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Molecular phylogeny of Phyllariaceae, Halosiphonaceae and Tilopteridales (Phaeophyceae)

HIDEAKI SASAKI[1], ANTONIO FLORES-MOYA[2], ERIC C. HENRY[3], DIETER G. MÜLLER[4] AND HIROSHI KAWAI[5]*

[1]*Graduate School of Natural Sciences, Kobe University, Rokkodai, Kobe 657-8501, Japan*
[2]*Department of Plant Biology, Faculty of Science, University of Malaga, Campus de Teatinos s/n, E-29071 Malaga, Spain*
[3]*Department of Botany and Plant Pathology, Oregon State University, Cordley Hall 2082, Corvallis, OR 97331-2902*
[4]*Faculty of Biology, University of Konstanz, D-78434 Konstanz, Germany*
[5]*Kobe University Research Center for Inland Seas, Rokkodai, Kobe 657-8501, Japan*

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Phylogenetic relationships of problematic members of the Laminariales (*Halosiphon* and Phyllariaceae) and Tilopteridales were studied comparing Rubisco gene (*rbc*L and spacer) and ribosomal DNA (5.8S, ITS2 and a part of 26S) sequence data covering all species of these taxa and 'primitive' Laminariales. Molecular phylogenetic trees were constructed by use of maximum parsimony (MP), maximum likelihood (ML) and neighbour-joining (NJ) methods. The *rbc*L data supported a monophyletic Tilopteridales and its close affiliation with the Phyllariaceae and *Halosiphon,* contrary to conventional taxonomy on the basis of life history patterns and morphological features. *Halosiphon,* Phyllariaceae and Tilopteridales formed a sister group to a clade consisting of Desmarestiales and Sporochnales in the ML and NJ analyses, although the bootstrap values supporting the relationship were not high. This larger clade, including all the taxa mentioned above, formed a sister lineage to a group including *Akkesiphycus,* Pseudochordaceae, Chordaceae and the 'advanced' Laminariales (Alariaceae, Laminariaceae and Lessoniaceae). The *rbc*L + Rubisco spacer sequences, as well as the 5.8S + ITS2 + 26S rDNA sequences, supported the independence of existing taxa of the Phyllariaceae and suggested early divergence of *Saccorhiza* within the family.

INTRODUCTION

The order Laminariales has traditionally included four families: Chordaceae, Alariaceae, Laminariaceae and Lessoniaceae (Setchell & Gardner 1925; Bold & Wynne 1985). However, various aspects of the taxonomy of the order at ranks of family and above have been challenged in the last two decades. Kawai & Kurogi (1985) created a new family Pseudochordaceae within the order to accommodate *Pseudochorda nagaii* (Tokida) Inagaki, and later a second species, *P. gracilis* Kawai & Nabata (Kawai & Nabata 1990), was added to the genus. Henry & South (1987) suggested that reappraisal of the family Phyllariaceae was not necessary, because of the presence of some primitive characteristics in its members, as will be mentioned below. The Pseudochordaceae, Phyllariaceae and Chordaceae have been considered to be primitive within the Laminariales on the basis of the following characteristics (Henry & Cole 1982; Maier 1984; Kawai & Kurogi 1985; Henry 1987; Henry & South 1987; Kawai & Nabata 1990; Kogame & Kawai 1996; Flores-Moya & Henry 1998): (1) the relatively simple organization of the sporophytes, which have no differentiation between blade and stipe (except Phyllariaceae) and also lack a meristematic rhizoidal holdfast; (2) the annual nature of the sporophytes and the lack of a distinct intercalary meristem [except for *Chorda filum* (Linnaeus) Stackhouse and Phyllariaceae]; (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 occurrence of monoecious [*Halosiphon tomentosus* (Lyngbye) Jaasund and *Saccorhiza dermatodea* (Bachelot de la Pylaie) J. Agardh] or dioecious but monomorphic gametophytes [*P. nagaii, Phyllariopsis brevipes* (C. Agardh) E.C. Henry & South and *P. purpurascens* (C. Agardh) E.C. Henry & South], with the notable exception of *S. polyschides* (Lightfoot) Batters. In addition, *Akkesiphycus lubricum* Yamada & Tak. Tanaka has been shown to have the closest phylogenetic affinity with the Pseudochordaceae (Kawai 1986), and hence an independent family Akkesiphycaceae has recently been established within the Laminariales on the basis of life history and molecular phylogenetic studies (Kawai & Sasaki 2000).

In contrast, the phylogenetic relationships of *H. tomentosus* (Lyngbye) Jaasund (= *Chorda tomentosa* Lyngbye) and the Phyllariaceae to other members of the Laminariales (Pseudochordaceae, Chordaceae and the Alariaceae/Laminariaceae/Lessoniaceae group) are thought to be rather distant, on the basis of physiological and morphological characters (Maier 1984; Henry & South 1987; Kogame & Kawai 1996), as well as molecular phylogenetic data (Peters 1998; Boo *et al.* 1999; Kawai & Sasaki 2000); this supports the resurrection of the Halosiphonaceae (Kawai & Sasaki 2000).

The relatively close systematic relationships between the Desmarestiales, Sporochnales and Laminariales have been repeatedly discussed, on the basis of morphological, physiological and molecular phylogenetic studies (Clayton 1984; Müller *et al.* 1985; Kawai 1992; Tan & Druehl 1996; Peters 1998; Boo *et al.* 1999; de Revier & Rousseau 1999). However, phylogenetic studies have not yet clarified these relationships, owing to the insufficient resolution of 18S rDNA data, as well

* Corresponding author (kawai@kobe-u.ac.jp).

as the lack of information about the cytology and physiology of primitive members of the Laminariales and related taxa.

The ordinal assignment of the family Tilopteridaceae (*Tilopteris* Kützing, *Haplospora* Kjellman and *Phaeosiphoniella* R.G. Hooper, E.C. Henry & Kuhlenkamp) has also been controversial. It has been placed in the Ectocarpales (Parke & Dixon 1976; Kornmann & Sahling 1977), Dictyosiphonales (Jaasund 1965), Tilopteridales *sensu stricto* (usually including only the three monospecific genera: Oltmanns 1922; Taylor 1937; Fritsch 1945; Kuhlenkamp & Müller 1985; Hooper *et al.* 1988) or Tilopteridales *sensu lato* (including the taxa usually comprising the Dictyosiphonales: Christensen 1980; Pedersen 1984). In contrast to all the previous proposals, however, Kawai & Sasaki (2000) suggested a relatively close phylogenetic relationship between *Halosiphon* Jaasund (Halosiphonaceae) and *Haplospora* (Tilopteridales), based on Rubisco gene sequence data. Furthermore, despite the great morphological similarities, *Phaeosiphoniella* has been suggested to be phylogenetically distant from *Tilopteris* and *Haplospora,* on the basis of 18S and 26S rDNA gene sequence data (de Reviers & Rousseau 1999).

Both 18S and 26S rDNA sequence data have frequently been used for elucidating phylogeny at higher taxonomic levels (family and above) in the Phaeophyceae, but these molecules have provided rather limited resolution, owing to their highly conserved nature (Saunders & Druehl 1992; Tan & Druehl 1993, 1996; Boo *et al.* 1999). In contrast, the spacer sequences of rDNA (ITS1 and ITS2) are much too variable and can scarcely be aligned between families in the Phaeophyceae (Peters 1998). The coding and spacer region sequences of the Rubisco gene show intermediate rates of divergence, lying between the rates for the coding and spacer sequences of rDNA, and hence appear to provide better resolution for discussing familial and ordinal relationships within the brown algae (Siemer *et al.* 1998; Kogame *et al.* 1999; Kawai & Sasaki 2000).

Therefore, we analysed phylogenetic relationships among the Tilopteridales, Phyllariaceae, Halosiphonaceae and other 'primitive' Laminariales, by comparing Rubisco (almost complete *rbc*L gene and its spacer region between *rbc*L and *rbc*S) and rDNA (5.8S, ITS2 and 26S rDNA) sequences, which are encoded in the plastid and nuclear genomes, respectively.

MATERIAL AND METHODS

The origins of specimens used for DNA extraction and the sequence data used for the analyses are listed in Table 1. The specimens used for the present study are deposited at Kobe University Research Center for Inland Seas. Cultures were grown in polystyrene Petri dishes containing 50 ml PESI medium (Tatewaki 1966) illuminated by daylight-type white fluorescent lighting of approximately 50 μmol m^{-2} s^{-1} (long days, viz. 16:8 h light: dark) at 10°C or 15°C. For DNA extraction, the culture materials were frozen in liquid nitrogen. Field-collected material was rapidly desiccated in silica gel. Air-dried herbarium vouchers were also used. Approximately 40 mg of algal tissue powder ground in liquid nitrogen were used for genomic DNA extractions, which were performed with use of a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification of the Rubisco large subunit gene (*rbc*L), the spacer region between the *rbc*L and *rbc*S, and rDNA (5.8S, ITS2 and part of the 26S rDNA) were carried out by use of GeneAmp PCR Systems 2400 and 9700 (Perkin Elmer, Foster City, California, USA) and a TaKaRa Ex Taq (Takara Shuzo, Shiga, Japan) reaction kit (total reaction volume of 25 μl was 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 including 0.5–1.0 μg DNA). Except where specified, primers (Table 2) were designed on the basis of 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; Kogame *et al.* 1999). The profile of PCR conditions was as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C (for 18S, 5.8S and 26S) or at 42°C or 58°C (for *rbc*L and spacer) for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products were directly sequenced by use of the Cy5 Auto Cycle Sequencing Kit (Pharmacia Biotech AB, Uppsala, Sweden) and ALF Express DNA sequencer (Pharmacia Biotech) or the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The Clustal W program (Thompson *et al.* 1994) was used for preliminary DNA sequence alignment, followed by manual final alignment. The aligned sequences were subjected to maximum parsimony (MP) analyses in a general heuristic search with use of PAUP v. 4.0b3a (Swofford 1999). Fifteen random taxon addition replicates were performed in each heuristic search, by using the option TBR branch swapping. Gaps were not taken into account in MP analysis. From the same alignment, two-parameter distances (Kimura 1980) between taxa were estimated, and a phylogenetic tree was constructed with the neighbour-joining (NJ) method, by using PAUP. Maximum likelihood (ML) analyses were also performed by use of PAUP in a general heuristic search with a substitution model (transition/transversion ratio = 2 and empirical base frequencies, by use of the Hasegawa-Kishino-Yano model) and equal among-site rate variation. The robustness of the resulting phylogenies was tested by a bootstrap analysis with 1000 (MP and NJ) and 500 (ML) resamplings (Felsenstein 1985). In an additional MP analysis, gaps were recognized as a fifth base.

In the analysis using *rbc*L, *Botrydiopsis intercedens* and *Tribonema intermixum* (Xanthophyceae) were used as outgroups, because the Xanthophyceae has been suggested to be phylogenetically closest to the Phaeophyceae by an analysis using *rbc*L sequence data (Daugbjerg & Andersen 1997). In the *rbc*L + spacer analysis, *Pseudochorda nagaii, P. gracilis* and *A. lubricum* were chosen as outgroups on the basis of results from *rbc*L (Kawai & Sasaki 2000 and present data) and 18S sequence data (Boo *et al.* 1999). For the rDNA analysis, *Haplospora globosa* and *Phaeosiphoniella cryophila* were used as outgroups. *Halosiphon tomentosus* was also used as an outgroup in the latter two analyses, but the elucidated relationships were basically the same.

RESULTS

*rbc*L

The aligned *rbc*L sequences were 1409 base pairs in total. There were 420 parsimony-informative nucleotide positions. In all of the analyses, monophyletic clades with moderate to strong bootstrap support were formed by: (1) the Ectocarpales *sensu lato* (including Chordariales, Dictyosiphonales, Ectocarpales and Scytosiphonales); (2) the so-called advanced Laminariales, comprising the Alariaceae/Laminariaceae/Lessoniaceae (A/L/L) group and Chordaceae; (3) the Pseudochordaceae and Akkesiphycaceae; and (4) the Halosiphonaceae, Tilopteridales, Phyllariaceae, Sporochnales and Desmarestiales (Fig. 1). Phylogenetic trees constructed with the MP, NJ and ML analyses showed essentially similar topologies, although, in the MP tree, the four clades listed above derived from a polychotomy (Fig 1a). In contrast, in the NJ and ML trees, the A/L/L group and Chordaceae clustered with the Akkesiphycaceae and Pseudochordaceae clade (Fig. 1b). The clade containing these groups next joined with the clade including the Halosiphonaceae, Tilopteridales, Phyllariaceae, Sporochnales and Desmarestiales, with the Sporochnales and Desmarestiales together forming a sister group to the clade including the Halosiphonaceae, Tilopteridales and Phyllariaceae. The larger clade including all these taxa was sister to the clade including the A/L/L group and Chordaceae and Pseudochordaceae + Akkesiphycaceae. The Ectocarpales *sensu lato* was basal to these within the Phaeophyceae. In an additional analysis, based on amino acid sequence and the DNA sequence data with use of only the first and second codon positions (trees not shown), the monophyly of the clade comprising the Halosiphonaceae, Tilopteridales and Phyllariaceae was supported by high (88–100%) bootstrap values in all of the analyses. This clade tended to cluster with a the clade containing the Sporochnales and Desmarestiales, although the bootstrap support was not always high.

*rbc*L + spacer

In order to elucidate more detailed phylogenetic relationships within the Phyllariaceae and Tilopteridales, *rbc*L gene sequences were analysed in combination with the spacer sequence between *rbc*L and *rbc*S. The aligned sequences of *rbc*L and spacer totalled 1707 sites. There were 404 parsimony-informative nucleotide positions. The sequence divergence within the Phyllariaceae was 3.0%. Divergences within species and subspecies were 1.0% (*S. polyschides*), 1.6% (*Phyllariopsis brevipes* ssp. *brevipes*), 0.2% (*P. brevipes* ssp. *pseudopurpurascens*), and 0.3% (*P. purpurascens*). Divergence between *P. brevipes* ssp. *brevipes* and *P. purpurascens* was 2.4% and between *P. brevipes* ssp. *brevipes* and *P. brevipes* ssp. *pseudopurpurascens,* 2.3%.

Tree topologies were essentially the same for all of the phylogenetic analyses (Fig. 2), although there were some discrepancies among trees with respect to the branching order of closely related taxa (within species). Monophyly of the Tilopteridales and Phyllariaceae, and independence of each species and subspecies within the Phyllariaceae, were confirmed and supported by moderate to high bootstrap values. Within the Phyllariaceae, the genus *Phyllariopsis* formed a monophyletic group and the two species of *Saccorhiza* Bachelot de la Pylaie (*S. polyschides* and *S. dermatodea*) branched before *Phyllariopsis* E.C. Henry & South in the NJ and ML trees. Within the genus *Phyllariopsis, P. brevipes* spp. *brevipes* clustered with ssp. *pseudopurpurascens*; *P. purpurascens* was basal to these two taxa. *Phyllariopsis brevipes* from Italy and Spain clustered first in all of the analyses.

5.8S + ITS2 + partial 26S rDNA

In order to clarify the intrafamilial relationships within the Phyllariaceae, 5.8S + ITS2 + partial 26S rDNA sequences were analysed. The sequence divergence within the Phyllariaceae was 10.8%. Divergences within species and subspecies were 0.8% (*S. polyschides*), 1.8% (*P. brevipes* ssp. *brevipes*), 0.3% (*P. brevipes* ssp. *pseudopurpurascens*), and 0.1% (*P. purpurascens*). Divergence between *P. brevipes* ssp. *brevipes* and *P. purpurascens* was 4.4% and between *P. brevipes* ssp. *brevipes* and *P. brevipes* ssp. *pseudopurpurascens* was 3.0%.

For phylogenetic analyses, *Haplospora globosa* and *Phaeosiphoniella cryophila* were used as outgroups. The data set including *Tilopteris mertensii* and using *Halosiphon tomentosus* as outgroup was also analysed, but there was difficulty in aligning sequences from *Tilopteris* and the *Haplospora/Phaeosiphoniella* group, and so the analyses excluding *Tilopteris* and *Halosiphon* are shown here. In each case, the tree topology within the Phyllariaceae was essentially the same, differing somewhat in the branching order of *S. dermatodea* and *S. polyschides* (Fig. 3). The aligned sequences were 1313 sites in total and contained 320 parsimony-informative nucleotide positions. Tree topologies were the same in all of the analyses, and bootstrap support for most branches was high. Within the family, specimens of the two subspecies of *Phyllariopsis brevipes* formed monophyletic clades, although the bootstrap value for the node uniting ssp. *brevipes* from Italy and ssp. *brevipes* from Spain showed only moderate support (72–81 %). The monophyletic clade of *Phyllariopsis purpurascens* was a sister group to the *P. brevipes* clade. In the NJ and ML trees (Fig. 3b), the monophyletic clade of *S. polyschides* formed a sister group to the *Phyllariopsis* clade and *S. dermatodea* was basal to these taxa, whereas *S. polyschides* was basal in the MP tree (Fig. 3a).

DISCUSSION

Molecular phylogeny of 'kelps' and 'pseudo-kelps'

The present molecular phylogenetic analyses, including all species of 'primitive' kelps (Chordaceae, Phyllariaceae, *Halosiphon,* Pseudochordaceae, *Akkesiphycus* Yamada & Tak. Tanaka), as well as representatives of Tilopteridales, Sporochnales and Desmarestiales, confirmed a relationship between Tilopteridales and Phyllariaceae and their close association with *Halosiphon,* Sporochnales and Desmarestiales, rather than with other members of Laminariales (A/L/L group, Chordaceae, *Akkesiphycus* and Pseudochordaceae), contrary to conventional taxonomy.

Members of the order Tilopteridales had not been considered to have a close phylogenetic relationship with 'kelps' (Laminariales) or 'pseudo-kelps' (e.g. Sporochnales and Desmarestiales) before the preliminary report by Kawai & Sasaki (2000). Laminariales, Sporochnales and Desmarestiales differ

Table 1. Origin of samples and sequence data used for molecular analyses, including their database accession numbers.

Species (taxonomic position)	Collection site	Reference (source)	DDBJ accession no. for *rbc*L and spacer gene	DDBJ accession no. for 5.8S, ITS and 26S 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	
Dictyosiphon foeniculaceus (Hudson) Greville		Siemer *et al.* (1998)	AF055397	
Desmarestiales				
Desmarestia latifrons Kützing		Kawai & Sasaki (2000), present study	AB037139, AB045239[1]	
Desmarestia sp.		Kawai & Sasaki (2000), present study	AB037141, AB045241[1]	
D. tabacoides Okamura		Kawai & Sasaki (2000), present study	AB037140, AB045240[1]	
Ectocarpales				
Ectocarpus siliculosus (Dillwyn) Lyngbye		Valentin & Zetsche (1990)	X52503	
Pilayella littoralis (Linnaeus) Kjellman		Assali *et al.* (1990)	X55372	
Laminariales				
[Akkesiphycaceae]				
Akkesiphycus lubricum Yamada & Tak. Tanaka		Kawai & Sasaki (2000)	AB036038	
[Alariaceae]				
Undaria peterseniana (Kjellman) Okamura		Kawai *et al.* (2001)	AB035794	
[Chordaceae]				
Chorda filum (Linnaeus) Stackhouse		Kawai *et al.* (2001)	AB035786	
C. rigida Kawai & Arai		Kawai *et al.* (2001)	AB035788	
[Halosiphonaceae]				
Halosiphon tomentosus (Lyngbye) Link [Pacific material]		Kawai & Sasaki (2000), present study	AB036137, AB045242[1]	
H. tomentosus [Atlantic material]		Kawai & Sasaki (2000), present study	AB036136, AB045243[1]	
H. tomentosus [Atlantic material]		Peters (1998)		Z98565
H. tomentosus [Atlantic material]		Rousseau & Reviers (1999)		AF071156
[Laminariaceae]				
Agarum clathratum Dumortier		Kawai *et al.* (2001)	AB035791	
Kjellmaniella crassifolia Miyabe		Kawai *et al.* (2001)	AB035792	
Thalassiophyllum clathrus (J.F. Gmelin) Postels & Ruprecht		Kawai *et al.* (2001)	AB035793	
[Phyllariaceae]				
Phyllariopsis brevipes (C. Agardh). E.C. Henry & South [Italy]	Strait of Messina, Italy	E.C. Henry, culture	AB045244[1]	AB045261[1]
P. brevipes [Spain 1]	Punta Carnero, Algeciras, Spain	A. Flores-Moya, field plant (silica gel)	AB045245[1]	AB045262[1]
P. brevipes [Spain 2]	Isla de Tarifa, Cadiz, Spain	A. Flores-Moya, field plant (silica gel)	AB045246[1]	AB045263[1]
P. brevipes ssp. pseudopurpurascens Pérez-Cirera, Cremades, Bárbara & López [Spain 1]	Cabo Vilano, La Coruña, Spain	I.B. Criado, field plant (silica gel)	AB045247[1]	AB045264[1]
P. brevipes ssp. pseudopurpurascens [Spain 2]	Cabo de la Buitra, La Coruña, Spain	I.B. Criado, field plant (silica gel)	AB045248[1]	AB045265[1]
P. purpurascens (C. Agardh) E.C. Henry & South [Spain 1]	Playa de las Dunas, Punta Paloma, Tarifa, Spain	I.B. Flores-Moya, field plant (silica gel)	AB045249[1]	AB045266[1]
P. purpurascens [Spain 2]	Playa del Rodeo, Marbella, Spain	A. Flores-Moya, field plant (silica gel)	AB045250[1]	AB045267[1]
P. purpurascens [Spain 3]	Isla de Tarifa, Cádiz, Spain	A. Flores-Moya, field plant (silica gel)	AB045251[1]	AB045268[1]
Saccorhiza dermatodea (Bachelot de la Pylaie) J. Agardh [NFLD]	Newfoundland, Canada	E.C. Henry, culture	AB045252[1]	AB045269[1]

Table 1. Continued.

Species (taxonomic position)	Collection site	Reference (source)	DDBJ accession no. for *rbc*L and spacer gene	DDBJ accession no. for 5.8S, ITS and 26S rDNA
S. polyschides (Lightfoot) Batters [France]	Roscoff, Brittany, France	H. Kawai, field plant (silica gel)	AB045256[1]	AB045273[1]
S. polyschides [Spain 1]	Bolonia, Cádiz, Spain	A. Flores-Moya, field plant (silica gel)	AB045253[1]	AB045270[1]
S. polyschides [Spain 2]	Isla de Tarifa, Cádiz, Spain	A. Flores-Moya, field plant (silica gel)	AB045254[1]	AB045271[1]
S. polyschides [Man]	Port Erin, Isle of Man	H. Kawai, field plant (silica gel)	AB045255[1]	AB045272[1]
[Pseudochordaceae]				
Pseudochorda gracilis Kawai & Nabata		Kawai *et al.* (2001)	AB035790, AB041867	
P. nagaii (Tokida) Inagaki		Kawai *et al.* (2001)	AB035789, AB041875	
Scytosiphonales				
Chnoospora implexa J. Agardh		Kogame *et al.* (1999)	AB022231	
Scytosiphon lomentaria (Lyngbye) Link		Kogame *et al.* (1999)	AB022238	
Sporochnales				
Carpomitra costata (Stackhouse) Batters	Hiroshima Pref., Japan	H. Kawai, culture	AB045257[1]	
Sporochnus scoparius Harvey		Kawai & Sasaki (2000), present study	AB037142, AB045394[1]	
Tilopteridales				
Haplospora globosa Kjellman	Helgoland, Germany	Kawai & Sasaki (2000), present study	AB037138, AB045258[1]	AB045274[1]
Phaeosiphoniella cryophila R.G. Hooper, E.C. Henry & Kuhlenkamp	Newfoundland, Canada	E.C. Henry, culture	AB045259[1]	AB045275[1]
Tilopteris mertensii (Turner *in* Smith) Kützing	Helgoland, Germany	D.G. Müller culture	AB045260[1]	
PHAEOTHAMNIOPHYCEAE				
Phaeothamnion confervicola Lagerheim		Bailey *et al.* (1998)	AF064746	
XANTHOPHYCEAE				
Botrydiopsis intercedens Vischer & Pascher		Daugbjerg & Andersen (1997)	AF015587	
Tribonema intermixum Pascher		Daugbjerg & Andersen (1997)	AF015588	

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

Table 2. List of primers used for PCR. Annealing positions correspond to the sequences of *Scytosiphon lomentaria* (18S, 5.8S and 25S of rDNA, accession number D16558; Kawai *et al.* 1995) and those of *Ectocarpus siliculosus* (*rbc*L and *rbc*S, accession number X52503; Valentin & Zetsche 1990).

Code	Direction	Sequence (5′ to 3′)	Annealing position
18F1	Forward	AAGGTGAAGTCGTAACAAGG	18S (1768–1787)
5.8F-1	Forward	ACGCAGCGAAATGCGATACG	5.8S (47–66)
25F1[1]	Forward	CCGCTGAATTTAAGCATAT	26S (27–45)
rbc-F0	Forward	ATCGAACTCGAATAAAAAGTGA	*rbc*L (20–41)
rbc-F1	Forward	CGTTACGAATCWGGTG	*rbc*L (43–58)
rbc-F2	Forward	AGGTTCWCTWGCTAA	*rbc*L (342–356)
PRB-F2[2]	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)
26R-1	Reverse	GTTAGTTTCTTTTCCTCCGC	26S (69–50)
25R1[1]	Reverse	CTTGGTCCGTGTTTCAAGAC	26S (616–635)
rbc-R1	Reverse	TTAGCWAGWGAACCT	*rbc*L (356–342)
rbc-R2	Reverse	CGCATGAATGGTTGTG	*rbc*L (650–635)
PRB-R2[2]	Reverse	CCTTTAACCATTAAGGGATC	*rbc*L (1040–1021)
PRB-R3[2]	Reverse	GTAATATCTTTCCATAAATCTAA	*rbc*L (1406–1384)
DP*rbc*L7[3]	Reverse	AAASHDCCTTGTGTWAGTYTC	*rbc*S (23–3)
RSPR[2]	Reverse	AATAAAGGAAGACCCCATAATTCCCA	*rbc*S (167–142)

[1] Rousseau & Reviers (1997).
[2] Kogame *et al.* (1999).
[3] Daugbjerg & Andersen (1997).

from Tilopteridales in most basic characters: they have an obviously heteromorphic life history, alternating between elaborate sporophytes and minute oogamous gametophytes, whereas the Tilopteridales have relatively simple, filamentous or terete thalli and a virtually isomorphic or direct type of life history, which lacks sexual reproduction (Bold & Wynne 1985; Kuhlenkamp & Müller 1985; Hooper *et al.* 1988). However, considerable (and presumably rapidly evolved) changes in life history patterns and sexual reproductive structures have been reported in some brown algal taxa. For example, there is a striking reduction of the gametophytes in some species of *Syringoderma* Levring (Henry 1984; Kawai & Yamada 1990), monoecious and dioecious gametophytes are both found in closely related species of the Laminariales and Desmarestiales (Ramirez *et al.* 1986; Henry 1987; Henry & South 1987; Peters *et al.* 1997), and there has been evolution from anisogamy to oogamy in primitive Laminariales (Kawai 1986; Kawai & Sasaki 2000) as well as in the sphacelarialean genus *Halopteris* Kützing (Lindauer *et al.* 1961; Womersley 1987; Kawai & Prud'homme van Reine 1998). Significantly, despite the relatively simple morphology of the erect thalli, the chloroplast morphology of the Tilopteridales – numerous chloroplasts lacking pyrenoids – differs strikingly from that of the Ectocarpales *sensu lato* (including the Ectocarpales, Dictyosiphonales, Chordariales and Scytosiphonales), in which there are prominent pyrenoids irrespective of the number of chloroplasts (Kawai 1992). The Tilopteridalean chloroplast morphology is widely distributed among advanced brown algal orders (Sphacelariales, Dictyotales, Cutleriales, Laminariales, Desmarestiales, Sporochnales, Fucales, etc.). Moreover, the intercalary growth mode appears to be similar in the Tilopteridales, Sporochnales and Desmarestiales, viz. trichothallic in Tilopteridales and Desmarestiales, and terminated with phaeophycean hairs in Sporochnales (Fritsch 1945).

It is also noteworthy that the Halosiphonaceae, Tilopteridales and Phyllariaceae are restricted to relatively cold-water regions of the North Atlantic and Mediterranean (Henry & South 1987; Lüning 1990), except for the occurrence of *H. tomentosus* at St. Lawrence Island, the Bering Sea (Kawai *et al.* 2001). This may indicate a recent spread of *Halosiphon* from the Atlantic into the Pacific, since it is apparently still restricted to this northernmost part of the Pacific. It is likely that the Tilopteridales and Phyllariaceae share a common ancestor with Halosiphonaceae and that they evolved in the Atlantic.

The life histories of the Tilopteridales appear to be asexual, since meiosis and sexual fusion are lacking. However, the presence of nonfunctional sexual structures (including oogonia, antheridia and sperm that may be released but do not swim) implies that the present life history pattern has originated from a sexual life history by reductive evolution (Kuhlenkamp & Müller 1985; Kuhlenkamp *et al.* 1993). Most *Haplospora* populations show an alternation between presumptive gametophyte and sporophyte phases, whereas *Tilopteris* displays only the gametophyte phase. *Phaeosiphoniella* is reported to propagate exclusively by a special type of vegetative fragmentation in the field; however, it occasionally forms nonfunctional structures that appear to be equivalent to antheridia, oogonia or plurilocular sporangia. The fact that these species still form such nonfunctional structures may indicate the recent loss of sexual reproduction within the group. It is also noteworthy that the oogonium-like and antheridium-like structures both tend to be formed on the same individuals, implying a monoecious ancestor. Monoecy is commonly seen in the clade containing *Halosiphon,* Phyllariaceae, Sporochnales and Desmarestiales, whereas the A/L/L group, Chordaceae, Pseudochordaceae and *Akkesiphycus* are dioecious throughout. These features favour the notion that Tilopteridales, *Halosiphon* and Phyllariaceae evolved from a common ancestor and experienced reduction of sexual reproduction, possibly as an adaptation to extremely cold habitats (Kuhlenkamp & Hooper 1995; Kuhlenkamp 1996).

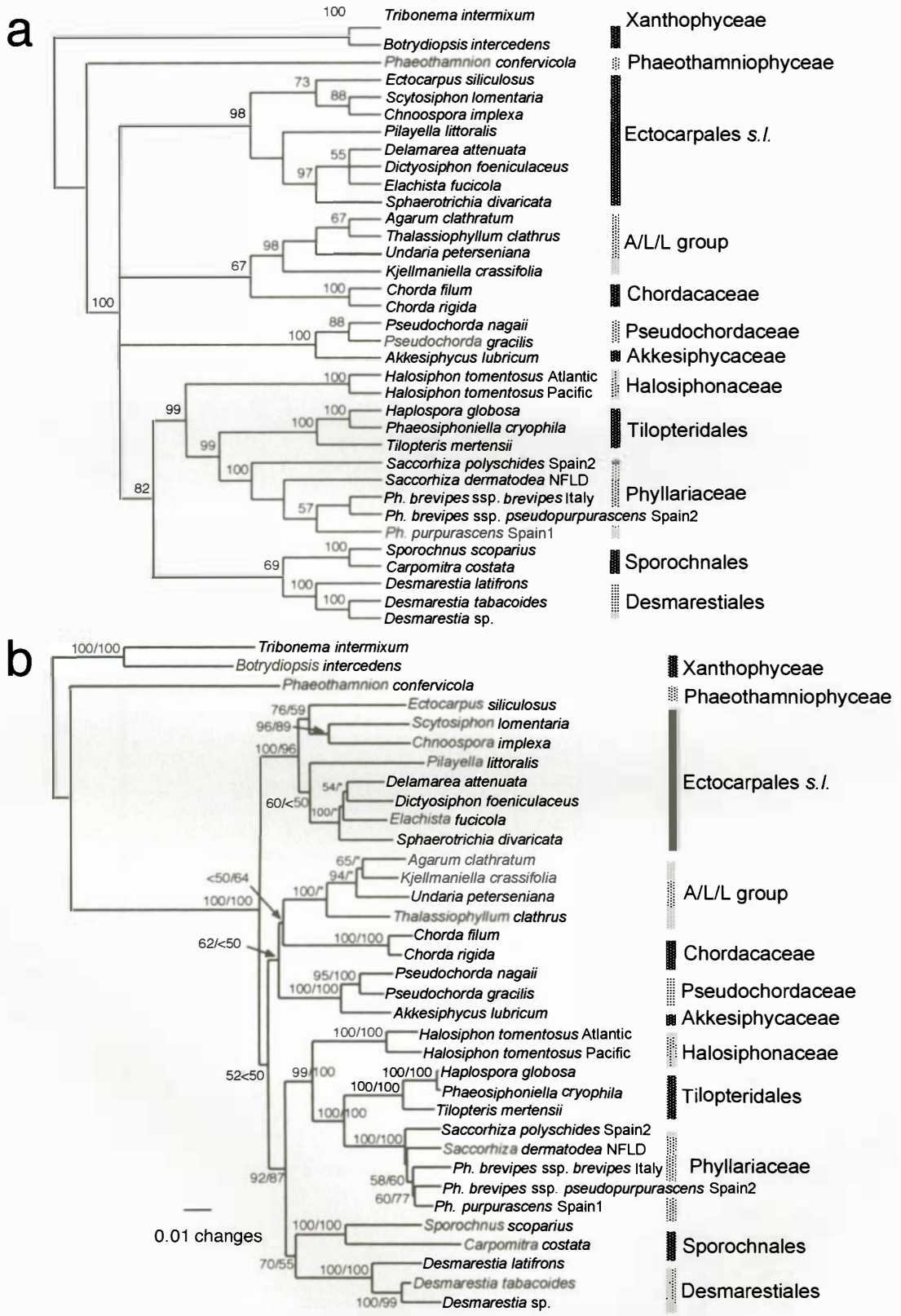

Fig. 1. Molecular phylogenetic trees based on *rbc*L sequences. a. MP analysis, strict consensus tree of four most-parsimonious trees. In the MP analysis, four equally parsimonious trees of 1703 steps were obtained with a consistency index (CI) of 0.4846 and a retention index (RI) of 0.6587. b. NJ and ML analyses. In the ML tree, −Ln likelihood was 11511.30887. Bootstrap values indicate % on the basis of 1000 (MP and NJ) and 500 (ML) replicates (in b, NJ/ML). '*' in the ML bootstrap value position indicates that the branching orders at the nodes were not consistent with those of the NJ tree, and hence the bootstrap values are not shown.

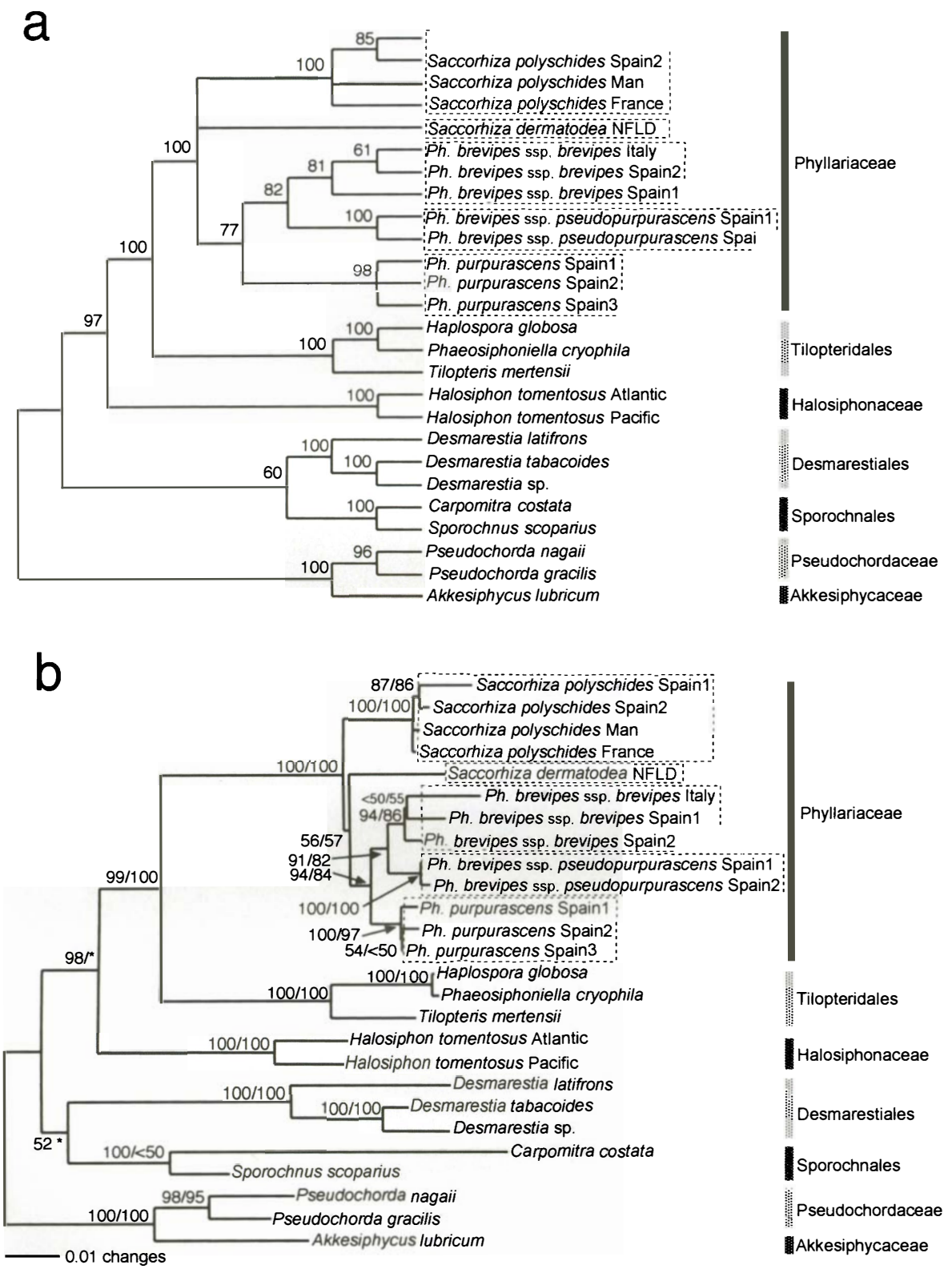

Fig. 2. Molecular phylogeny trees based on *rbc*L and spacer region sequences. a. MP analysis, strict consensus tree of four most-parsimonious trees. In the MP analysis, four most parsimonious trees of 1027 steps were obtained with a CI of 0.6987 and an RI of 0.8279. b. NJ and ML analyses. In the ML tree, −Ln likelihood was 8085.54711. Bootstrap values indicate % on the basis of 1039 (MP and NJ) and 500 (ML) replicates (in b, NJ/ML). '*' in the ML bootstrap value position indicates that the branching orders at the nodes were not consistent with those of the NJ tree, and hence the bootstrap values are not shown.

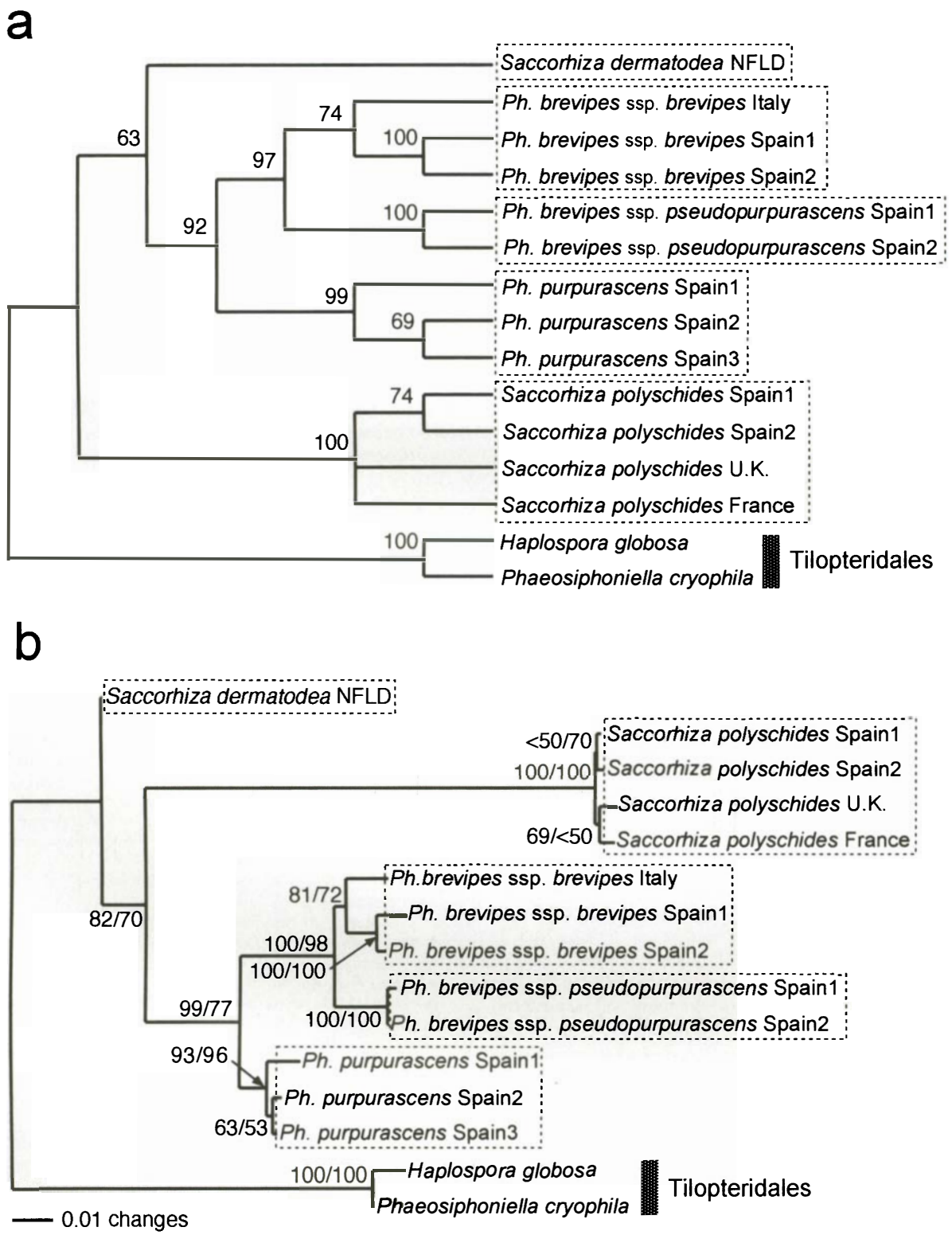

Fig.3. Molecular phylogeny trees based on 5.8S, ITS2 and 26S rDNA region sequences. a. MP analysis, strict consensus tree of two most parsimonious trees. In the MP analysis, two most parsimonious trees of 518 steps were obtained with a CI of 0.9208 and an RI of 0.9506. b. NJ and ML analyses. In the ML tree, −Ln likelihood was 4389.83989. Bootstrap values indicate % on the basis of 1000 (MP and NJ) and 500 (ML) replicates (in b, bootstrap values: NJ/ML).

Systematics of *Phyllariopsis* and *Saccorhiza*

Perez-Cirera *et al.* (1991) described a new subspecies of *Phyllariopsis brevipes* (spp. *pseudopurpurascens*) from the north-west of the Iberian Peninsula, on the basis of morphological and ecological characteristics. Flores-Moya *et al.* (1993) studied the geographical distribution of *P. purpurascens* and the two subspecies of *P. brevipes,* and showed that *P. purpurascens* and *P. brevipes* ssp. *brevipes* have wide distributional ranges along the Iberian Peninsula, whereas *P. brevipes* ssp. *pseudopurpurascens* is restricted to the north-west coast. In our molecular analyses, all three taxa are monophyletic in both the Rubisco and rDNA trees, in agreement with current taxonomy (Figs 2, 3). However, in view of the relatively large genetic divergence among the specimens of ssp. *brevipes,* it will be necessary to examine more specimens from a wider area in order to determine whether the two subspecies should be distinguished at the species level or not.

In contrast to the close similarities of the three taxa of *Phyllariopsis* (Figs 2, 3), the two species of *Saccorhiza* (*S. dermatodea* and *S. polyschides*) were shown to be genetically divergent. Since there are also significant morphological differences between the two species (the presence of bulbous holdfast only in *S. polyschides*; monoecious vs dioecious gametophytes), it may be necessary to re-examine the generic assignment of *S. dermatodea* (Norton & Burrows 1969; Norton 1972; Henry & South 1987).

Ordinal assignment of 'kelps' and 'pseudo-kelps'

Kawai & Sasaki (2000) suggested inclusion of Akkesiphycaceae in the Laminariales and emended the definition of the order to include anisogamy. The present study indicates the presence of a large monophyletic group that is sister group to Laminariales and includes the Sporochnales, Desmarestiales, Tilopteridales, Halosiphonaceae and Phyllariaceae. There are several options for taxonomic treatment of these entities: (1) inclusion of all these taxa in Laminariales; (2) recognition of two orders – Laminariales (including the A/L/L group, Chordaceae, Pseudochordaceae and Akkesiphycaceae) and Tilopteridales (including the Tilopteridales *sensu stricto,* Halosiphonaceae, Phyllariaceae, Desmarestiales and Sporochnales); (3) recognition of three orders – Laminariales (including the A/L/L group, Chordaceae, Pseudochordaceae and Akkesiphycaceae), Tilopteridales (including Tilopteridales *sensu stricto,* Halosiphonaceae and Phyllariaceae) and Desmarestiales (including Desmarestiales and Sporochnales); or (4) a four-order system, similar to (3) but with retention of the Sporochnales as distinct from the Desmarestiales. The names Laminariales Kylin (1917) and Tilopteridales Kylin (1917) would have nomenclatural priority over Desmarestiales Setchell & Gardner (1925) and Sporochnales Sauvageau (1926), should the latter two be merged with the former orders. Among the possibilities listed above, we currently favour option (4), for the following reasons: the bootstrap values are not high for the nodes separating the A/L/L group, Chordaceae, the Pseudochordaceae/Akkesiphycaceae group and the large clade consisting of Halosiphonaceae, Phyllariaceae, Tilopteridales, Sporochnales and Desmarestiales. However, the monophyly of the Halosiphonaceae, Tilopteridales and Phyllariaceae appears to be proved, and their genetic divergence, deduced from Rubisco and rDNA sequence data, is comparable to that within the Laminariales, including the A/L/L group, Chordaceae and the Pseudochordaceae/Akkesiphycaceae group. Although a close phylogenetic relationship between the Sporochnales and Desmarestiales has been suggested based on morphological and physiological characters – sometimes they have even been combined, as Desmarestiales (Parke & Dixon 1976) – very few members have been subjected to molecular studies, and the bootstrap values for nodes connecting the two groups are not high (52–70%). It remains to be seen whether the substantial taxonomic rearrangement suggested here, based on molecular data, can be substantiated and confirmed with additional evidence from other disciplines.

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