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Molecular phylogeny of Laminariales (Phaeophyceae) inferred from small subunit ribosomal DNA sequences

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SUMMARY

In order to elucidate the molecular phylogeny of the order Laminariales, complete small subunit (SSU) rDNA sequences were determined for 11 species, including representatives from all the families of the order: *Pseudochorda gracilis* Kawai et Nabata and *P. nagaii* (Tokida) Inagaki of the Pseudochordaceae, *Chorda filum* (L.) Stackhouse of the Chordaceae, *Alaria fistulosa* Postels et Ruprecht, *Ecklonia cava* Kjellman and *Egregia menziesii* (Turner) Areschoug of the Alariaceae, *Agarum clathratum* f. *yakishiriense,* I. Yamada, *Kjellmaniella crassifolia* Miyabe and *Laminaria japonica* Areschoug of the Laminariaceae and *Lessonia nigrescens* Bory and *Postelsia palmaeformis* Ruprecht of the Lessoniaceae. The published data of *Halosiphon tomentosus* (Lyngbye) Jaasund and *Saccorhiza polyschides* (Lightfoot) Batters were included for tree construction. Our SSU rDNA sequences show that the Pseudochordaceae and Chordaceae are clearly separated from the strongly monophyletic group consisting of the Alariaceae, Laminariaceae and Lessoniaceae. The sequence data also show that the Pseudochordaceae is monophyletic and is distant from *H. tomentosus* and *S. polyschides*. Considering our molecular data and the reported morphology, life history and sex pheromones of the family, it appears likely that the Pseudochordaceae might have branched off first from the laminarialean lineage that leads, through the Chordaceae, to the advanced Laminariales (i.e. the Alariaceae, Laminariaceae and Lessoniaceae). The limited resolution that results from the close similarities among the SSU rDNA sequences gives a need for more informative molecular markers in order to resolve the circumscription and phylogeny of the Laminariales.

Key words: Laminariales, molecular phylogeny, Phaeophyceae, Pseudochordaceae, small subunit rDNA.

INTRODUCTION

The Laminariales is one of the most advanced orders in the Phaeophyceae. The order has a strongly heteromorphic, diplohaplontic life cycle, with an alternation between highly differentiated diploid sporophytes and microscopic haploid gametophytes (van den Hoek *et al.* 1995). The Laminariales currently has six families: Pseudochordaceae, Chordaceae, Phyllariaceae, Alariaceae, Laminariaceae and Lessoniaceae and two members of uncertain familial assignment, *Halosiphon tomentosum* and *Akkesiphycus lubricus* (Setchell and Gardner 1925; Tilden 1935; Kawai and Kurogi 1985; Kawai 1986; Henry and South 1987; Peters 1998). Even though the SSU rDNA sequence data support a phylogenetic position close to the Sporochnales and Desmarestiales (Tan and Druehl 1996), there are few molecular phylogenetic studies on circumscription of the Laminariales and the interfamilial relationships of all the families within the Laminariales.

Members of the Pseudochordaceae and Chordaceae, *Halosiphon tomentosum* and *Akkesiphycus lubricus* are generally regarded as 'primitive'. They share some plesiomorphic features: (i) diffuse meristematic activity on thalli of annual sporophytes, except for *Chorda filum* and *Pseudochorda gracilis* (Kogame and Kawai 1996); (ii) presence of stigmata in zoospores; and (iii) monoecious, or dioecious but sexually monomorphic gametophytes; except for *C. filum* and *Pseudochorda gracilis* (Kawai and Kurogi 1985; Kawai and Nabata 1990). The Chordaceae, represented only by *C. filum*, is characterized by an intercalary meristem and the nature of the sexual pheromones and gametophytes (Maier 1995; Kogame and Kawai 1996). *Chorda tomentosa* Lyngbye was reinstated as the monospecific genus *Halosiphon* Jaasund by Peters (1998) on the basis of ribosomal DNA sequences. The Phyllariaceae also exhibit some of the primitive features of the Pseudochordaceae and Chordaceae (Henry 1987a,b; Kogame and Kawai 1996), but they have foliose sporophytes and conducting elements called solenocysts and allelocysts (Sauvageau 1918).

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The Alariaceae, Laminariaceae and Lessoniaceae are regarded as advanced families in the Laminariales compared with the previously discussed primitive families. These three families share many common features: absence of a stigma in zoospores; dioecious and sexually dimorphic gametophytes; lamoxirene as the sexual pheromone; presence of an intercalary meristem; conducting systems in the sporophytes; and unicellular paraphyses. However, published morphological (Setchell and Gardner 1925) and molecular data (Druehl 1989; Druehl and Saunders 1992) are contradictory in regard to the interfamilial relationships as well as the taxonomic positions of the individual taxa.

In the present study, we aimed to elucidate the familial phylogeny of the Laminariales using SSU rDNA sequence data. Together with published data of *Saccorhiza polyschides* of the Phyllariaceae and *Halosiphon tomentosus* (Tan and Druehl 1996), we studied two *Pseudochorda* species of the Pseudochordaceae, *Chorda filum* of the Chordaceae, three species each of the Alariaceae and Laminariaceae and two species of the Lessoniaceae to include all the families, although *Akkesiphycus* could not be included. According to Saunders and Druehl (1992), the SSU rDNA sequences are so conserved that it is rather difficult to resolve the phylogenetic relationships among members of the advanced Laminariales, but the same gene system, nevertheless, provides adequate sequence divergence to distinguish *S. polyschides* of the Phyllariaceae and *H. tomentosus* from the advanced Laminariales (Tan and Druehl 1996).

MATERIALS AND METHODS

A list of species investigated in this study, their taxonomic positions and collection sites are provided in Table 1. Thalli dried in air were used for DNA extraction, except for *Pseudochorda gracilis*, where fresh sporophytes were used. Genomic DNA was extracted using a modification of a hexadecyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Approximately 0.02 g algal tissue powder was incubated in 500 µL 2% CTAB buffer (with the addition of 2% β-mercaptoethanol) at 60°C for 45 min. Extractions with chloroform-isoamyl alcohol (CIA, 24:1 v/v) were performed repeatedly until complete removal of the interphase was achieved. DNA was precipitated with 1000 µL of 100% cold ethanol for 30 min at −70°C, pelleted by centrifugation (10 min, 10 000 *g*, 4°C), washed with 70% ethanol twice, air dried and dissolved in 150 µL Tris-EDTA.

The SSU rDNA gene was amplified as two fragments using the primers SR1 + SR7 and SR4 + SR12 (Nakayama *et al.* 1996) in an automated thermocycler (MJ Research, Watertown, MA, USA). The profile of the polymerase chain reaction (PCR) conditions was an initial denaturation at 95°C for 2 min annealing at 45°C for 0.5 min and extension at 72°C for 2 min, followed by 28 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 0.5 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The reaction mixtures of Nakayama *et al.* (1996) were used. A negative control without a target DNA template was included in each set of reactions. The amplified DNA was purified using a GeneClean II kit (Bio 101, La Jolla, CA, USA).

For *C. filum* and two *Pseudochorda* species, the SSU rDNA was amplified as two fragments using the primers LD 1 + LDC and LDD + LDF (Saunders and Druehl 1992). The amplification profile was an initial denaturation at 94°C for 3 min, annealing at 55°C for 1 min and extension at 72°C for 3 min, followed by 25 cycles of denaturation at 94°C for 0.75 min, annealing at 55°C for 1 min, extension at 72°C for 3 min and final extension at 72°C for 10 min. Reaction mixtures described by Kawai *et al.* (1995) were used. Polymerase chain reaction products were purified by gel filtration using Sephacryl S-200 HR resin (Amersham Life Science, Cleveland, OH, USA).

The sequence of each SSU rDNA was analyzed using the BigDye™ Terminator Cycle Sequencing Kit [Applied Biosystems (ABI), Perkin-Elmer Cetus, Norwalk, CT, USA] following the manufacturer's recommendations. Reactions were electrophoresed and the sequence data were collected with the ABI Model PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Both strands of SSU rDNA were completely sequenced, using the PCR and nested primers of Nakayama *et al.* (1996) and newly designed primers of forward direction (S34, 5′ CAAGTCTGGCAATTGGAATGAGA 3′; S56, 5′ AGCATGGAATAATGAGATAGG G 3′; S78, 5′ ACCAGGAGTGGAGCCTGCGGCTT 3′; S11, CGAGGAATTCCTAGTAAACG 3′). However, SSU rDNA from *C. filum* and the two *Pseudochorda* species were sequenced using the primers of Saunders and Druehl (1992) and newly designed primers of forward direction (KA1, 5′ TAGCATGGAATAATGAGATAG 3′) and of reverse direction (KA2, 5′ CCGCACGCGCGCTACACTGATG 3′). There were no differences between the *Laminaria japonica* sequences obtained with primers of Nakayama *et al.* (1996) and those of Kawai *et al.* (1995).

The SSU rDNA sequences were aligned using SeqPup, a multisequence editing program (Gilbert 1995). Final alignment was done manually. Two-parameter distances (Kimura 1980) between taxa were estimated and phylogenies were inferred with the neighbor-joining method using the NEIGHBOR program of PHYLIP (Felsenstein 1995). The maximum-likelihood (ML) tree was constructed using the DNAML program of PHYLIP in which the global rearrangement option was given. Maximum parsimony (MP) analysis was carried out using the PAUP program (version 3.1.1, Swofford 1993). To provide rough estimates of support for topological elements in all analyses, bootstrapping (Felsenstein 1985) was done with 1000 resamplings in both

Table 1. List of species investigated in this study, including their taxonomic position, collection site and GenBank accession number

Order/family/species	Collection site	Reference and GenBank accession no.
Laminariales		
Family uncertain		
Halosiphon tomentosus (Lyngbye) Jaasund		Tan and Druehl 1996, L43056
Chordaceae		
Chorda filum (L.) Stackhouse	Oshoro, Japan	New sequence, AF123585
Phyllariaceae		
Saccorhiza polyschides (Lightfoot) Batters		Tan and Druehl 1996, L43059
Pseudochordaceae		
Pseudochorda gracilis Kawai et Nabata	Isoya, Japan	New sequence, AF123583
P. nagaii (Tokida) Inagaki	Hanasaki, Japan	New sequence, AF123584
Alariaceae		
Alaria fistulosa Postels et Ruprecht	Kamchatka, Russia	New sequence, AF123578
Ecklonia cava Kjellman	Cheju, Korea	New sequence, AF123579
Egregia menziesii (Turner) Areschoug	Oregon, USA	New sequence, AF123580
Laminariaceae		
Agarum clathratum f. *yakishiriense* I. Yamada	Kangreung, Korea	New sequence, AF123576
Kjellmaniella crassifolia Miyabe	Kangreung, Korea	New sequence, AF123577
Laminaria japonica Areschoug	Sinnam, Korea	New sequence, AF123575
Lessoniaceae		
Lessonia nigrescens Bory	Ilo, Peru	New sequence, AF123581
Postelsia palmaeformis Ruprecht	Oregon, USA	New sequence, AF123582
Desmarestiales		
Desmarestiaceae		
Desmarestia ligulata (Lightfoot) Lamouroux		Tan and Druehl 1996, L43060
Scytosiphonales		
Scytosiphonaceae		
Scytosiphon lomentaria (Lyngbye) Link		Kawai *et al.* 1995, D16558
Sporochnales		
Sporochnaceae		
Sporochnus comosus C. Agardh		Tan and Druehl 1996, L43016

the MP and NJ analyses but 100 resamplings in the ML analysis. As the Desmarestiales and Sporochnales have been shown to be relatives of the Laminariales by morphology and SSU rDNA sequences (van den Hoek *et al.* 1995), published sequences of *Desmarestia ligulata* and *Sporochnus comosus* were included for data analyses. In all cases, *Scytosiphon lomentaria* of the Scytosiphonales was used as an outgroup.

RESULTS

Complete SSU rDNA sequences were determined for 11 representatives of the Laminariales (Table 1). The total length of the SSU rDNA sequences of the species treated here ranged from 1715 to 1722 b.p., which were aligned with a total of 1726 positions (alignment available on request). Although most of the positions were the same among the sequences, there were 101 variable positions between 613 and 619, 666 and 670 and 1657 and 1685.

Phylogenetic trees constructed from the MP, ML and NJ analyses showed a similar topology. There were 34 parsimony-informative nucleotide positions in our alignment. The MP analysis retained 48 trees, but most of the branches collapsed in the 50% majority-rule consensus tree (tree not shown).

As is seen in both the ML and NJ trees (Figs 1,2), *Saccorhiza polyschides* was basal to the clade comprising all the taxa treated here, in which *Halosiphon tomentosus* branched off and then *Sporochnus comosus* and *Desmarestia ligulata* followed. However, most of these branches were weakly supported by low bootstrap values.

The ML and NJ trees showed that members of the Chordaceae, Pseudochordaceae, Alariaceae, Laminariaceae and Lessoniaceae form a single monophyletic clade, even though the clade was supported by 58% (ML tree) and 48% (NJ tree) bootstrap values. The two members of the Pseudochordaceae formed a single clade with 85% (ML tree) and 97% (NJ tree) bootstrap values, and were shown to be sisters of the Chordaceae. The Pseudochordaceae is also distant from *Halosiphon tomentosus* and *Saccorhiza tomentosus*. The members of the Alariaceae, Laminariaceae and Lessoniaceae produced a strong clade with 94% (ML tree) and 97% (NJ tree) bootstrap values. The members of the three families formed branches that overlapped with each other and the bootstrap supports were also weak.

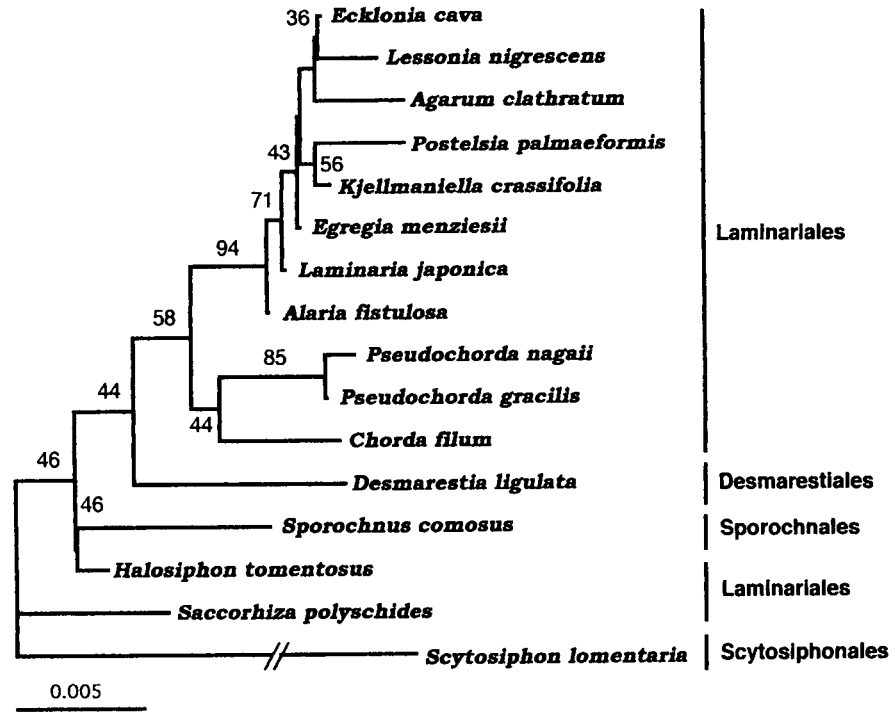

Fig. 1. Phylogenetic tree using maximum-likelihood analysis. Numbers above branches are bootstrap values (100 resamplings).

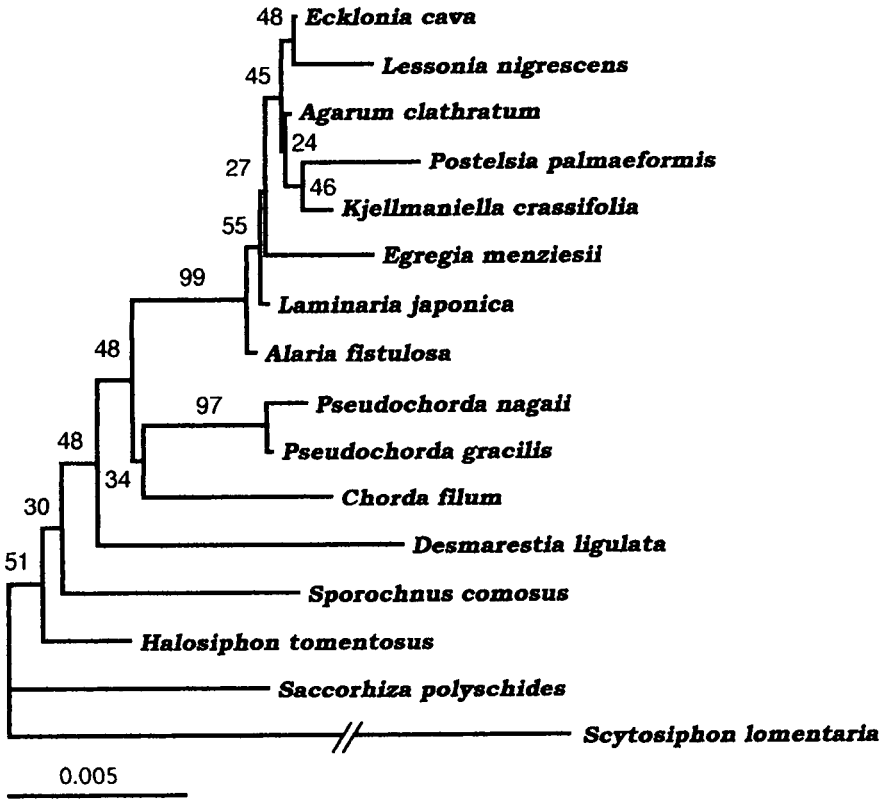

Fig. 2. Phylogenetic tree using neighbor-joining analysis. Numbers above the branches are bootstrap values (1000 resamplings).

DISCUSSION

In the molecular analyses based on complete SSU rDNA gene sequences, both the Pseudochordaceae and Chordaceae are clearly separated from the strongly monophyletic group consisting of the Alariaceae, Laminariaceae and Lessoniaceae. The Pseudochordaceae and Chordaceae show a sister relationship in the trees constructed in this study, so either family might have branched off first within the laminarialean lineage.

As mentioned previously, the Chordaceae (Kogame and Kawai 1996) and Pseudochordaceae (Kawai and

Kurogi 1985) Kawai and Nabata share some basic morphological features (e.g. terete sporophyte organization and early developmental processes of sporophytes). The Chordaceae also shares closest morphological similarity with the Alariaceae, Laminariaceae and Lessoniaceae in that it has: (i) unicellular paraphyses; (ii) trumpet-shaped hyphae; (iii) a well developed intercalary meristem; and (iv) sexually dimorphic gametophytes. Therefore, the close phylogenetic relationship of the Pseudochordaceae, Chordaceae, Alariaceae, Laminariaceae and Lessoniaceae based on the SSU rDNA molecular data agree with the morphological features.

The Pseudochordaceae, including only *Pseudochorda gracilis* and *P. nagaii*, shows a strong monophyly. Although *P. nagaii*, which has dioecious but sexually monomorphic gametophytes, is different from *P. gracilis*, which has dioecious and dimorphic gametophytes, the close similarity of the SSU rDNA supports the identity of the Pseudochordaceae proposed by Kawai and Kurogi (1985). Kawai and Kurogi (1985) suggested that the Pseudochordaceae should be regarded as the most primitive Laminariales based on morphology and life history. However, it appears likely that the Pseudochordaceae might have branched off first from the laminarialean lineage that leads, through the Chordaceae, to the advanced Laminariales.

The current SSU rDNA sequences also show that *Saccorhiza polyschides* of the Phyllariaceae and *Halosiphon tomentosus* are quite distantly related to the other families of the Laminariales. These results are in accordance with those of Peters (1998) who showed that *H. tomentosus* is separated from the Chordaceae and Tan and Druehl (1996) who showed that *S. polyschides* of the Phyllariaceae is related to both *H. tomentosus* and *Sporochnus comosus*. As described earlier, the Phyllariaceae have also been regarded as primitive on the basis of morphology, life history and sex pheromones. Given the present molecular data together with other published data, *S. polyschides* appears quite different from the Laminariales, even though the Phyllariaceae show some similarities to the Laminariales (e.g. Flores-Moya and Henry 1998).

The members of the Alariaceae, Laminariaceae and Lessoniaceae are the most strongly grouped in the trees constructed in the present study. This result suggests that these families might have evolved closely to each other, but separately from the other families of the Laminariales. This relationship is shown in the published SSU rDNA (Saunders and Druehl 1992; Tan and Druehl 1996) and ITS data (Saunders and Druehl 1993a,b; Druehl *et al.* 1997). These three families share many features, such as: (i) differentiation between blade and stipe; (ii) presence of mucilaginous structures; (iii) lack of a stigma in zoospores; (iv) the usually perennial nature of the sporophytes; and (v) lamoxirene as the common sexual pheromone. These characteristics are generally regarded as advanced features in the Laminariales. Our current molecular data and the above-discussed data support the view that the Alariaceae, Laminariaceae and Lessoniaceae are the most recently evolved (advanced) groups in the Laminariales.

The present SSU rDNA sequences do not show clear separation among the advanced families of the Laminariales because of the very similar sequences and a small number of the informative sites. The close relationship among the advanced families is also represented in the data of internal transcribed spacer (ITS) sequences (Saunders and Druehl 1993a; Druehl *et al.* 1997), which are generally known to be much more variable than those of the 18S region in the same ribosomal gene and to be fast-evolving in the Laminariales (Saunders and Druehl 1992, 1993b) as well as in other algae (Olsen 1990). For example, Saunders and Druehl (1993a) concluded that both the Alariaceae and Lessoniaceae are polyphyletic and Druehl *et al.* (1997) further established that the Laminariaceae is not a natural group.

Our current sequence data support a view that the Laminariales may not be monophyletic if *Saccorhiza polyschides* of the Phyllariaceae and *Halosiphon tomentosus* are included in the order, consistent with the reports of both Tan and Druehl (1996), based on SSU rDNA data, and Rousseau *et al.* (1997), based on large subunit rDNA sequences. However, because of the limited resolution from the small number of informative sites and low bootstrap values, circumscription of the order Laminariales needs further study. As is mentioned by Peters (1998), even though the sequences of additional members may be analyzed, both the informative and variable sites may not significantly increase. This rather confuses phylogenetic relationships, especially among the advanced families. Therefore, more informative markers should be analyzed in order to improve the molecular taxonomy and phylogeny of the Laminariales. Both the ITS regions in nuclear ribosomal genes (Druehl *et al.* 1997; Peters 1998) and the plastid-encoded RuBisCo large subunit and spacer region, which are more informative within the Ectocarpales *sensu lato* (Siemer *et al.* 1998) and in the family Alariaceae (Yoon and Boo, in press), may prove to be recommendable markers for an improved molecular phylogeny of the Laminariales.

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