Molecular phylogeny of the brown algal genera *Akkesiphycus* and *Halosiphon* (Laminariales), resulting in the circumscription of the new families Akkesiphycaceae and Halosiphonaceae

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Phylogenetic relationships of *Akkesiphycus lubricum* with the Laminariales and related taxa were assessed by molecular phylogenetic analyses based on the Rubisco large subunit gene (*rbc*L), internal transcribed spacer regions (ITS1 and ITS2), and 18S ribosomal DNA (18s rDNA) sequence data. Among the three data sets, *rbc*L gene sequences gave the best resolution for indicating familial and ordinal relationships within the Laminariales and related taxa. Using *rbc*L data, *Akkesiphycus* showed the closest phylogenetic relationship to the Pseudochordaceae (Laminariales) and this was also supported by ITS and 18S rDNA data. The clade of *Akkesiphycus* and the Pseudochordaceae formed a sister group to the Alariaceae/Laminariaceae/Lessoniaceae group and the Chordaceae of the Laminariales in maximum parsimony and neighbour-joining analyses. Morphologically, *Akkesiphycus* differs significantly from other Laminariales in having gametophytes with anisogamous planogametes. However, considering that some laminarialean species have residual flagella in eggs and the close phylogenetic relationship with the Pseudochordaceae, as demonstrated in the present study, we propose to establish a new family, the Akkesiphycaceae Kawai & Sasaki *fam. nov.,* to accommodate *A. lubricum*; we place it in the order Laminariales. The Akkesiphycaceae is distinct in having foliose sporophytes with multicellular paraphyses and monomorphic dioecious gametophytes that form anisogametes. As a result of inclusion of the Akkesiphycaceae, the ordinal definition of the Laminariales has to be emended to include anisogamy. On the other hand, the distant phylogenetic relationship between *Halosiphon* and Chordaceae, suggested previously by ITS and 18S rDNA data, was confirmed by *rbc*L data, and hence, a second new family, the Halosiphonaceae Kawai & Sasaki *fam. nov.* is described, although its ordinal assignment needs further discussion. The family Halosiphonaceae is characterized by having simple, cord-shaped sporophytes with multicellular assimilators and monoecious oogamous gametophytes.

INTRODUCTION

Akkesiphycus lubricum Yamada & Tak. Tanaka, a rare brown alga described from northern Japan and the only known species of *Akkesiphycus* (Yamada & Tanaka 1944; Kurogi 1968), has relatively large foliose thalli resembling nondigitate *Laminaria* J.V. Lamouroux species in appearance. However, the species had been placed in the Coilodesmaceae (Yamada & Tanaka 1944) or Punctariaceae (Wynne 1982) of the Dictyosiphonales or in the Scytosiphonales (Chihara 1979), based on anatomical similarities. Kawai (1986) studied the species in culture and showed that it has a heteromorphic life history, alternating between parenchymatous sporophytes and sexually monomorphic, microscopic gametophytes. In addition to this basic life history pattern, similarities in the germination and developmental patterns of young sporophytes and in chloroplast morphology (the presence of many discoid chloroplasts without pyrenoids in each cell) suggested a systematic position closer to the Laminariales than to the Dictyosiphonales and Scytosiphonales (Kawai 1986). However, *Akkesiphycus* differs from oogamous Laminariales in having anisogamous flagellated gametes. The phylogenetic relationship of *Akkesiphycus* to other brown algal orders has been studied using 5S ribosomal RNA sequence data (Lim *et al.* 1986), but this molecule does not provide sufficient resolution for taxonomic

comparisons within the brown algae. Therefore, the systematic position of *Akkesiphycus* at higher taxonomic ranks (i.e. familial and ordinal) has remained unresolved.

The order Laminariales currently includes six families: the Pseudochordaceae, Chordaceae, Phyllariaceae, Alariaceae, Laminariaceae and Lessoniaceae. Table 1 summarizes the major distinguishing characters of these families. *Halosiphon tomentosum* (Lyngbye) Jaasund (= *Chorda tomentosa* Lyngbye) is considerably different from *Chorda filum* (Linnaeus) Stackhouse in lacking intercalary meristems and paraphyses and in its possession of monoecious gametophytes (Maier 1984; Peters 1998; Kawai *et al.* in press), so that *H. tomentosum* cannot be classified in the Chordaceae. The families Alariaceae, Laminariaceae and Lessoniaceae (the A/L/L group) do possess these features, however, and can therefore be grouped together. The Pseudochordaceae, Chordaceae, *Halosiphon* Jaasund, and Phyllariaceae are generally regarded as primitive, because they possess the following characteristics (Kawai & Kurogi 1985; Henry & South 1987; Kawai & Nabata 1990): (1) relatively simply organized sporophytes, without differentiation between blade and stipe (except Phyllariaceae), and lack of a meristematic rhizoidal holdfast; (2) sporophytes that are annuals and that lack distinct intercalary meristems (except for *C. filum*); (3) a lack of mucilaginous organs (e.g. mucilage gland cells, mucilage ducts) and mucilage caps on paraphyses; (4) the presence of eyespots in zoospores; and (5) the occur-

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Table 1. Comparisons of major taxonomic features of *Akkesiphycus, Halosiphon* and families of the Laminariales.[1]

Feature	*Akkesiphycus*	Pseudochordaceae	Chordaceae	*Halosiphon*	Phyllariaceae	Alariaceae, Laminariaceae, Lessoniaceae
Sporophyte						
Annual/perennial	annual	annual	annual	annual	annual	perennial
Gross morphology	foliose	terete	terete	terete	foliose	foliose
Intercalary meristem	absent	absent	present	absent	present	present
Hyphae	rhizoidal	rhizoidal/trumpet-shaped	trumpet-shaped	absent	solenocyst/allelocyst	trumpet-shaped
Mucilaginous organs	absent	absent	absent	absent	absent	present
Paraphyses	multicellular	multicellular	unicellular	multicellular	unicellular	unicellular
Eyespot in zoospore	present	present	present	present	present	absent
Gametophyte						
Monoecism/dioecism	dioecious, monomorphic	dioecious, monomorphic/dimorphic	dioecious, dimorphic	monoecious	monoecious/dioecious, monomorphic/dimorphic	dioecious, dimorphic
Female gamete	macrogamete, flagellated	egg	egg	egg	egg	egg
Antheridia	clustered	clustered/single-chambered	single-chambered	single-chambered	clustered/single-chambered	single-chambered
Sperm flagellation	normally flagellated[2]	*Chorda* type[3]	*Chorda* type[4]	*Chorda* type?	*Chorda* type?	*Laminaria* type[5]
Chloroplasts in sperm	associated with flagellar base	reduced, not associated with flagellar base	reduced, not associated with flagellar base	reduced, not associated with flagellar base	reduced, not associated with flagellar base	reduced, not associated with flagellar base
Sexual pheromone	? (not studied)	? (ectocarpene isolated, but not confirmed by bioassay)	similar to, but different from lamoxirene; sperm sensitive to lamoxirene	(+)-multifidene; sperm not sensitive to lamoxirene	? (not studied); sperm not sensitive to lamoxirene	lamoxirene

[1] Table 1 was prepared from Henry (1986), Henry & Cole (1982), Kawai (1986), Kawai & Nabata (1990), Kawai *et al.* (in press), and Maier (1984).
[2] Sperm with longer anterior and shorter posterior flagella as commonly seen in brown algal zoospores.
[3] Sperm with shorter anterior and longer posterior flagella without deep flagellar gullets.
[4] From Kawai *et al.* (in press).
[5] Sperm with shorter anterior and longer posterior flagella with deep flagellar gullets.

rence of monoecious [*Halosiphon, Saccorhiza dermatodea* (de la Pylaie) J. Agardh] or dioecious but sexually monomorphic gametophytes [*Pseudochorda nagaii* (Tokida) Inagaki, *Phyllariopsis* E.C. Henry & South spp.]. However, a rather distant phylogenetic relationship between *Halosiphon* and Phyllariaceae and other members of the Laminariales (Pseudochordaceae, Chordaceae and the A/L/L group) has been suggested based on 18S ribosomal DNA (rDNA) data (Peters 1998; Boo *et al.* 1999) and some nonmolecular features, such as sexual pheromones and the presence of solenocysts and allelocysts (Maier 1984; Henry & South 1987; Kogame & Kawai 1996).

In addition, relatively close systematic relationships between the Desmarestiales, Sporochnales and Laminariales have been repeatedly discussed (Clayton 1984; Müller *et al.* 1985a; Kawai 1992a; Tan & Druehl 1996; Peters 1998; Boo *et al.* 1999; Reviers & Rousseau 1999). However, due to relatively limited knowledge of the primitive members of Laminariales and the insufficient resolution of 18S rDNA data, which have been used for studying the problem, their relationships have remained unresolved.

In order to elucidate the phylogenetic relationship between *Akkesiphycus* and related taxa and to obtain more information for discussing the ordinal boundary of the Laminariales, we analysed molecular data from *Akkesiphycus* and members of the Laminariales and related taxa using almost complete sequences of three different frequently used DNA regions of different taxonomic resolution encoded in both the nuclear and plastid genomes. These were the Rubisco large subunit gene (*rbc*L), the internal transcribed spacers (ITS1 and ITS2) of ribosomal DNA, and the 18S coding region of ribosomal DNA.

MATERIAL AND METHODS

For DNA extraction for *Akkesiphycus lubricum,* we used a unialgal culture strain (AL-1) established from a sporophyte collected on 26 July 1983 at Daikoku Island, Akkeshi (43°02′N, 144°52′E), Hokkaido, Japan, as used in a previous life history study (Kawai 1986). For comparison, the following specimens were also used: *Halosiphon tomentosus* (E. Henry, culture strain from Newfoundland, Canada; silica gel-dried material from St. Lawrence Island, Bering Sea, USA); *Desmarestia latifrons* Kützing (E. Henry, culture strain from Harris Beach, Oregon, USA); *Desmarestia tabacoides* Okamura (frozen sporophyte from Maiko, Kobe, Hyogo Prefecture, Japan); *Desmarestia* J.V. Lamouroux sp. (H. Kawai, culture strain from Kamagari Island, Hiroshima Prefecture, Japan); *Sporochnus scoparius* Harvey (frozen sporophyte from Imagoura, Kasumi, Hyogo Prefecture, Japan); and *Haplospora globosa* Kjellman (D.G. Müller, culture strain from Helgoland, Germany).

Cultures were grown in polystyrene Petri dishes containing 50 ml PESI medium (Tatewaki 1966) and illuminated by daylight-type white fluorescent lighting of approximately 50 μmol m^{-2} s^{-1} at 10°C, with long days (16 : 8-h light : dark cycle). Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The material was ground in liquid nitrogen and approximately 40 mg of algal tissue powder was used. The intactness of extracted total DNA was assessed on ethidium bromide-stained 1.0% agarose gels.

Polymerase chain reaction (PCR) amplification of 18S, ITS1 and ITS2 regions of rDNA and the *rbc*L were carried out using a GeneAmp PCR System 2400 and 9700 (Perkin–Elmer, California, USA) and TaKaRa Ex Taq™ (Takara Shuzo, Shiga, Japan) reaction kit (total volume of 25 μl, composed of 2.5 μl 10× Ex Taq™ buffer, 5.0 μM dNTP mixture, 0.1 μM of each primer, 0.625 units TaKaRa Ex Taq™ and 2.0 μl DNA solution containing 0.5–1.0 μg DNA). Primers (Table 2) were designed based on known sequences of the corresponding regions reported for related taxa (Assali *et al.* 1990; Valentin & Zetsche 1990; Saunders & Druehl 1992; Tan & Druehl 1993, 1996; Kawai *et al.* 1995; Daugbjerg & Andersen 1997; Stache-Crain *et al.* 1997).

The PCR sequence was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 42–58°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. Polymerse chain reaction products were checked on ethidium bromide-stained 1.0% agarose gels. Polymerase chain reaction products were directly sequenced using the Cy5™ Auto Cycle™ Sequencing Kit (Pharmacia Biotech AB, Uppsala, Sweden), following manufacturer's instructions. Reactions were electrophoresed, and the sequence data were collected with the ALFExpress™ DNA sequencer (Pharmacia Biotech).

For phylogenetic comparisons, published sequence data for related taxa were used along with our own data (Table 3). Sequences were aligned for phylogenetic analyses either using the Clustal W computer program (Thompson *et al.* 1994) or manually and were subjected to maximum parsimony (MP) analyses in a general heuristic search, using PAUP versions 3.1.1 and 4.0.2b (Swofford 1993, 1999). From the same alignment data, two-parameter distances (Kimura 1980) between taxa were estimated and phylogenetic trees were constructed with neighbour-joining (NJ) analyses, using PAUP version 4.0.2b. Maximum likelihood (ML) analyses were performed using PAUP version 4.0.2b in a general heuristic search, with substitution model (transition : transversion ratio = 2) base frequencies (empirical frequencies using Hasegawa–Kishino–Yano model) and among-site rate variation (equal rate for all sites). The robustness of the trees was tested by bootstrap analyses with 1000 (MP and NJ) or 500 (ML) replications, using PAUP version 4.0.2b. Gaps were not taken into account in every analysis (Felsenstein 1985). In an additional MP analysis, a gap was recognized as a fifth base, but the tree topology was basically the same (data not shown). *Botrydiopsis intercedens* Vischer & Pascher and *Tribonema intermixum* Pascher were used as outgroups for *rbc*L trees, *Desmarestia aculeata* (Linnaeus) J.V. Lamouroux and *D. ligulata* (Lightfoot) J.V. Lamouroux for ITS trees, and *Tribonema aequale* for 18S rDNA trees. In ITS trees, any sequence regions in which the alignment was too difficult because of the presence of long insertions or deletions were removed from analyses.

RESULTS

Rubisco large subunit gene

The tree topologies were essentially the same in all of the analyses (MP, NJ and ML trees) using *rbc*L sequence data.

Table 2. List of primer sequences and their annealing positions.[1]

Code	Direction	Sequences (from 5' to 3')	Annealing position
rbc-F0	forward	ATCGAACTCGAATAAAAAGTGA	*rbc*L (20–41)
rbc-F1	forward	CGTTACGAATCWGGTG	*rbc*L (43–58)
rbc-F2	forward	AGGTTCWCTWGCTAA	*rbc*L (342–456)
rbc-F2.5	forward	TTCCAAGGCCCAGCAACAGGT	*rbc*L (454–474)
rbc-F3	forward	CACAACCATTCATGCG	*rbc*L (635–650)
rbc-F4	forward	GTAAATGGATGCGTA	*rbc*L (953–967)
rbc-F5	forward	ATTTGGTGGTGGTACTATTGG	*rbc*L (1212–1232)
rbc-F6	forward	TTAGATTTATGGAAAGATATWAC	*rbc*L (1384–1406)
rbc-R1	reverse	TTAGCWAGWGAACCT	*rbc*L (359–342)
rbc-R2	reverse	CGCATGAATGGTTGTG	*rbc*L (650–653)
rbc-R3	reverse	CCTTTAACCATTAAGGGATC	*rbc*L (1040–1021)
rbc-R4	reverse	GTAATATCTTTCCATAAATCTAA	*rbc*L (1406–1384)
rbc-R5	reverse	AAASHDCCTTGTGTWAGTYTC	*rbc*S (23–3)
rbc-R6	reverse	AATAAAGGAAGACCCCATAATTCCCA	*rbc*S (167–142)
18F-1	forward	AAGGTGAAGTCGTAACAAGG	18S (1768–1787)
18R-1	reverse	CCTTGTTACGACTTCACCTT	18S (1787–1768)
5.8F-1	forward	ACGCAGCGAAATGCGATACG	5.8S (42–61)
5.8R-1	reverse	CGTATCGCATTTCGCTGCGT	5.8S (61–42)
26R-1	reverse	GTTAGTTTCTTTTCCTCCGC	26S (69–50)
LD2	forward	TAGTCATACGCTTGTCTCAA	18S (21–40)
LDA	forward	CGATTCCGGAGAGGGAGCCTG	18S (377–397)
LDB	forward	GTCTGGTGCCAGCAGCCGCGG	18S (558–578)
LDG	forward	TAGCATGGAATAATGAGATAG	18S (813–833)
LDD	forward	CAGAGGTGAAATTCTTGGAT	18S (914–933)
LD7	forward	CTGAAACTTAAAGAAATTGACGG	18S (1145–1167)
LDH	forward	CGCACGCGCGCTACACTGATG	18S (1473–1493)
LD4	reverse	TCAGGCTCCCTCTCCGG	18S (397–377)
LD5	reverse	CCGCGGCAGCTGGCACCAGAC	18S (578–558)
LD6	reverse	ATCCAAGAATTTCACCTCTG	18S (933–914)

[1] Mixtures: D = A + G + T; H = A + C + T; S = C + G; W = A + T; Y = C + T.

The tree topologies were also basically the same in analyses based on amino acid sequences (trees not shown). There were two large clades: (1) the Ectocarpales *sensu lato* (including the Chordariales, Dictyosiphonales, Ectocarpales and Scytosiphonales); and (2) the Laminariales and related taxa (including the Desmarestiales, *Halosiphon,* Sporochnales and Tilopteridales). *Akkesiphycus* clustered with the monophyletic family Pseudochordaceae and these relationships were supported by high bootstrap values (99–100%) (Fig. 1a–c). The Pseudochordaceae/*Akkesiphycus* clade formed a weakly supported sister group to the Laminariaceae and Chordaceae. *Halosiphon, Haplospora* Kjellman (Tilopteridales), *Desmarestia* (Desmarestiales) and *Sporochnus* C. Agardh (Sporochnales) formed a moderately supported cluster and this clade was a sister group to the other Laminariales.

Internal transcribed spacer regions

In general, the tree topologies were similar in all of the trees (except for the position of *Halosiphon*), but the bootstrap values were relatively low in the branches connecting families (except for the relationships between the Alariaceae and Laminariaceae). In the MP tree (Fig. 2a), monophyly of the Pseudochordaceae, Chordaceae and Alariaceae/Laminariaceae was supported by high bootstrap values (95–100%). *Akkesiphycus* grouped with terete members of the Laminariales (Chordaceae, Pseudochordaceae and *Halosiphon*), but the branching order within this cluster was not resolved. This clade formed a sister group to the Alariaceae and Laminariaceae. In the NJ analysis (Fig. 2b), monophyly of the Pseudochordaceae, Chordaceae and Alariaceae/Laminariaceae was also supported by

100% bootstrap values. *Akkesiphycus* clustered first with the Pseudochordaceae, although the bootstrap support was relatively weak (52%). This clade in turn clustered with the Chordaceae and then with *Halosiphon,* but the bootstrap values were low (< 50%). In the ML tree (Fig. 2c), *Akkesiphycus* clustered with the clade containing the Pseudochordaceae and Chordaceae, but the bootstrap value was low (< 50%). *Halosiphon* was basal to all other Laminariales and *Akkesiphycus.*

18S ribosomal DNA

In general, *Akkesiphycus* showed close relationships to the Pseudochordaceae, as was the case in the two other data sets, and the general tree topologies were similar in all of the analyses. However, the bootstrap values for the branches connecting families were generally low. In the MP tree (Fig. 3a), monophyly was demonstrated for the Pseudochordaceae and the A/L/L group (86 and 94% respectively). *Akkesiphycus* clustered with the Pseudochordaceae, and this clade was basal to other Laminariales (the A/L/L group, Chordaceae, *Halosiphon, Saccorhiza* Bachelot de la Pylaie) and to the Desmarestiales and Sporochnales, although the bootstrap values were low (< 50%). *Akkesiphycus* also clustered with the Pseudochordaceae in the NJ tree (Fig. 3b); together these formed the sister group to other Laminariales. In the ML tree (Fig. 3c), *Akkesiphycus* again clustered with the Pseudochordaceae, but other branching patterns were rather different than in other trees. The Ectocarpales and Scytosiphonales branched from *Akkesiphycus,* and *Chorda filum* was basal to other Laminariales and related taxa, but bootstrap values of both branches were low (< 50%).

Table 3. Origin of samples and sequence data used for molecular analyses, with database accession numbers for the sequences.

Species (taxonomic position)	Origin	DDBJ accession no. for *rbc*L gene	DDBJ accession no. for ITS rDNA	DDBJ accession no. for 18S rDNA
Phaeophyceae				
Chordariales				
Elachista fucicola (Velley) Areschoug	Siemer *et al.* (1998)	AF055398		
Sphaerotrichia divaricata (C. Agardh) Kylin	Siemer *et al.* (1998)	AF055412		
Dictyosiphonales				
Delamarea attenuata (Kjellman) Rosenvinge	Siemer *et al.* (1998)	AF055396		
Dicytosiphon foeniculaceus (Hudson) Greville	Siemer *et al.* (1998)	AF055397		
Desmarestiales				
Desmarestia aculeata (Linnaeus) J.V. Lamouroux	Peters *et al.* (1997)		Z84446, Z84447	
D. latrifrons Kützing[1]	present paper	AB037139		
D. ligulata (Lightfoot) J.V. Lamouroux	Tan & Druehl (1996)			L43060
D. ligulata	Peters *et al.* (1997)		Z84440, Z84441	
D. tabacoides Okamura[1]	present paper	AB037140		
D. sp.[1]	present paper	AB037141		
Ectocarpales				
Ectocarpus siliculosus (Dillwyn) Lyngbye	Tan & Druehl (1996)			L43062
E. siliculosus	Valentin & Zetsche (1990)	X52503		
Pilayella littoralis (Linnaeus) Kjellman	Assali *et al.* (1990)	X55372		
Laminariales				
Akkesiphycus lubricum Yamada & Tak. Tanaka[1]	present paper	AB036038	AB036037	AB036036
Halosiphon tomentosus (Lyngbye) Jaasund	Tan & Druehl (1996)			L43056
H. tomentosus	Peters (1998)		Z98565	
H. tomentosus [North Pacific material][1]	present paper	AB036137		
H. tomentosus [North Atlantic material][1]	present paper	AB036136		
Alariaceae				
Alaria fistulosa Postels & Ruprecht	Boo *et al.* (1999)			AF123578
A. praelonga Kjellman	Yotsukura *et al.* (1999)		AB022813, AB022814	
Ecklonia cava Kjellman	Boo *et al.* (1999)			AF123579
Egregia menziesii (Turner) Areschoug	Boo *et al.* (1999)			AF123580
Undaria peterseniana (Kjellman) Okamura	Kawai *et al.* (in press)	AB035794		
Chordaceae				
Chorda filum (Linnaeus) Stackhouse	Boo *et al.* (1999)			AF123585
C. filum	Kawai *et al.* (in press)	AB035786	AB035763	
C. rigida Kawai & Arai	Kawai *et al.* (in press)	AB035788	AB053777	
Laminariaceae				
Agarum clathratum Dumortier	Kawai *et al.* (in press)	AB035791		
A. cribrosum f. *yakishiriense* I. Yamada	Boo *et al.* (1999)			AF123576
Kjellmaniella crassifolia Miyae	Boo *et al.* (1999)			AF123577
K. crassifolia Miyabe	Kawai *et al.* (in press)	AB035792		
K. gyrata (Kjellman) Miyabe	Yotsukura *et al.* (1999)		AB022809, AB022810	
Laminaria diabolica Miyabe	Yotsukura *et al.* (1999)		AB022795, AB022796	
L. japonica Areschoug	Boo *et al.* (1999)			AF123575
L. yendona Miyabe	Yotsukura *et al.* (1999)		AB022807, AB022808	
Thalassiophyllum clathrus (Gmelin) Postels & Ruprecht	Kawai *et al.* (in press)	AB035793		
Lessoniaceae				
Lessonia nigrescens Bory	Boo *et al.* (1999)			AF123581
Postelsia palmaeformis Ruprecht	Boo *et al.* (1999)			AF123582
Phyllariaceae				
Saccorhiza polyschides (Lightfoot) Batters	Tan & Druehl (1996)			L43059
Pseudochordaceae				
Pseudochorda gracilis Kawai & Nabata	Boo *et al.* (1999)			AF123583
P. gracilis	Kawai *et al.* (in press)	AB035790	AB035780	
P. nagaii (Tokida) Inagaki	Boo *et al.* (1999)			AF123584
P. nagaii	Kawai *et al.* (in press)	AB035789	AB035779	
Scytosiphonales				
Chnoospora implexa J. Agardh	Kogame *et al.* (1999)	AB022231		
Scytosiphon lomentaria (Lyngbye) Link	Kawai *et al.* (1995)			D16558
S. lomentaria	Kogame *et al.* (1999)	AB022238		
Sporochnales				
Sporochnus comosus C. Agardh	Tan & Druehl (1996)			L43061
S. scoparius Harvey[1]	present paper	AB037142		

Table 3. Continued.

Species (taxonomic position)	Origin	DDBJ accession no. for *rbc*L gene	DDBJ accession no. for ITS rDNA	DDBJ accession no. for 18S rDNA
Tilopteridales				
Haplospora globosa Kjellman[1]	present paper	AB037138		
Phaeothamniophyceae				
Phaeothmnion confervicola Lagerheim	Bailey *et al.* (1998)	AF064746		
Xanthophyceae				
Botrydiopsis intercedens Vischer & Pascher	Daugbjerg & Andersen (1997)	AF015587		
Tribonema aequale Pascher	Ariztia-Carmona *et al.* (1991)			M55286
Tribonema intermixum Pascher	Daugbjerg & Andersen (1997)	AF015588		

[1] New sequence data published in the present paper.

DISCUSSION

In our molecular phylogenetic analyses, the closest phylogenetic relationship of *Akkesiphycus* was to the family Pseudochordaceae in all of the data sets (*rbc*L gene, 18S and ITS rDNA). The *rbc*L data set (Fig. 1) was the most suitable for resolving phylogenetic relationships at the ordinal and familial levels in the Laminariales and related taxa, judging from the following: the tree topologies were essentially identical in all of the analyses (MP, NJ and ML trees), and the bootstrap values connecting the families were generally high.

The ITS1 and ITS2 data sets appeared to be too variable for familial-level comparisons within Laminariales and alignment was difficult between species of different families. Nevertheless, the close relationship between *Akkesiphycus* and the Pseudochordaceae, as well as the monophyly of the families Chordaceae, Pseudochordaceae and the A/L/L group, were supported by relatively high bootstrap values (Fig. 2).

18S ribosomal DNA was of limited value in elucidating the phylogenetic relationships below familial level in brown algae (Fig. 3), as has been discussed repeatedly (Saunders & Druehl 1992; Tan & Druehl 1993, 1996; Boo *et al.* 1999). The bootstrap values of the branches connecting the families in each analysis were generally low, reflecting this tendency. However, even in the 18S trees, a close relationship of *Akkesiphycus* to the Pseudochordaceae was revealed, regardless of the analytical method used. Although the bootstrap support was weak (< 50%) in these trees, the results were consistent throughout.

The Chordaceae formed a monophyletic clade with the A/L/L group in the *rbc*L trees, with moderate bootstrap support (61–65%), whereas it clustered with the Pseudochordaceae and *Akkesiphycus* in the ITS trees (and also with *Halosiphon* in the MP tree). The branching position of the Chordaceae was not consistent in the 18S trees. Although the relationships suggested by the various molecular data sets were not the same, judging from the reliability of *rbc*L data for the other comparisons mentioned above, we consider that the Chordaceae is the closest relative of the A/L/L group, as has been traditionally believed. These two groups have a similar elaborate intercalary meristem (primary and secondary meristem) in the sporophytes (Kylin 1917; South & Burrows 1967; Kogame & Kawai 1996), and they share characteristic trumpet-shaped hyphae. The Phyllariaceae also develop an intercalary meristem, but the developmental processes are different (Sauvageau 1918); furthermore, the solenocysts and allelocysts, with their multinuclear cytoplasm, are peculiar and indicate a rather distant phylogenetic relationship to other Laminariales (Henry & South 1987). The only other multinucleate structures known in brown algae are the conducting channels of the Ascoseirales (Clayton & Ashburner 1990). In contrast, the Pseudochordaceae (Tokida 1938; Kawai & Kurogi 1985; Kawai & Nabata 1990) and *Akkesiphycus* (Kurogi & Yamada 1970; Kawai 1986) also have inner hyphae in the medullary layer of the sporophytes; these are typically trumpet-shaped in *Pseudochorda nagaii* (Tokida 1938), suggesting a closer affinity to the Chordaceae and the A/L/L group.

A close relationship between the Chordaceae and the A/L/L group is further supported by sexual pheromone analyses. Species of the A/L/L group have lamoxirene as their common and specific sexual pheromone. Among related taxa, *Halosiphon tomentosum* has been shown to use (+)-multifidene as the sexual pheromone and is not sensitive to lamoxirene (Maier 1984, 1995; Müller *et al.* 1985b; Maier & Müller 1986). The Phyllariaceae too are not sensitive to lamoxirene but their sexual pheromones have not yet been identified (Müller *et al.* 1985b). In contrast, although the sexual pheromone of *Chorda filum* has not been fully identified, it is known to have a chemical structure similar to that of lamoxirene, containing oxygen (Müller *et al.* 1985b; Kawai *et al.*, unpublished results). Furthermore, the sperm of *Chorda filum* shows moderate sensitivity to lamoxirene. The Desmarestiales and Sporochnales have their own specific sexual pheromones – desmarestene and caudoxirene, respectively (Müller *et al.* 1982; Maier 1995). On the other hand, in a preliminary experiment, mature eggs of *Pseudochorda nagaii* were shown to secrete ectocarpene as the dominant pheromone-like substance (Kawai & Müller, unpublished results), although its effectiveness for attracting sperm has not been fully analysed. The sexual pheromone of *Akkesiphycus* has not yet been studied. Therefore, based on existing data about sexual pheromones, *Chorda filum* shows the closest affinity to the A/L/L group among related taxa.

The *rbc*L data also suggest a close relationship between the clade containing *Akkesiphycus* and the Pseudochordaceae and that containing the A/L/L group and the Chordaceae. Al-
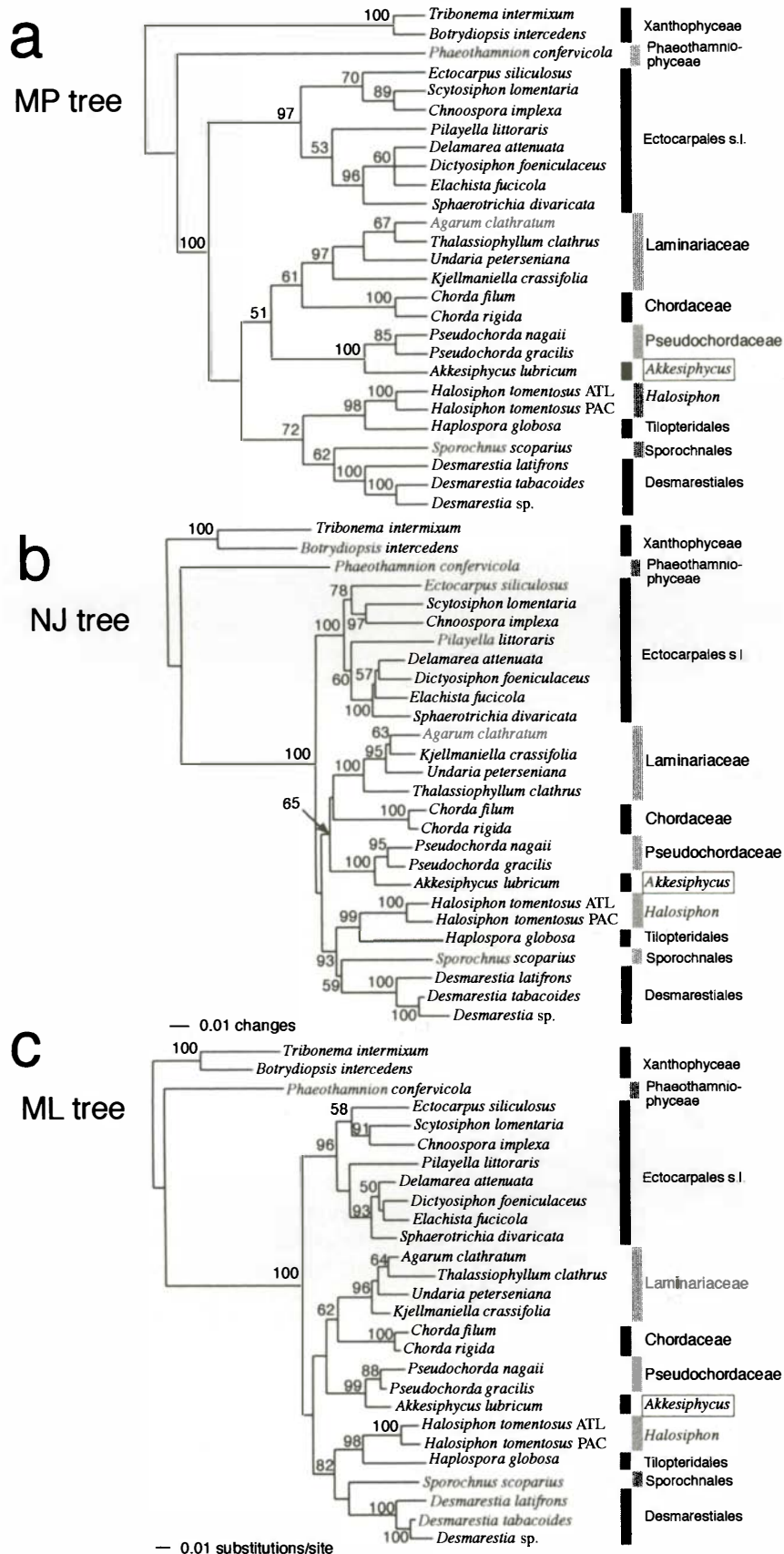
Fig. 1. Molecular phylogenetic trees based on *rbc*L region sequences. a) Maximum parsimony (MP) analysis (strict consensus); b) neighbour-joining (NJ) analysis; c) maximum likelihood (ML) analysis. Bootstrap values indicate % based on 1000 (a, b) or 500 (c) replicates. In the MP analysis, two equally parsimonious trees of 1488 steps were obtained with a consistency index (CI) of 0.4987 and a retention index (RI) of 0.6129. There were 393 parsimony-informative sites within the 1409 positions aligned. In the ML tree, –Ln likelihood was 10163.12034.
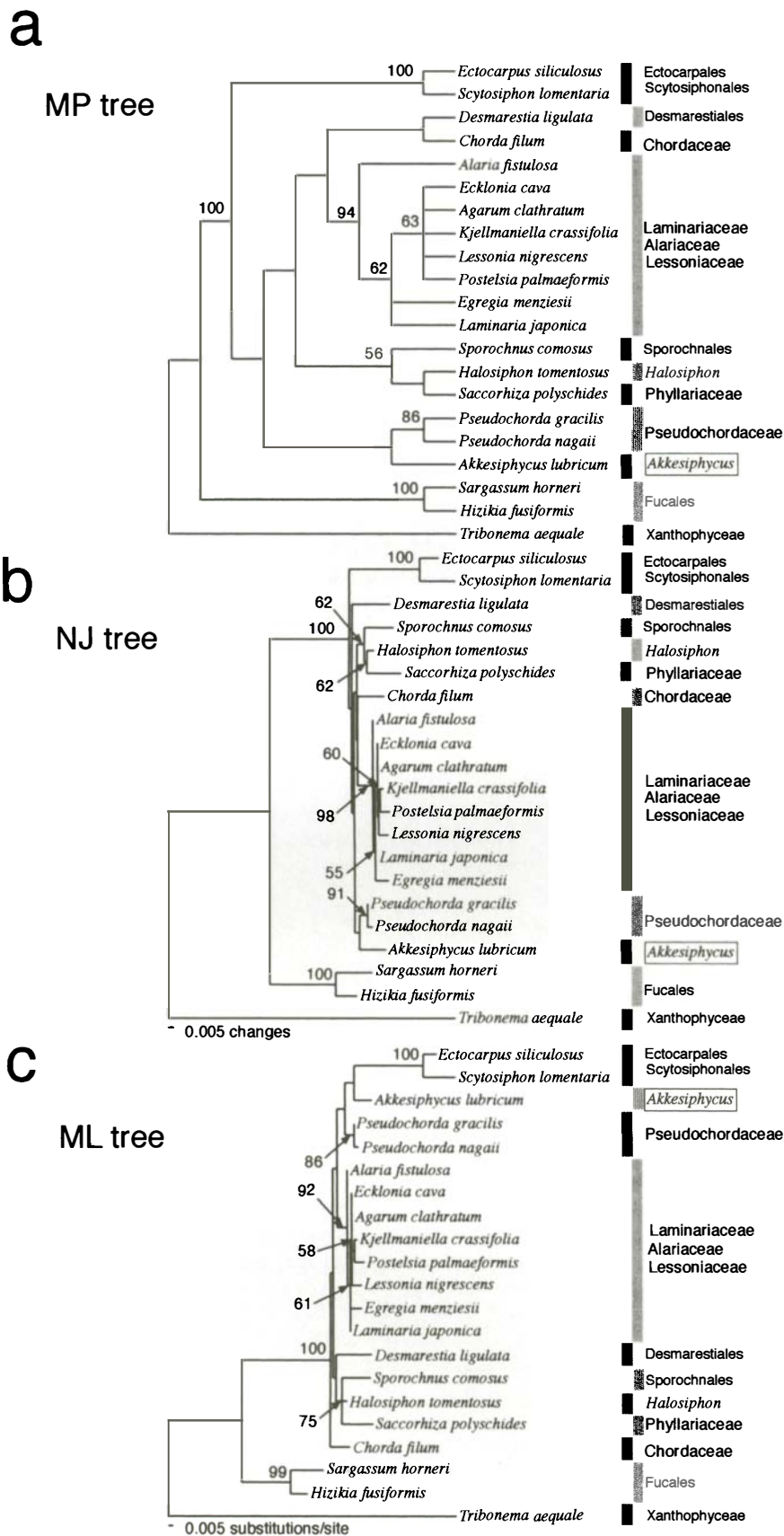
a MP tree

b NJ tree

c ML tree
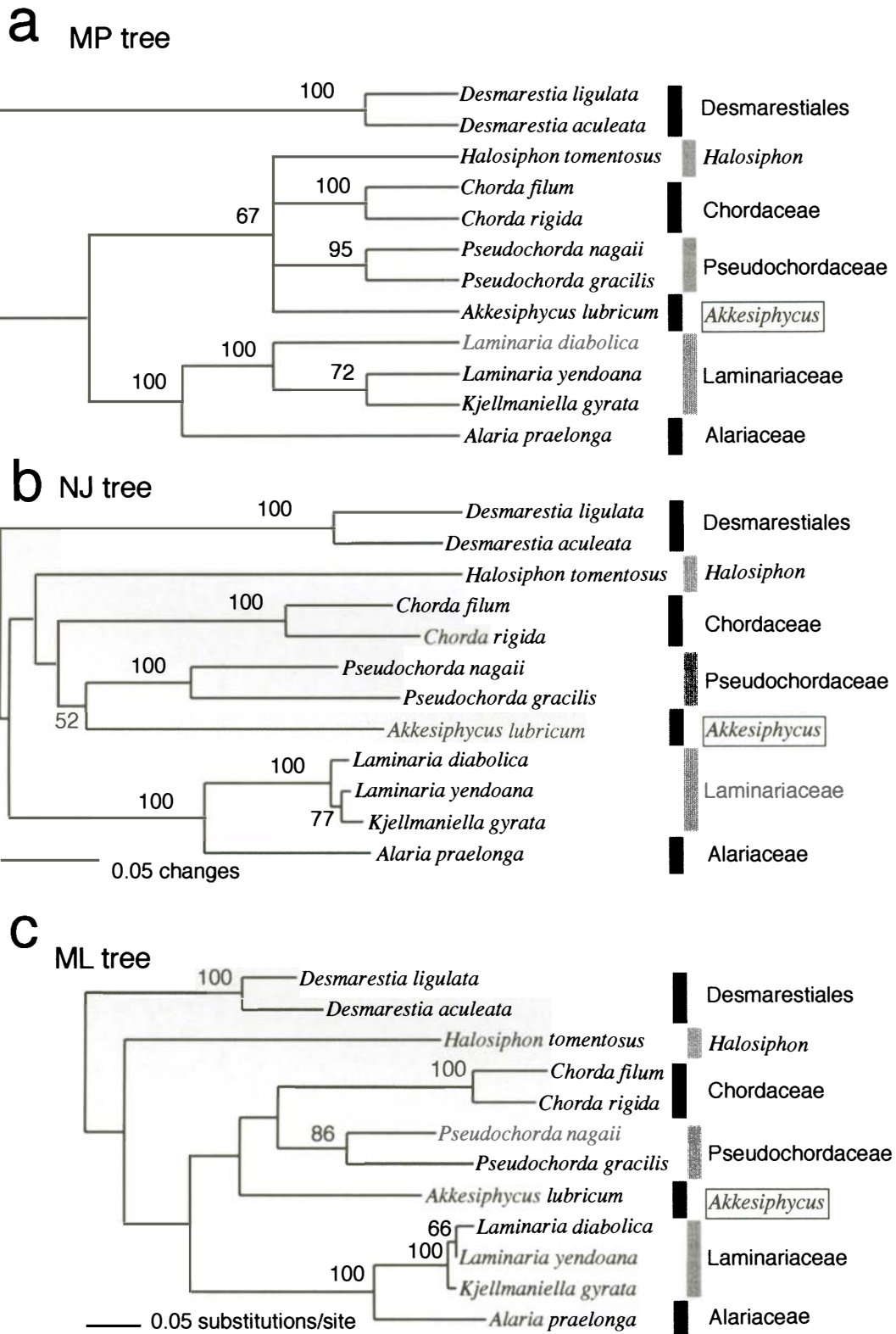
Fig. 2. Molecular phylogeny trees based on ITS1 and ITS2 rDNA sequences. a) MP analysis (strict consensus); b) NJ analysis; c) ML analysis. Bootstrap values indicate % based on 1000 replicates. In the MP analysis, four most-parsimonious trees of 741 steps were obtained with a consistency index (CI) of 0.6827 and a retention index (RI) of 0.6737. There were 238 parsimony-informative sites in the 613 positions aligned. In the ML tree, −Ln likelihood was 34937.11546.

Fig. 3. Molecular phylogeny trees based on 18S (SSU) rDNA region sequences. a) MP analysis (strict consensus); b) NJ analysis; c) ML analysis. Bootstrap values indicate % based on 1000 replicates. In the MP analysis, four most-parsimonious trees of 365 steps were obtained with a consistency index (CI) of 0.8548 and a retention index (RI) of 0.7782. There were 110 parsimony-informative sites within the 1764 positions aligned. In the ML tree, –Ln likelihood was 4619.77559.

though bootstrap support was not very high (51 and 65% in the MP and NJ trees, respectively, and below 50% in the ML tree), the tree topologies were consistent in all of the analyses and appear to be relatively reliable. In addition, *Halosiphon, Haplospora* (Tilopteridales), Desmarestiales and Sporochnales also formed a clade; the tree topology was again consistent in all of the analyses and bootstrap support was generally high (59–100%). A close relationship between *Haplospora* and these groups has not been suggested previously, but the links between the Desmarestiales, Sporochnales and *Halosiphon* were already evident from 18S rDNA data (Boo *et al.* 1999; this study). Therefore, we recognize two large groups within the Laminariales and its related taxa: one comprising the A/L/L group, Chordaceae, *Akkesiphycus* and the Pseudochordaceae, and the other comprising the Desmarestiales, Sporochnales, and *Halosiphon* (and possibly also the Tilopteridales, although this requires investigation of additional taxa). The ordinal boundary of Laminariales is still controversial, because there appear to be four options: (1) the A/L/L group only; (2) A/L/L + Chordaceae; (3) A/L/L + Chordaceae + Pseudochordaceae + *Akkesiphycus*; or (4) all the above-mentioned taxa. At present, we suggest expanding the boundary at least to the limits of item (3) above by including *Akkesiphycus* in the order Laminariales. Although *Akkesiphycus* differs from other members in some essential morphological characters, the boundary between the two taxa is not clear, as will be discussed below.

Akkesiphycus is anisogamous, gametes of both sexes being flagellate. Regarding female reproductive organs, *Akkesiphycus* forms flagellated female gametes, each with a longer anterior and a shorter posterior flagellum; these gametes each contain several chloroplasts and an eyespot. This clearly contrasts with the other members of Laminariales, which show oogamy. However, Motomura & Sakai (1988) reported the occurrence of residual flagella in the eggs of *Laminaria angustata* Kjellman, although a thorough survey on the occurrence of this phenomenon in the Laminariales has not been done. Therefore, at least part of the oogamy of Laminariales is in the strict sense aplano-anisogamy, and the gap between the anisogamy of *Akkesiphycus* and the oogamy of the Laminariales now assumes less taxonomic importance than has hitherto been assumed.

As to the morphology of male reproductive organs, *Akkesiphycus* forms characteristic clustered plurilocular male gametangia. Male gametes have a longer anterior and a shorter posterior flagellum and contain a single chloroplast and an eyespot. In contrast, typical antheridia in the Laminariales are single-chambered, and their sperm have a longer posterior flagellum and contain several small chloroplasts but no eyespots (Henry & Cole 1982; Motomura 1989). Some primitive members of the Laminariales, such as *Pseudochorda* Yamada, Tokida & Inagaki *in* Inagaki, have plurilocular antheridia resembling the male gametangia of *Akkesiphycus,* but even so, the sperm of *Pseudochorda* has a longer posterior flagellum and contains several small chloroplasts. Members of the Phyllariaceae also form clustered antheridia (Henry 1986; Flores-Moya & Henry 1998) and their sperm also lack eyespots, but the cellular fine structure has not been studied.

A close phylogenetic relationship between *Akkesiphycus* and the Pseudochordaceae is biogeographically understandable because their distributions overlap, both being endemic to the northwest Pacific Ocean. The derived (advanced) Laminariales (A/L/L group) are distributed in the Pacific and Atlantic Oceans. Among the taxa presumed to be primitive, *Chorda* Stackhouse has a relatively wide distributional range in both the Pacific and Atlantic Oceans (Druehl 1970; Lüning 1985, 1990) but, as mentioned above, the Pseudochordaceae are only known from the Pacific. Because of the rich occurrence of laminarialean taxa in the Pacific and the restriction of the Pseudochordaceae to this area, the Laminariales have been considered to have originated in the Pacific (Lüning & tom Dieck 1990). Previously, the occurrence of the Phyllariaceae and *Halosiphon* only in the Atlantic Ocean (Lüning 1985, 1990; Lüning & tom Dieck 1990) seemed to contradict this notion. However, *Halosiphon* has now been shown to be distributed also in the Pacific Ocean (this study), whereas the Phyllariaceae are unlikely to have been ancestral to the Chordaceae and the A/L/L group, as mentioned above. Therefore, although it is premature to come to firm conclusions about the palaeo-phytogeography of the entire order Laminariales, the present data favour a Pacific origin.

Although *Akkesiphycus* has been shown to have a close phylogenetic relationship to the Pseudochordaceae, it differs from this family in the foliose morphology of the sporophyte and the formation by the gametophytes of anisogamous male and female planogametes, which have longer anterior and shorter posterior flagella and perform phototaxis (they are provided with eyespot and flagellar swelling and exhibit green flagellar autofluorescence: Kawai 1988, 1992b).

In conclusion, we suggest the establishment of two new families: the Akkesiphycaceae to accommodate the monotypic genus *Akkesiphycus* and the Halosiphonaceae for the genus *Halosiphon.* The distant phylogenetic relationship of *Halosiphon* to the Chordaceae has been suggested by ITS and 18S rDNA data (Peters 1998; Boo *et al.* 1999). However, the authors avoided making taxonomic changes because the relationship with the Pseudochordaceae was unclear and the resolution of the data was insufficient. However, we believe the problem has been solved in the present study. The familial name Halosiphonaceae was once used by Christensen (1962) in an early version of his textbook, but this term was nomenclaturally invalid since it lacked a Latin diagnosis; he stopped using the name in the later version, although his reasons for doing so are unclear (Christensen 1966; Silva 1980). For the ordinal assignments of the families Akkesiphycaceae and Halosiphonaceae, as discussed above, we suggest inclusion of only the Pseudochordaceae and Akkesiphycaceae in the Laminariales. We leave Halosiphonaceae as *incertae sedis,* because it is more likely to be grouped with the Phyllariaceae, Desmarestiales and Sporochnales (and possibly Tilopteridales too) in a separate order. Because of the inclusion of *Akkesiphycus* in the Laminariales and the finding of residual flagella in some eggs (Motomura & Sakai 1988), the definition of the Laminariales must be emended to include anisogamy in addition to oogamy.

If it is supposed that *Akkesiphycus,* the Pseudochordaceae and the A/L/L group form a monophyletic group and that *Halosiphon* and the Phyllariaceae are not included in this clade, the evolution of morphological and physiological characters, as summarized in Table 1, can be hypothesized, as in Fig. 4a. The tree topology shown in Fig. 4b, as directly extrapolated from *rbc*L trees, is less likely, because the tree re-
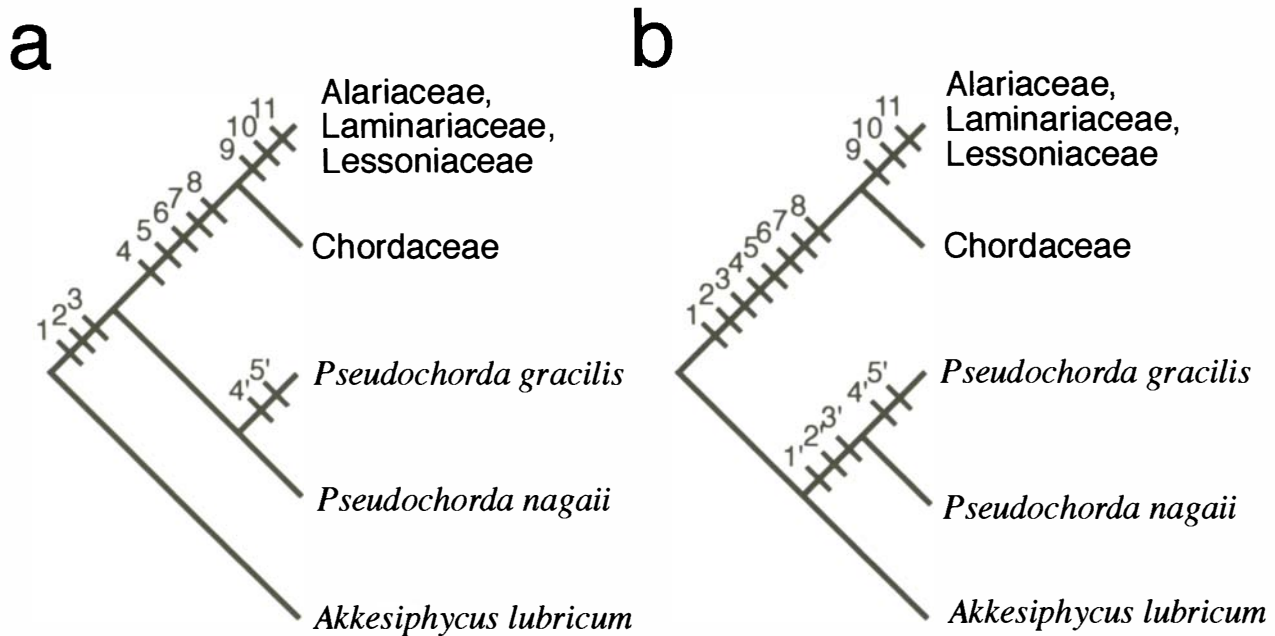
Fig. 4. Cladograms indicating the hypothetic transformations of morphological and physiological characters in the course of the evolution within Laminariales. Tree topology a is more parsimonious than that of b. 1: oogamy; 2: longer posterior flagellum in sperm; 3: several small chloroplasts in sperm; 4: dimorphic gametophyte; 5: uniseriate antheridium; 6: intercalary meristem; 7: unicellular paraphysis; 8: trumpet-shaped hypha; 9: mucilaginous structure (i.e. mucilage cap, secretory cell and duct); 10: loss of phototaxis in zoospore; 11: perennial sporophyte.

quires many more parallel evolutionary changes (e.g. oogamy, morphological changes in antheridia and sperm, and dimorphism in gametophytes). Nevertheless, dimorphism of the gametophytes is considered to have occurred twice, once during the evolution of the Chordaceae and the A/L/L group and once within the Pseudochordaceae. Such a parallel evolutionary trend is not surprising because similar examples are found in the Desmarestiales (Ramirez *et al.* 1986; Peters *et al.* 1997), the Phyllariaceae (Henry 1987; Henry & South 1987) and the Syringodermatales (Henry 1984; Kawai & Yamada 1990). Our preferred tree (Fig. 4a) contains the suggestion that oogamy is derived within the Laminariales. In contrast, the anisogamy of *Notheia* Harvey & Bailey (Notheiaceae, Fucales) appears to have been derived from oogamy, judging by molecular phylogenetic analyses using 18S and 28S rDNA sequences (Saunders & Kraft 1995; Rousseau & Reviers 1999), since *Notheia* nests within a clade of oogamous relatives (*Durvillaea* Bory + Fucales). However, the reliability of the branches connecting *Durvillaea* and *Notheia* to other oogamous fucalean members is not high, and the monophyly of oogamy in *Durvillaea* and Fucales is still controversial (Maier & Clayton 1989, from cytological data). Furthermore, considering that the flagellar basal system is one of the most complex structures within cells, consisting of more than a hundred polypeptides and elaborate rootlets, it is highly unlikely that flagellated female gametes evolved from eggs that had lost these structures. This notion needs further discussion in relation to the evolution of morphological features.

Akkesiphycaceae Kawai & Sazaki *fam. nov.*

Sporophyton macroscopicum foliaceum parenchymaticum, cellulas medullosas capiens, paraphysibus multicellularibus instructum, sine operculis mucosis, sine glandicellulis muciferis. Sporangium unil-

oculare anguste ovatum sessile. Gametophyton minutum dioecium monomorphum; gametangia plurilocularia anisogametas facientia. Cellulae sporophyticae et gametophyticae chloroplastos numerosos discoideos sine pyrenoidibus capientes.

TYPE: *Akkesiphycus* Yamada & Tanaka (1944, pp. 61, 62, pl. 8)

Sporophyte macroscopic, foliose, parenchymatous, containing medullary cells and bearing multicellular paraphyses, without mucilage caps and without mucilage gland-cells. Unilocular sporangia sessile, narrowly ovate. Gametophyte minute, dioecious and monomorphic, with plurilocular gametangia, which produce anisogametes. Sporophytic as well as gametophytic cells containing many disc-shaped chloroplasts without pyrenoids.

Halosiphonaceae Kawai & Sasaki *fam. nov.*

Sporophyton macroscopicum simplex filiforme, parenchymaticum, cellulas medullosas capiens, filis assimilantibus multicellularibus instructum, sine glandicellulis muciferis. Sporangium uniloculare anguste ovatum sessile. Gametophyton minutum monoecium sexualis reproductio oogama; antheridium solitarium. Cellulae sporophyticae et gametophyticae chloroplastos numerosos discoideos sine pyrenoidibus capientes.

TYPE: *Halosiphon* Jaasund (1957, pp. 211–212).

Sporophyte macroscopic, simple, cord-shaped, parenchymatous, with medullary cells and multicellular assimilators, without mucilage gland cells. Unilocular sporangia sessile, narrowly ovate. Gametophyte minute, monoecious; sexual reproduction oogamous. Antheridium solitary. Sporophytic and gametophytic cells containing many disc-shaped chloroplasts without pyrenoids.

providing cultures of *Chorda tomentosa* and *Desmarestia latifrons,* to Dr D.G. Müller for providing cultures of *Haplospora globosa,* and to Dr Akira Peters for his helpful suggestions.

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