with members of Group 3 forming a paraphyletic grade leading into Groups 1 and 2. Parsimony analysis recovered 18 trees with a length of 879, a CI of 0.5836 and an RI of 0.5892. The consensus tree had the Type II topology, however, the backbone received no support with bootstrap analysis. NJ also produced a Type II backbone topology that was not supported by bootstrap analysis. The removal of *Egregia* (see below) from the alignment resulted in a parsimony consensus tree with a Type I topology, which was not supported by bootstrap, but had little effect on NJ or Bayesian tree topologies. In all cases, both Groups 1 and 2 were monophyletic in analyses of nuclear data (Fig. 4).

Mitochondrial alignment. The model specified by Modeltest for the mitochondrial region was the TVM + I + G and there was an A-T bias (A = 0.2899, C = 0.1725, G = 0.209, T = 0.3286) in the base frequency. Sequences from the *nad*6 region showed evidence of saturation, with divergence values between 40% and 61% for second codon positions and rates between 195% and 258% (indicating >1substitution per site) for the third codon position when the model generated by Modeltest was used to compare ALL taxa to the outgroup. Even among ingroup taxa, divergence was as high as 80% at the third codon position. However, the A-T bias of the third codon position (76%) was no worse than the *rbc*S third position. Owing to the extremely high levels of sequence divergence in the mitochondrial region when the outgroup was included, phylogenetic analyses conducted solely with this data set showed low levels of support for the backbone topology of the ingroup taxa (data not shown). While none of the supported relationships were in conflict with our other analyses, we determined that these data alone were of little use for analyses directed at resolving the backbone structure of the ALL families.

Congruency tests for combined alignments. The pairwise ILD test values for both the Combined Total (mitochondrial/RUBISCO = 0.193, mitochondrial/nuclear = 0.176, RUBISCO/nuclear = 0.186) and Combined Ingroup (mitochondrial/RUBISCO = 0.025, mitochondrial/nuclear = 0.074, RUBISCO/nuclear = 0.017) alignments were within the acceptable range for combining the data sets when the P value of 0.01 was used (Cunningham 1997). Values for the Combined Ingroup alignment were much higher when the fairly invariable LSU was removed from the nuclear data (mitochondrial/nuclear = 0.115, RUBISCO/nuclear = 0.537). However, removal of the LSU from the data did not change the topology of our trees.

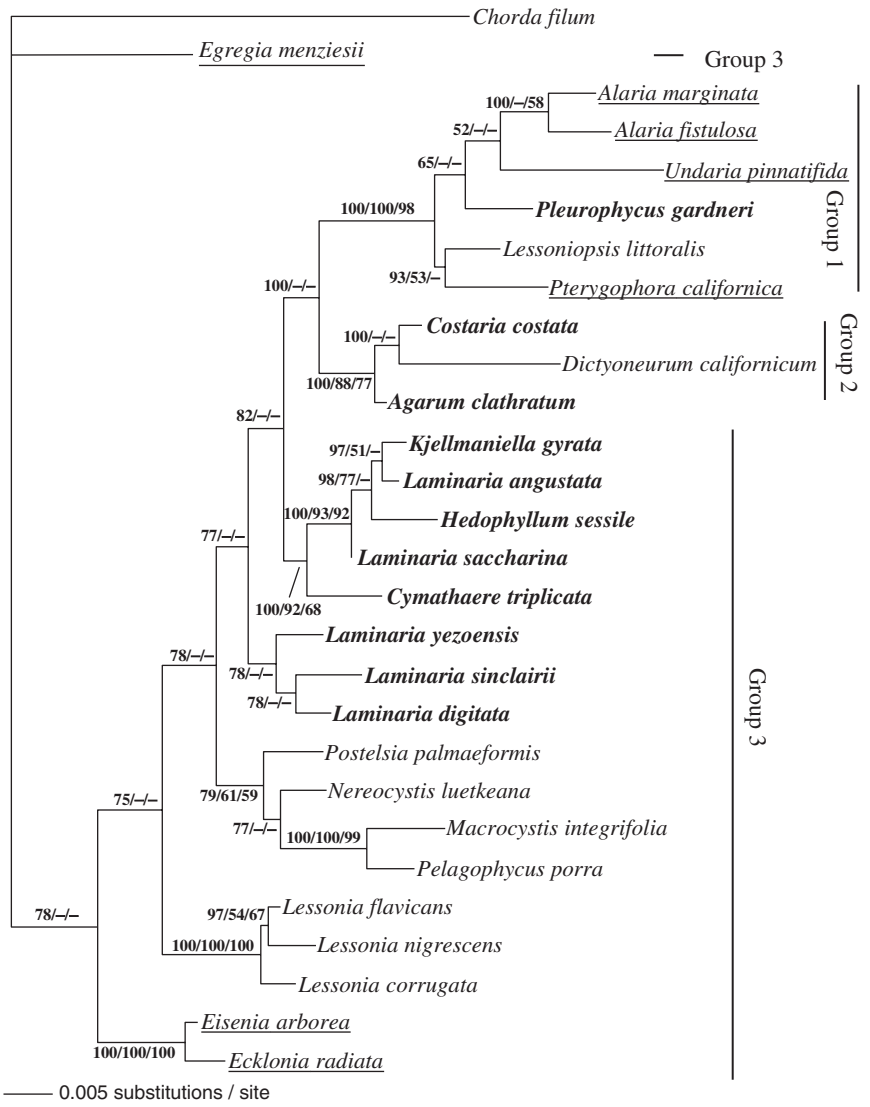

FIG. 4. Bayesian consensus tree for the Nuclear alignment. Nuclear data produced trees with a Type II backbone topology, in which Group 3 is paraphyletic leading to Groups 1 and 2. Symbols and the order of support values are as in Fig. 3.

Combined total alignment. A slight nucleotide bias occurred in the 5873 bp of the Combined Total alignment toward A/T, at the expense of C (A = 0.2624, C = 0.2098, G = 0.2520, T = 0.2758). The model estimated by Modeltest for analysis of these data was the TrN + I + G. NJ analysis produced a tree with a Type II topology and *Egregia* as the earliest divergence among the ingroup taxa, but only weakly (bootstrap support of 57). Parsimony recovered two trees with a length of 1903, a CI of 0.572 and an RI of 0.571. Bootstrap analysis supported the Type II backbone topology for parsimony analysis, with *Egregia* sister to the other ingroup taxa. Bayesian analysis (with partitioning as outlined in the Materials and Methods) resulted in a tree with a $-\ln L$ of 18797.03242 and a Type II backbone topology (Fig. 5a). However, when *Egregia* was removed from the alignment, both parsimony (<50 bootstrap support) and Bayesian (posterior probability score of 86) analyses produced Type I topologies (Fig. 5b). Further analysis of the Combined Total alignment using a covarion model yielded a Type I topology resolving *Egregia* in Group 3 with moderate support (Fig. 6). When *Egregia* was removed and the covarion option invoked, support for the major clade either remained the same (100% for Groups 1 and 2) or increased (from 83% to 96% for Group 3) (Fig. 6).

Subsequently, each of the other ALL taxa in the alignment were individually removed and parsimony analyses were performed for each permutation of the alignment to determine whether the removal of other taxa would change the tree topology. The only taxon, besides *Egregia*, to change the backbone topology when removed was *Agarum clathratum*. The removal of *Agarum* caused Group 2 to move to an association with *Lessonia*, but this arrangement was not supported by bootstrap analysis. However, removing *Egregia* turned the entire tree inside out (Fig. 5), indicating probable branch attraction (Felsenstein 1978, Hendy and Penny 1989) between *Egregia* and our outgroup,

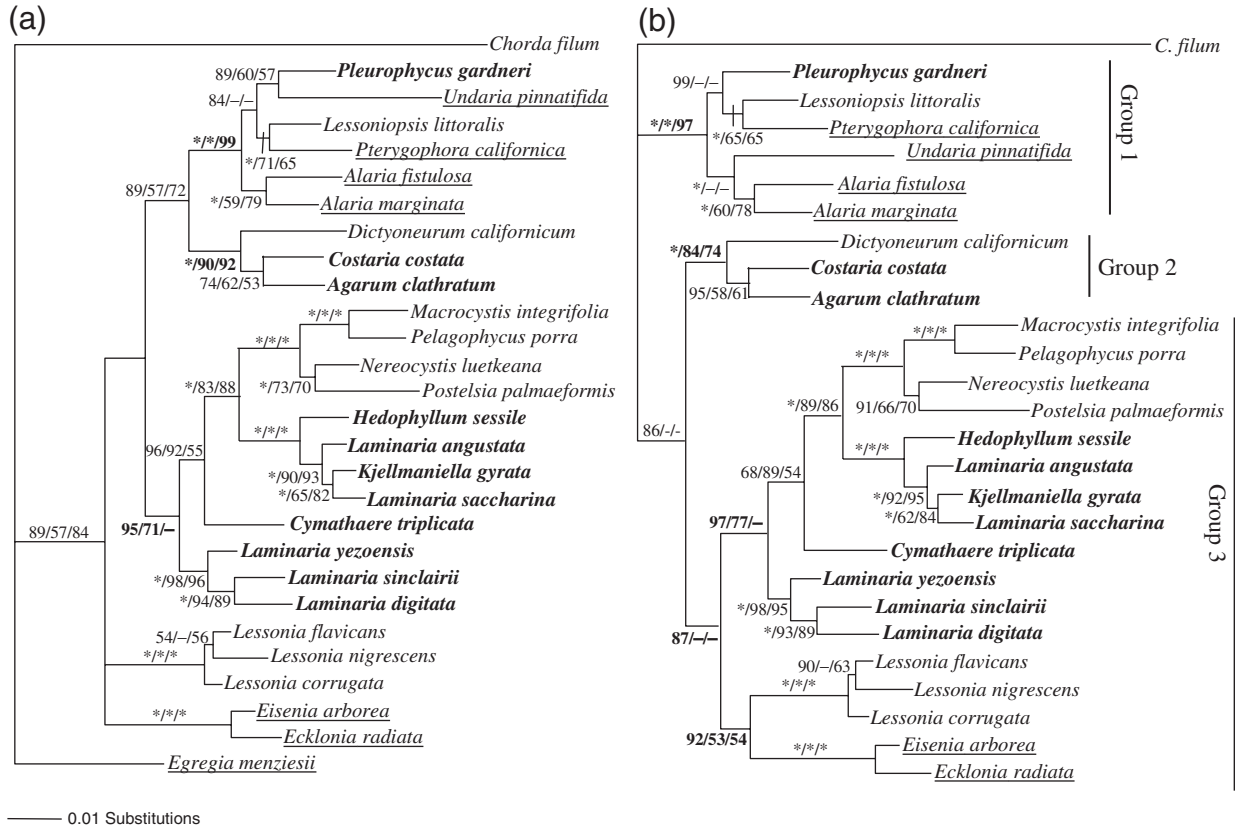

FIG. 5. Bayesian consensus tree from the combined total alignment (nuclear, RUBISCO, and 2nd codon positions from the nad6 region of the mitochondrion) both (a) with, and (b) without *Egregia menziesii* included in the analysis. The removal of *E. menziesii* from the data set changes the topology from a Type II to a Type I under Bayesian analyses. Support values are presented in the same order as Fig. 3 (Bayes/NJ/pars). *100% support and "-" indicates <50% support for each analysis. Support values in bold indicate major clades.

Chorda filum. To further investigate branch attraction in our data both the SH and Templeton tests were performed using the Combined Total alignment. When the conflicting Type I and II topologies were tested using the SH test their likelihood was not significantly different ($P = 0.398$). The Templeton test also found no significant difference between the topologies ($P = 0.138$) under parsimony.

The attraction of an ingroup taxon with a long branch to the outgroup is a common problem and has been studied with both simulation and analytical studies (cf. Holland et al. 2003). In particular, Holland et al. (2003) found that all the tree estimation methods they studied, which included the methods used in this study, were biased toward pairing the outgroup taxon with an ingroup taxon in situations where the outgroup has a long branch compared with the branches among the ingroup taxa. Further support for branch attraction as an explanation for the Type II topology was harnessed when a covarion model was used for analysis (Fig. 6) or when additional *Chorda* sequences were added to an *rbc*L alignment (selected because of availability of data in GenBank). With more *Chorda* sequences to break up the outgroup branch the tree changed from a Type II to a Type I backbone topology (not shown).

In light of the previous discussion, we removed the outgroup for a series of subsequent analyses to reduce the attraction artifact and rooted the tree along the branch between Group 1 and the remaining two Groups, i.e. a Type I topology.

Combined ingroup alignment. The base frequency in the Combined Ingroup alignment was fairly equal (A = 0.2663, C = 0.2017, G = 0.2516, T = 0.2804), but reflected the same slight A/T bias as our Combined Total alignment, (i.e. *Chorda filum* included). The model estimated by Modeltest was the GTR + I + G. The Combined Ingroup alignment produced trees with consistent, strong support in all analyses (Fig. 7). The $-\ln L$ of our Bayesian tree was 20046.57258, while parsimony yielded three trees with a length of 1943, a CI of 0.556 and an RI of 0.577. In all cases Groups 1 and 2 were solidly monophyletic and sister to a reasonably supported Group 3, which consisted of two distinct clades, one we continue to label Group 3, and the other, including *Ecklonia*, *Eisenia*, *Egregia* and *Lessonia*, which we here designate "Group 4."

Group 3: ITS Alignment. All of our analyses indicated a split between members of the genus *Laminaria*, thus we expanded our sampling for this genus using new and published ITS sequences available in

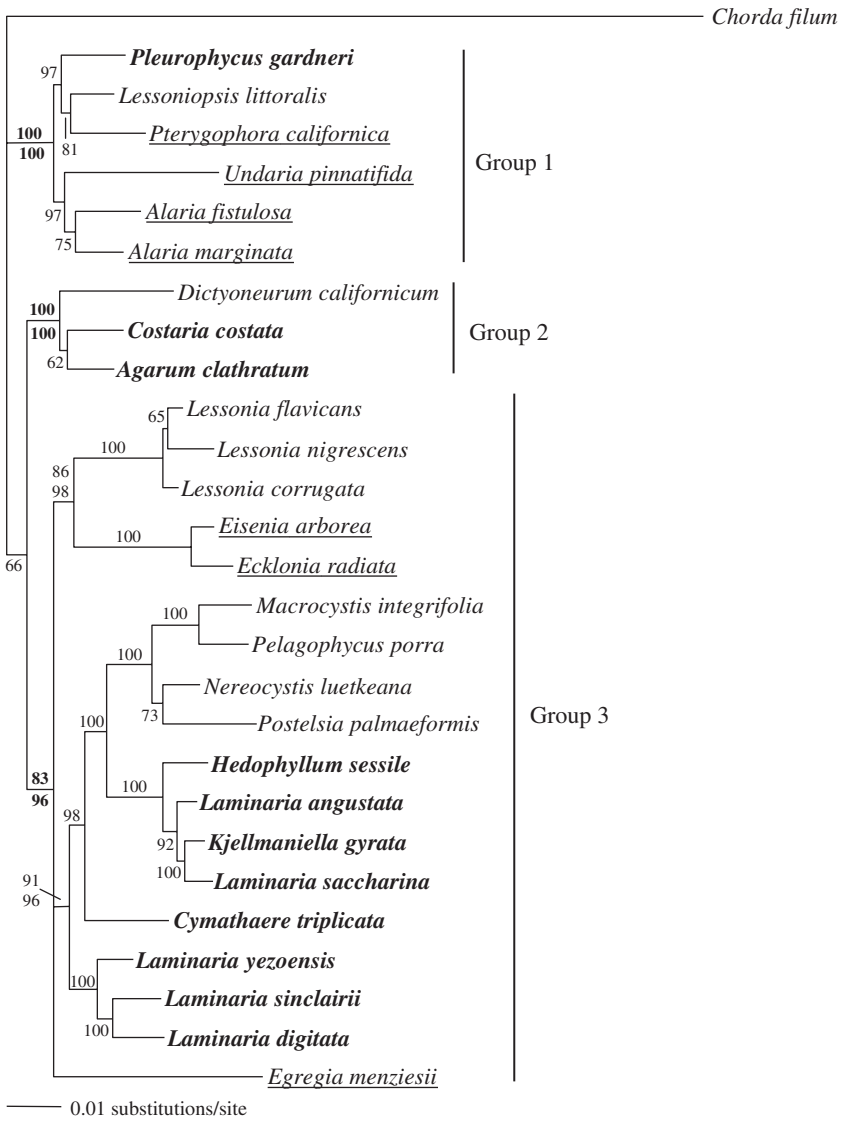

FIG. 6. Phylogeny resulting from analysis of the combined total alignment with the covarion option in MrBayes. Implementation of the covarion model produced a Type I topology, resolving *Egregia menziesii* as a member of Group 3. At nodes with two posterior probability values, the bottom number is the value found when Egregia is excluded from the analysis. Support values in bold indicate major clades.

GenBank. Parsimony analysis recovered three trees with a length of 205, a CI of 0.727 and an RI of 0.781, while the Bayesian tree had a $-\ln L$ of 2087.89591. All three methods of analyses supported what our previous alignments indicated: species of *Laminaria* fall into two genetically distinct clades (Fig. 8a). One of the two clades (Clade 1) includes *L. digitata*, the type of the genus, and an assemblage of species with diverse holdfast and blade morphologies. The second clade (Clade 2) is comprised of several *Laminaria* species, as well as representatives of other genera, including *Cymathaere*, *Hedophyllum* and *Kjellmaniella* (Fig. 8a).

In addition, several *Laminaria* ITS sequences in GenBank were identical, indicating identification errors, a large degree of over-classification in this genus, or a combination of the two. Sequences from *Laminaria digitata* (this study) and *L. hyperborea* (GenBank #AF319015) were identical, however, they differed from other sequences of *L. hyperborea* in GenBank (AY441771–3) by 20 bp. Therefore, AY441773 was used to represent *L. hyperborea* in our analyses. For the second, more problematic clade, our *Laminaria japonica* ITS sequence was identical to the *L. longissima* (AB022801, AB022802), *L. diabolica* (AB022795, AB022794) and *L. longipedalis* (AB022797, AB022798) sequences of Yotsukura et al. (1999) and only one difference separated *L. japonica* from *L. religiosa* (AB022791, AB022792) and *L. ochotensis* (AB022793, AB022794). It has been previously suggested that these taxa are conspecific (Yoon et al. 2001) and our analyses support this conclusion. In addition, our *L. saccharina* ITS sequence was identical to *L. coriacea* (AB022803, AB022804), *L. cichorioides* (AB022805, AB022806), and *L. yendoana* (AB022806, AB022807) from Yotsukura et al. (1999), and *L. longicuris* from our own collections. Deciphering between over-classification and misidentification of these taxa

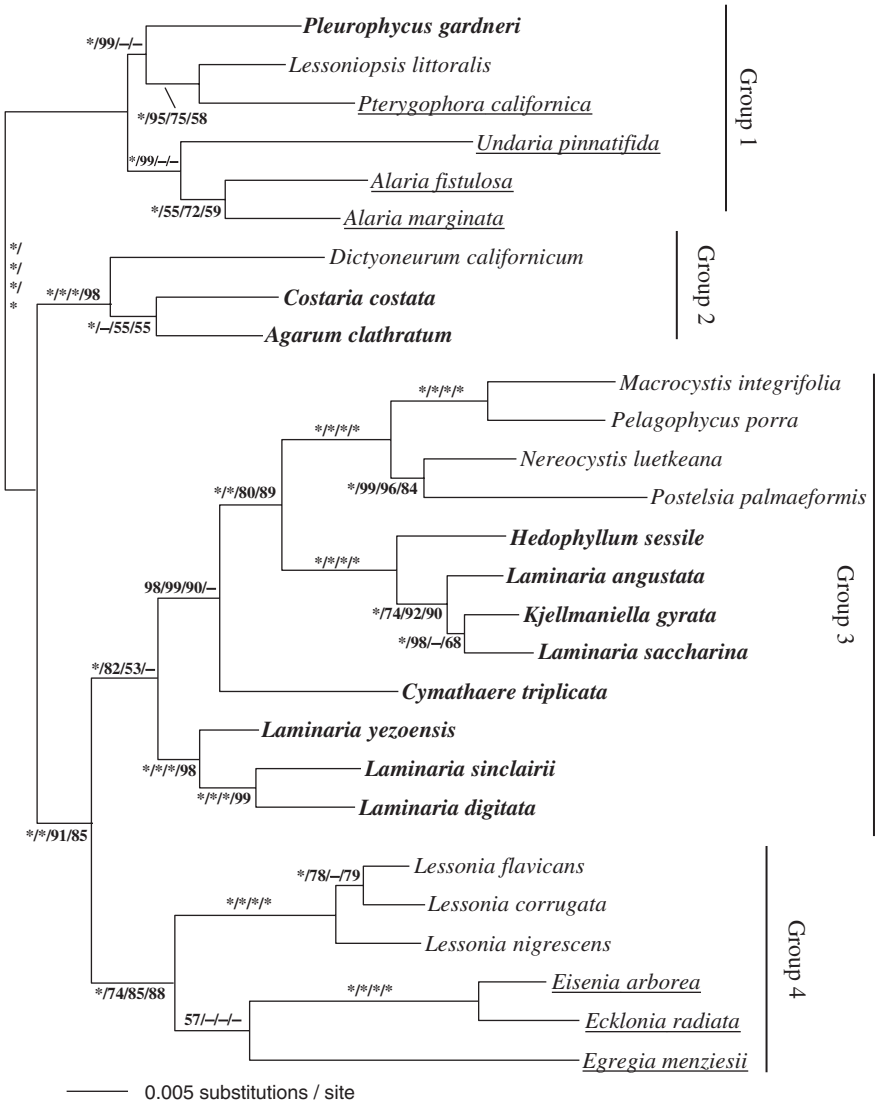

FIG. 7. Bayesian consensus tree from the combined ingroup alignment (nuclear, RUBISCO, and 1st and 2nd codon positions from the nad6 region of the mitochondrion). Text styles are consistent with Fig. 3. Support values are presented as Bayesian posterior probabilities, posterior probabilities under a covarion model, Neighbor-Joining bootstrap and parsimony bootstrap, respectively. *, indicates full support at a node, whereas -, indicates $<50\%$ support in a particular analysis.

will require a thorough examination of species from both clades of *Laminaria*.

Group 4: rbcSp Alignment. Sequence data from the *rbc*Sp were available for six additional members of Group 4 from GenBank, increasing the representation for this lineage to 12 taxa. A clear A–T bias ($A = 0.3453$, $C = 0.1354$, $G = 0.167$, $T = 0.3523$) in the nucleotide composition was evident. Modeltest chose the K81uf + I model. Analyses of the Group 4-*rbc*Sp alignment produced two trees in parsimony, with a length of 105, a CI of 0.914 and an RI of 0.932. The $-\ln L$ of the Bayesian tree was 1441.16711. A nearly identical topology was recovered by all analyses (Fig. 8b), with only the position of *Lessonia corrugata* ambiguous among analyses. All of the *Lessonia* sequences form a strongly supported clade, as do the species of *Ecklonia, Eckloniopsis*, and *Eisenia* (Fig. 8b). Neither *Ecklonia* nor *Eisenia* are monophyletic in our trees, with *Eckloniopsis* positioned among species of *Ecklonia*.

DISCUSSION

Kelp biologists have used the classification system of Setchell and Gardner (1925), based on morphological characters, for over 75 years. However, our data indicate that a rearrangement of familial level taxonomy among the ALL genera is requisite. This is not a new revelation (Saunders and Druehl 1993b, Druehl et al. 1997, Yoon and Boo 1999, Yoon et al. 2001), but previous molecular analyses of the ALL families have only weakly resolved relationships among the component genera; generally resulting in a conservative approach to taxonomic revision. Here we present the most comprehensive phylogenetic analyses for the ALL taxa published to date.

Few hypotheses regarding the evolution of the ALL families of the Laminariales have been presented in the literature and, owing to their predominantly North Pacific distribution, there are even fewer biogeographical hypotheses that are relevant at the family level.

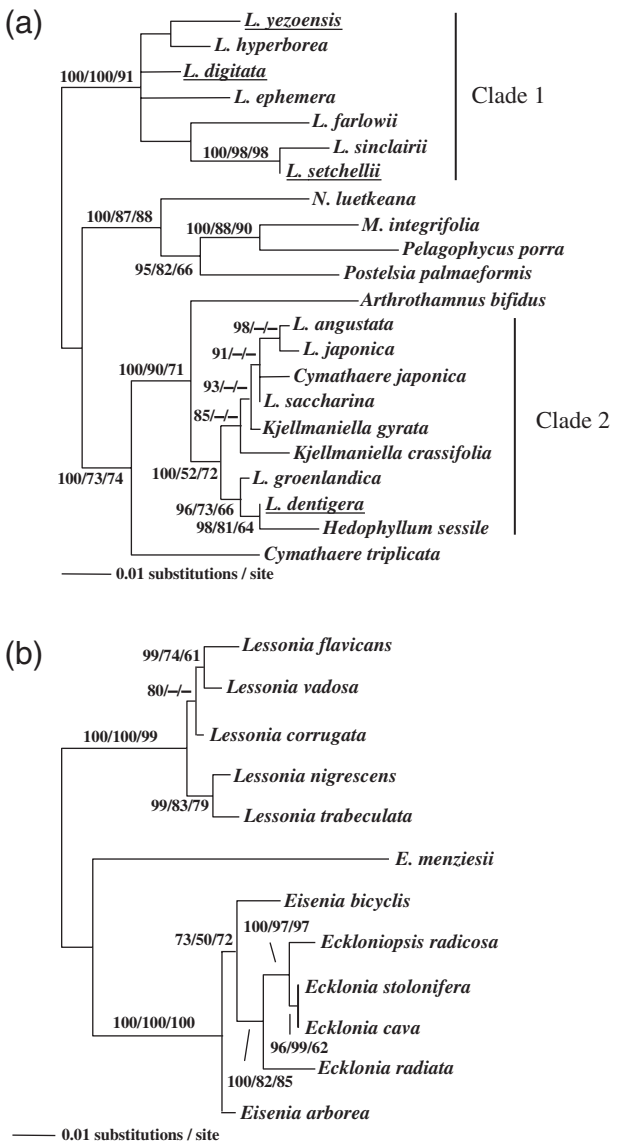

FIG. 8. Bayesian consensus trees of ingroup alignments from under-represented areas of the Combined data sets. (a) Internal transcribed spacer regions data from members of the two clades of *Laminaria* and the related members of Group 3. Underlined taxa produce divided blades, a character previously used to establish the section Digitatae (Agardh, 1867). (b) RUBISCO spacer and flanking coding regions for representatives of Group 4. Symbols and the order of support values for both trees are as in Fig. 3.

Our results provide a solid foundation for a meaningful assessment of such issues, particularly for the reevaluation of morphological evolution in the Laminariales, as they indicate parallel evolution of several gross morphological features. In light of our trees we can conclude, for example, that sporophylls have evolved multiple times, at least once each in Groups 1, 3 and 4, and should not be considered homologous structures. Sporophylls are borne from the stipe in the Alariaceae, whereas they are divergent variations of the blade (above the transition zone) in Groups 3 and 4 (e.g. *Macrocystis* and *Eisenia*, respectively). Splitting has also evolved multiple times the Laminariales, both at the transition zone [e.g. *Lessoniopsis* (Group 1) and *Nereocystis* (Group 3)] and in the blade (e.g. *Laminaria digitata* and *L. dentigera* representing divergent clades of *Laminaria* in Group 3). In fact, Setchell and Gardner's Laminariaceae was based on a single morphological trait (a simple blade), which probably represents the plesiomorphic condition, its distribution on the tree in part possibly the result of parallel reversals to this state. The data presented here establish the groundwork for meaningful family-level comparative studies within the Laminariales considering more detailed aspects of anatomy and biochemistry in both the sporophyte and gametophyte generations.

We can now account for the discrepancies in formerly published molecular phylogenies of the Laminariales, which are likely because of the rapid radiation of northern Pacific kelps (Estes and Steinberg 1988) combined with some long branches in the tree. A further challenge to resolving a molecular phylogeny for the ALL families is the genetic distance between them and the five species among the "ancestral" families, which are difficult to acquire because most inhabit remote localities in northern Japan and Siberia. *Chorda filum* was the only species available to us for DNA extraction, and as our single outgroup, arguably created branch attraction artifacts in our trees. Individual DNA-region phylogenies produced conflicting hypotheses with variable levels of resolution and for all of our data sets *C. filum* pulled members of Group 4 (almost always *Egregia*) to the base of the tree in some, or all analyses. We believe this arrangement (Type II topology) is a long-branch artifact based on the following evidence: (1) the removal of *Egregia* from our analyses consistently results in a Type I topology; (2) removing any other taxon in the alignment does not result in supported changes in the backbone structure of the tree; (3) using a covarion model, which takes into account changing evolutionary rates over time (Lockhart et al. 1998, Huelsenbeck 2002), on the Combined Total alignment produced a Type I topology; (4) and, whereas an *rbc*L-only alignment with *Chorda filum* as the sole outgroup produces a Type II topology, diversifying the outgroup by the addition of more *Chorda* sequences from GenBank draws Group 1 into a sister association with the remaining ALL taxa and places *Egregia* in an unresolved polytomy with a monophyletic Group 2 and members of Groups 3 and 4.

We thus strongly believe we are correct in selecting the Type I topology for the rooting of our Ingroup analyses, which enabled us to remove distant outgroups and include more positions in our analyses. This decision, however, only affects the taxonomy of Group 4 and the hypothesis of the pattern in which the Groups have evolved, i.e. Groups 1, 2 and 3 as designated here are monophyletic in both Type I and II topologies (Fig. 5). While at first glance the tree topologies of Yoon et al. (2001) and Druehl et al. (1997) appear substantially different, as do our own Type I

and II topologies resolved here in the Combined Total alignment, the position of the outgroup is the only major discrepancy between them (positioned within Group 4 in the Type II topologies and along the branch separating Group 1 from the other Groups in the Type I topology). Thus, if we are wrong in our argument that the Type I topology is correct, either a paraphyletic Group 4 will continue to be recognized as a family, or multiple families will have to be established for the various lineages (probably three, including one each for *Egregia*, *Lessonia*, and the *Ecklonia/Eisenia* complex) to restore monophyletic taxa, but Groups 1, 2 and 3 remain monophyletic (Fig. 5).

Our Combined Ingroup alignment produced trees with solid support for Groups 1–3 of Druehl et al. (1997) and for Groups 1, 2, and 4 proposed here. Our Group 3 received full support with Bayesian analysis (moderate support with the covarion model), but low support in NJ and parsimony bootstrap analyses (Fig. 7). Interestingly, the short (468 bp) and variable ITS1 data set of Saunders and Druehl (1993b) and Druehl et al. (1997) gave a similar tree to the data presented here.

Group 1: Alaria, Lessoniopsis, Pleurophycus, Pterygophora, Undaria (Undariella). Group 1 consistently forms a well-supported clade in all our molecular analyses. Saunders and Druehl (1993b) had previously emended the Alariaceae to include only the genera *Alaria*, *Lessoniopsis* and *Pterygophora*, all of which have a midrib on the blade and stipe-derived sporophylls. However, in a subsequent publication (Druehl et al. 1997), *Pleurophycus* was resolved in Group 1, while *Undaria* was hypothesized to belong to this assemblage owing to its stipe-derived sporophylls, which occur as opposite frilled margins on the stipe (Yendo 1911, Okamura 1915). This last hypothesis was supported by all of the relevant analyses presented here.

Pleurophycus is the only member of this clade that lacks specialized blades for the reproductive sori (Fig. 1c), producing the sorus on its midrib instead. *Pleurophycus* never falls at the base of Group 1 in our analyses and one hypothesis is to conclude that sporophylls were lost in the evolution of this genus from a sporophyll-bearing ancestor (Druehl et al. 1997). However, another possibility is that the "blade" of *Pleurophycus* may itself be an enlarged sporophyll; as with the sporophylls of *Alaria*, the blade has a distinct collar at the transition zone and is shed annually (Germann 1986). Further investigation of this hypothesis is warranted.

Lessoniopsis, *Pleurophycus*, and *Pterygophora* are all monotypic genera, but as many as 25 species have been described for *Alaria*. Currently, 12 species are accepted in the genus (Widdowson 1971, Lüning 1990), of which 11 are confined to the north Pacific. *Alaria esculenta* is the only species of *Alaria* in the North Atlantic and is distributed throughout the Arctic and northern cold temperate regions, except along the northwestern coast of North America (Widdowson 1971). *Alaria fistulosa* is distinguished from other species by the air bladders in its midrib, and it is quite distinct genetically from *A. marginata*. In fact, as much divergence occurs between these two species of *Alaria* as occurs between other genera in Group 1 (Fig. 7). An in-depth analysis of this genus is currently being prepared for a separate publication.

Group 2: Agarum, Costaria, Dictyoneurum (Dictyoneuropsis) Thalassiophyllum. *Agarum*, *Costaria*, and *Dictyoneurum* group solidly together in all analyses, generally have a flattened stipe and, along with *Thalassiophyllum*, have either a perforated or reticulated blade. Stipe shape can be variable in both *Agarum* (Setchell and Gardner 1925) and *Costaria*, with stipes in *Costaria* becoming terete in wave-sheltered areas (Obrien 1972), whereas the flattened stipe is a stable character in *Dictyoneurum* and *Thalassiophyllum*. While none of the analyses presented here include *Thalassiophyllum*, ITS and *rbc*Sp sequences clearly place it within this group (Yoon et al. 2001), however, an *rbc*L sequence in GenBank (Kawai and Sasaki 2000: AB035793) places it variously with *Agarum* or at the base of the ALL families (Kawai and Sasaki 2000, Fig. 1). We have studied this sequence and it is clearly a chimera between *Thalassiophyllum* and a member of the Ectocarpales [the fragment corresponding to primers rbc-F2 and rbc-R3 in Kawai and Sasaki (2000)]. We are thus confident that all available data are consistent with inclusion of *Thalassiophyllum* in Group 2.

Several earlier authors had affiliated *Agarum* and *Thalassiophyllum* based on the shared character of perforated blades (Kützing 1843, Rosenthal 1890, Setchell 1893, Reinke 1903) and, subsequently, on development in *Thalassiophyllum*, which "passes through an *Agarum*-like stage" (Setchell 1905, p. 125). In the same study, Setchell states that *Agarum fimbriatum* Harvey "forms a link between *Agarum turneri* (*A. clathratum* Domortier) and such forms as *Costaria*" (Setchell 1905, p. 125). As further indication of the relatedness of taxa in this group, when *Dictyoneuropsis reticulata* was originally described (Saunders 1895), it was placed in the genus *Costaria*, as *Costaria reticulata* Saunders.

In the year after the description of *Costaria reticulata*, Setchell (1896) considered Saunders' samples to be conspecific with *Dictyoneurum californicum*. Saunders' alga, however, has a midrib, unlike Ruprect's original description of *D. californicum*. Setchell and Gardner (1925) considered this feature to be two different stages of growth and not worthy of specific distinction. Further, they placed *Costaria* in the Laminariaceae because of its simple blade, and *Dictyoneurum* in the Lessoniaceae based on splitting in the transition zone. Thus, *Costaria costata* and *Costaria reticulata* were changed from two species of the same genus to species in two different families, over the course of 30 years. Smith (1942) later interpreted the presence or absence of a midrib in *Dictyoneurum* a generic character when he established *Dictyoneuropsis reticulata* for members of *Dictyoneurum* as that had a midrib on the blade.

In agreement with Fain (1986) and Saunders and Druehl (1993b), we found no difference in the sequence between *Dictyoneurum* and *Dictyoneuropsis,* even in the variable ITS region. While our molecular data suggest that Setchell and Gardner were incorrect in separating *Costaria* and *Dictyoneurum* into different families, we agree with their observation, that the midrib of *Dictyoneuropsis* "represents only a very slight modification of the blade" (Setchell and Gardner 1925, p. 623), and retain *Dictyoneuropsis* in synonym with *Dictyoneurum*.

Group 3: Cymathaere, Hedophyllum, Kjellmaniella, Laminaria, Macrocystis, Nereocystis, Pelagophycus, *and* Postelsia. Group 3 is the largest of the clades in the Laminariales and contains the largest genus, *Laminaria*. Numerous attempts have been made to divide *Laminaria* into sub-genera or sections based on morphology (Agardh 1867, Setchell 1900, Petrov 1974, Tokida et al. 1980, Druehl et al. 1988, Bhattacharya et al. 1991), but our data support two distinct clades of *Laminaria*, each including species with diverse morphologies (Figs. 3–8a). None of the traditionally emphasized taxonomic features of sporophyte morphology clearly separate these two groups. For example, ontogenetic blade splitting, a character previously given taxonomic value (Agardh 1867), occurs in both clades—*L. digitata, L. setchellii*, and *L. yezoensis* in Clade 1 and *L. dentigera* in Clade 2 (Fig. 8a). In all cases, species with blade splitting are sister to those lacking this attribute. Over 200 species or varieties have been recognized in *Laminaria* since Lamouroux established this genus in 1813. The majority of these species have been reduced to synonymy, indicating extensive morphological plasticity (Burrows 1964) and a "somewhat chaotic" (Kain 1979, p. 102) taxonomic process in *Laminaria*. The extensive phenotypic variation and plasticity within and between the included species makes it nearly impossible to outline morphological characters to distinguish between the two clades of *Laminaria* recovered from our data without a re-evaluation of taxonomically useful characters. However, all of our alignments clearly produce two distinct clades of *Laminaria*, regardless of the analyses used. While a comprehensive monograph of this genus is beyond the scope of this paper, we have included a geographically diverse sampling of *Laminaria* in our trees and, coupled with the species omitted based on sequence identity (above), this represents the most thorough molecular sampling of *Laminaria* published to date.

Species from three genera in addition to *Laminaria* are included in Clade 2, *Cymathaere japonica*, *Hedophyllum sessile*, *Kjellmaniella crassifolia*, and *K. gyrata* (Figs. 3–8a). *Kjellmaniella gyrata* and *H. sessile* were both originally described in the genus *Laminaria* and were each transferred to new genera based on single morphological characters. Setchell (1901) transferred *L. sessile* to *Hedophyllum* based on the lack of a stipe in this taxon. *Laminaria gyrata* was transferred to *Kjellmaniella* when *Kjellmaniella crassifolia* was described by Miyabe (1902) based only on the feature of fine carvings on the blade (Nagai 1940). Stipe size and blade morphology are variable characters at both the inter- and intra-specific level within the Laminariales, especially within and between species of *Laminaria* (Sundene 1962, Burrows 1964, Druehl et al. 1988), and are not dependable characters for classification at the generic level.

Blade morphology is another morphological character of dubious taxonomic usefulness that has been used to cluster species in Group 3. *Cymathaere japonica* was added to the genus based on the folds, or fascia, along its blade (Nagai 1940), a feature *Cymathaere* shares with several species of *Laminaria* (Druehl et al. 1988) and *Costaria* (Fig. 1e). However, *C. japonica* has 4-folds on its blade, whereas *C. triplicata* (Fig. 1l) has only three. In addition, *C. triplicata* is the only member of the genus that has a discoid holdfast whereas *C. japonica* has a simple hapteral holdfast, more common to members of *Laminaria*. Neither the morphological features of *C. japonica*, nor our sequence data, exclude it from *Laminaria*.

In contrast, *C. triplicata* was unique among species from six genera of kelp studied by Smith (1939) because the pits in the transverse walls of cells in the stipe were arranged indiscriminately rather than in a peripheral circle. Using electron microscopy, Henry and Cole (1982) discovered characteristic striated adhesion vesicles in the spores of *C. triplicata* unlike those in spores from any of the 17 species in 14 genera they studied, including *Postelsia palmaeformis*, *Hedophyllum sessile*, *Laminaria saccharina* and *Laminaria groenlandica*, species that group relatively close to *C. triplicata* in our trees (Fig. 8a). These unusual features for *C. triplicata* concur with its genetic distinctness shown here.

Group 3 also includes an assemblage of four genera, which make up two sister clades, with the perennial *Macrocystis* and *Pelagophycus* forming one, and the annual *Nereocystis* and *Postelsia* the other (Fig. 7). All four genera exhibit the characteristic ontogenetic splitting of the Lessoniaceae, which extends into the transition zone between the stipe and blade. Three of these genera are monotypic and are confined to the northeast Pacific. *Macrocystis* is the only exception and reportedly includes four species, which are found in both the Northern and Southern Pacific (Druehl 1970). The way in which splitting occurs has been a significant feature in the past for separating these taxa into the same clades we resolved in our trees (Figs. 3, 5–7). *Macrocystis* (Fig. 1i) and *Pelagophycus* were united in the tribe Macrocysteae by Kützing (1843) based on the scorpioid sympodial stipe resulting from unilateral splitting. Setchell and Gardner (1925) also placed a heavy emphasis on the unilateral splitting in *Macrocystis* and *Pelagophycus* when they used Kützing's Macrocysteae to unite these two genera to the exclusion of *Nereocystis* and *Postelsia*. The stipes of both *Nereocystis* and *Postelsia* (Fig. 1j) are hollow and terminate in a region of compressed, dichotomous splitting, which gives rise to blades. Setchell and Gardner (1925) stated that *Ne-*

reocystis and *Postelsia* are closely related and differed mainly in dimension and the lack of a large pneumatocyst in *Postelsia*.

Group 4: Ecklonia, Eckloniopsis, Egregia, Eisenia *and* Lessonia. All of the genera in this group include species in the Southern Hemisphere except *Egregia*, which is endemic to the Northeast Pacific. While *Lessonia* is confined to the Southern Pacific, *Eisenia* and *Ecklonia* are known from both hemispheres. Setchell and Gardner (1925) placed both *Eisenia* and *Ecklonia* within the Alariaceae because they considered that the sporophylls of these taxa were homologous to those of *Alaria*. However, our data support the conclusion of Saunders and Druehl (1993b) that sporophylls in these taxa are derived from the blade and not the stipe, and are therefore, more likely analogous to those of Group 1 taxa.

In all of our Combined Ingroup analyses this clade resolved as sister to Group 3 *sensu stricto*. However, in many of our Combined Total analyses, long branches in Group 4 interacted with the outgroup and caused serious artifacts in the resulting trees. Particularly problematic was the single long branch to *Egregia*, which caused it to be drawn to the base of the tree. When *Egregia* was pulled to the base, Groups 3 and 4 usually moved with it and a Type II (e.g. Figs. 4 and 5a) topology resulted.

The fact that *Egregia menziesii* was not closely allied to any genera in our molecular data was not surprising given its morphology (Fig. 1g), which includes a number of distinctive traits. The "stipe" and "blade" of *E. menziesii* are similar in appearance; both are strap-like and produce small blades and floats along their edges (Abbott and Hollenberg 1976). However, there is a narrowing of the thallus at the intercalary meristem, or transition zone, which separates stipe from blade. Rather than the characteristic Lessoniaceae-type branching at this transition zone, branching in *E. menziesii* occurs in the lower stipe region and is a direct development of proliferations (Setchell and Gardner 1925). Another unique feature of *E. menziesii* is that the reproductive sori are borne only on specialized blades grown from the floats along the thallus, both below and above the transition zone (Setchell and Gardner 1925).

Eisenia, *Ecklonia*, and *Eckloniopsis* form a fully resolved clade in all of our analyses (Figs. 3–7 and 8b) and their status as separate genera, at least as currently circumscribed, should be re-examined (Fig. 8b). The generic separation of *Eisenia* and *Ecklonia* was doubted, but upheld, by Setchell (1905, p. 129). Referring to *Eisenia bicyclis*, Setchell (1905) states "The closeness of the forms [of *E. bicyclis*] to those of the species of *Ecklonia*, particularly in the younger stages, is sufficient to cause some students to feel that they are not to be separated from the species of *Ecklonia* generically." Setchell's observation is supported by the fact that neither *Eisenia* nor *Ecklonia* are monophyletic in our trees. In addition, no differences were observed between the *rbc*Sp sequences of *Ecklonia cava* and *Ecklonia stolonifera* (Yoon et al. 2001), causing us to question the distinctiveness of these taxa (Fig. 8b). Our data indicate that the status of *Eckloniopsis*, in particular, should be reviewed because it falls among species of *Ecklonia* (Fig. 8b). However, without *Ecklonia maxima* (Osbeck) Papenfuss, the type of the genus, and a better representation of *Lessonia* and *Ecklonia* species, we are reluctant to recommend taxonomic modifications within Group 4 at this time.

Taxonomic conclusions: The most obvious conclusion from our data is that the Setchell and Gardner (1925) classification is artificial and requires modification. We recognize the three families of Setchell and Gardner for our Groups 1 (Alariaceae), 3 (Laminariaceae), and 4 (Lessoniaceae), although with radically altered generic composition in all cases, and establish a new family for Group 2, Costariaceae C. E. Lane, Mayes, Druehl *et* G. W. Saunders *fam. nov.* Further, we resurrect the genus *Saccharina* Stackhouse for the members of *Laminaria* in Clade 2 and subsume *Cymathaere japonica* and the genera *Hedophyllum* and *Kjellmaniella* into the construct.

Families: Alariaceae Postels *et* Ruprecht. Our concept of the Alariaceae includes three of the original genera (*Alaria*, *Pterygophora*, and *Undaria*) as well as *Lessoniopsis* (added by Saunders and Druehl 1993b), which was discussed as a taxon of questionable placement by Setchell and Gardner (1925), and *Pleurophycus*. In general, this group is characterized by stipe-derived sporophylls. The only exception is *Pleurophycus*, which has a blade that shares annual abscission features with the sporophylls of *Alaria*, and may be a homologous structure.

Laminariaceae Postels *et* Ruprecht. *Macrocystis*, *Nereocystis*, *Pelagophycus*, and *Postelsia* all resolve within our concept of the Laminariaceae, along with *Arthrothamnus*, *Cymathaere*, *Laminaria*, and the resurrected genus *Saccharina* (outlined below). Based on available information, *Streptophyllopsis* is likely a member of this family as well, but neither samples from this genus, nor published data were available for this study.

Lessoniaceae Postels *et* Ruprecht. Our concept of the Lessoniaceae is radically different from its original circumscription. We include only the genera *Ecklonia, Eckloniopsis* (pending further taxonomic evaluation), *Egregia, Eisenia*, and *Lessonia*. This group includes the only kelp genus confined to the Southern Hemisphere, *Lessonia*.

Costariaceae C. E. Lane, C. Mayes, Druehl et G. W. Saunders, fam. nov.

Diagnosis: *Membra Laminarialium generaliter cum stipitibus complanatus sed subinde teretibus, et alteruteris laminis perforatisve reticulatis vel ambo*.

Members of the Laminariales that generally have a flattened stipe but are occasionally terete, and either a perforated or reticulated blade, or both.

Type genus: *Costaria* Greville 1830, Algae Britannicae p. 39.

Additional genera: *Agarum*, *Dictyoneurum* and *Thalassiophyllum*.

Laminaria

It is evident from all of our trees (Figs. 3–8a) that the genus *Laminaria* consists of two independent clades, only one of which can remain *Laminaria*. As *Laminaria digitata* is the type of the genus members of Clade 1 (Fig. 8a) remain as in the genus *Laminaria* while members of Clade 2 must be transferred. The generic name *Saccharina* Stackhouse (1809) is available and predates *Laminaria* Lamouroux (1813), but the latter was conserved against it based on its common usage. In fact, many of the genera described by Stackhouse were commonly overlooked because most of the copies of his 1809 work perished in the 1812 burning of Moscow by Napoleon's army (Hughey et al. 2001). Because the type of Stackhouse's (1809, pp. 53, 65) genus, *S. plana* (current name: *Laminaria saccharina*), falls in Clade 2 (Fig. 8a), we resurrect *Saccharina* for this assemblage. To further complicate matters, the epithet "*plana*" is not the oldest valid name for this species. Linnaeus (1753) had earlier described this species as *F. saccharinus*, but a new combination under this name would result in a tautonym, which is prohibited by the International Code of Botanical Nomenclature (Greuter et al. 2000; Art. 23.4). However, Linnaeus (1753) also described *U. latissima* in his Species Plantarum, which was subsequently shown to represent what is presently called *L. saccharina* (see Silva 1952). Thus, the legitimate combination nomenclature for the type of the genus *Saccharina* is *S. latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl et G.W. Saunders *comb. nov.*

***Saccharina latissima* (Linnaeus) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Fucus saccharinus Linnaeus 1753, Species Plantarum Vol. 2, p. 1161; *Ulva latissima* Linnaeus 1753, Species Plantarum Vol. 2, p. 1163.

Most recent synonym: *Laminaria saccharina* (Linnaeus) Lamouroux

***Saccharina angustata* (Kjellman) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria angustata Kjellman *in* Kjellman *et* Petersen 1885, Vega-expeditionens vetenskapliga iakttagelser p. 266.

***Saccharina cichorioides* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria cichorioides Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 131.

***Saccharina coriacea* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria coriacea Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 132.

***Saccharina crassifolia* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Kjellmaniella crassifolia Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 134.

***Saccharina dentigera* (Kjellman) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria dentigera Kjellman 1889, Om Beringhafvets Algflora. Kungliga Svenska Vetenskapsakademiens Handlingar 23:p. 45.

***Saccharina diabolica* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria diabolica Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 131.

***Saccharina groenlandica* (Rosenvinge) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria groenlandica Rosenvinge 1893, Grönlands Havalger. Meddelelser om Grönlands 3:p. 847.

***Saccharina gyrata* (Kjellman) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria gyrata Kjellman 1892, Om en ny organisationstyp inom slägtet *Laminaria*. Bihang til Kongliga Svenska Vetenskaps-Akademiens Handlingar 18:p. 16.

Most recent synonym: *Kjellmaniella gyrata* (Kjell.) Miyabe

***Saccharina japonica* (Areschoug) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria japonica Areschoug 1851, Phyceae Capenses p. 29.

***Saccharina kurilensis* (Miyabe *et* Nagai) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, nom. nov.**

Basionym: Cymathaere japonica Miyabe *et* Nagai *in* Nagai 1940, Marine Algae of the Kurile Islands. Journal of the Faculty of Agriculture Hokkaido Imperial University 46:p. 87.

Etymology: The inclusion of *Laminaria japonica* in the clade of taxa being transferred to *Saccharina* necessitates changing the specific epithet of *Cymathaere japonica*. We have chosen *Saccharina kurilensis* because this taxon is endemic to the Kurile Islands north of Hokkaido, Japan.

***Saccharina longicruris* (Bachelot de la Pylaie) Kuntze 1891, Revisio generum plantarum p. 915.**

Basionym: Laminaria longicruris Bachelot de la Pylaie 1824. Quelques observations sur les productions de l'île de Terre Neuve et sur quelques Algues de la côte de France, appartement au genre *Laminaire*. Annales des Sciences Naturelles, Botanique 4: p.177, pl. 9: Figs A–B

***Saccharina longipedalis* (Okamura) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria longipedalis Okamura 1896, On *Laminaria* of Japan. Botanical Magazine, Tokyo 10: 89, pl. 7: Figs. 1–3

***Saccharina longissima* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria longissima Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 132.

***Saccharina ochotensis* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria ochotensis Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 130.

***Saccharina religosa* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria religosa Miyabe, *in* Okamura 1902, Nippon Sorui Meii p. 131.

***Saccharina sessile* (C. Agardh) *Saccharina sessile* (C. Agardh) Kuntze 1891, Revisio generum plantarum p. 915.**

Basionym: Laminaria sessile Agardh 1824, Systema Algarum p. 270.

Most recent synonym: Hedophyllum sessile (C. Ag) Setch.

***Saccharina yendoana* (Miyabe) C. E. Lane, C. Mayes, Druehl et G. W. Saunders, comb. nov.**

Basionym: Laminaria yendoana Miyabe, *in* Okamura 1936, Nippon Kaiso Shi (Descriptions of Japanese algae) pp. 253, 288.

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