Molecular phylogeny of crustose brown algae (Ralfsiales, Phaeophyceae) inferred from *rbc*L sequences resulting in the proposal for Neoralfsiaceae fam. nov.

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The order Ralfsiales was established to accommodate the brown algal taxa having a crustose thallus, an isomorphic life history, discoid early development of the thallus and containing a single, plate-shaped chloroplast without pyrenoids in each cell. However, the validity of the order has been questioned by many researchers because several exceptions to these criteria have been found within the order. Molecular phylogenetic analysis of the taxa assigned to the order, using *rbc*L DNA sequences, reveals that Ralfsiales is not a monophyletic group but is separated into two major groups, excluding Lithodermataceae, which were not included in the present analysis: clade I, comprising the members of Ralfsiaceae, Mesosporaceae, *Analipus japonicus* and *Heteroralfsia saxicola*; and clade II, consisting of *Diplura* species, sister to the Ishigeales clade. On the basis of these results, we propose emendment of the Ralfsiales to contain only species having (1) discoidal early development of the thallus; (2) intercalary plurilocular gametangia with terminal cells and terminal unilocular zoidangia; and (3) a crustose phase in the life history. Furthermore, we propose the establishment of the new family Neoralfsiaceae to accommodate the new genus *Neoralfsia*, on the basis of *Ralfsia expansa*.

KEY WORDS: Moleuclar phylogeny, *Neoralfsia*, Neoralfsiaceae, Ralfsiaceae, Ralfsiales, *rbc*L, Taxonomy

INTRODUCTION

The classification system of the algal class Phaeophyceae at the ordinal rank has been revised several times. Kylin (1933) classified the Phaeophyceae primarily on the basis of the life history patterns (isomorphic, heteromorphic, or lack of any alternation of generations) and the basic construction of the thalli (haplostichous or polystichous), and thereby he recognized 11 orders: Chordariales, Cutleriales, Desmarestiales, Dictyosiphonales, Dictyotales, Ectocarpales, Fucales, Laminariales, Sphacelariales, Sporochnales and Tilopteridales. Papenfuss (1951) basically followed Kylin's concept of classification and regarded Ectocarpales as the most primitive order in the class because of their simple thallus construction and the isomorphic life history. Most subsequent researchers generally followed Kylin's classification system (Wynne & Loiseaux 1976; van den Hoek & Jahns 1978; Clayton 1990). In 1972, Nakamura established a new order, Ralfsiales Nakamura to accommodate the crustose brown algal taxa, and included the families Lithodermataceae Hauck, Nemodermataceae Feldmann, and Ralfsiaceae Farlow. The main distinguishing features of the new order were (1) a single, parietal, plate-shaped chloroplast without pyrenoids in each cell; (2) isomorphic life history; and (3) discoid early development of the thallus. The distinction among the three families was mainly based on their reproductive structures: Lithoderma-

taceae have terminal unilocular and plurilocular zoidangia, Nemodermataceae have intercalary unilocular and lateral plurilocular zoidangia, and Ralfsiaceae have lateral unilocular and intercalary plurilocular zoidangia. Nakamura (1972) also implied the possibility of merging the three families into a single family, Ralfsiaceae.

However, the validity of the order Ralfsiales has been challenged by various authors. First of all, Nakamura (1972) provided no Latin description/diagnosis, so the name did not fulfill the requirement of the International Code of Botanical Nomenclature, and the order was not regarded as a valid publication. Tanaka & Chihara (1980a) and Silva & de Reviers (2000) basically accepted the order Ralfsiales and added a new family, Mesosporaceae, including *Mesospora, Hapalospongidion* and *Basispora* (Tanaka & Chihara 1982), but they emended the distinguishing features of the order to exclude the number of chloroplasts. In contrast, Nelson (1982) did not accept Ralfsiales and proposed including all crustose brown algae in a single family, Ralfsiaceae within Ectocarpales. Kawai (1989) showed that *Gobia saxicola* Okamura et Yamada, originally described as a chordarialean species, had a crustose gametophytic stage in the life history, and transferred the species to Ralfsiales as *Heteroralfsia saxicola* (Okamura et Yamada) Kawai. Because of this inclusion, Ralfsiales came to include both isomorphic and heteromorphic life history patterns.

In contrast, the first molecular study using 18S rDNA by Tan and Druehl (1994) showed that *Ralfsia fungiformis* (Gunnerus) Setchell et Gardner and *Analipus japonicus*

(Harvey) Wynne were phylogenetically close, but rather distant from Ectocarpales. Since then, several different DNA sequences have been examined to elucidate the molecular phylogenetics of Phaeophyceae, such as nuclear 18S and 26S rDNA, and Rubisco large subunit (rbc*L*) (Boo *et al.* 1999; Rousseau & de Reviers 1999a, b; Siemer *et al.* 1998; Draisma *et al*. 2001; Sasaki *et al.* 2001; Cho *et al.* 2004; Kawai & Sasaki 2004; Kawai *et al.* 2005). Among the DNA sequences, *rbc*L was considered to have the most suitable resolution for discerning the ordinal and familial phylogenetic relations within Phaeophyceae (Draisma *et al.* 2001; Sasaki *et al.* 2001; Kawai & Sasaki 2004).

In the most thorough work on the ordinal phylogeny of the Phaeophyceae, Draisma *et al*. (2001) showed that 11 orders were recognizable on the basis of the *rbc*L sequence data: Ascoseirales, Cutleriales, Desmarestiales, Dictyotales, Ectocarpales, Fucales, Laminariales, Scytothamnales, Sphacelariales, Syringodermatales and Tilopteridales. Unfortunately, no representatives of the orders Ralfsiales and Sporochnales were included in the analyses. Cho *et al.* (2004) added a new order, Ishigeales, on the basis of three plastid gene sequences *rbc*L, *psa*A and *psb*A. Later, on the basis of an *rbc*L molecular phylogenetic study, Kawai *et al.* (2007) reinstated the use of the order Discosporangiales to include Choristocarpaceae and Discosporangiaceae, and showed that the order branched first in the brown algal lineage. Hence, the objective of this study was to clarify the molecular phylogeny of the crustose brown algae using *rbc*L DNA sequence data and to reexamine the validity of the order Ralfsiales.

MATERIAL AND METHODS

Source of samples

For the present study, 18 new *rbc*L sequences of *Ralfsia, Mesospora, Diplura* and *Endoplura* species were determined for field-collected specimens and culture strains (Kobe University Macroalgal Culture Collection) listed in Table 1, collected from various localities. Their DNA database accession numbers are shown in Table 1 in boldface. The *rbc*L sequence data downloaded from the DDBJ (DNA Data Bank of Japan)/GenBank nucleotide sequence database were also used (*n* = 32) (Table 1). Several specimens of the taxa belonging to the family Lithodermataceae were also examined, but they were not included in the present study because of difficulties in their identifications.

Morphological observations

Light microscopical examinations were done on radial longitudinal sections, which were prepared by hand-sectioning using razor blades or by squash preparations. The specimens were stained with aniline blue and mounted in Karo corn syrup on glass slides.

The representative morphological features examined for identifying the species are as follows: thickness of the thallus; symmetry/asymmetry of the arrangement of the vegetative filaments; position of unilocular and plurilocular sporangia; presence/absence and number of terminal sterile cell(s); delimitation of cortex and medulla; number of chloroplast(s); and presence/absence of pyrenoids. Specimens were identified on the basis of the above-mentioned criteria used by Agardh (1848), Weber van Bosse (1913), Hollenberg (1969), Tanaka & Chihara (1980a, b, c; 1981a, b, c; 1982), Kawai (1989) and León-Alvarez & Norris (2005).

Molecular phylogenetic study

For DNA extraction, approximately 30–40 mg of each dried specimen was ground into powder in liquid nitrogen. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

Polymerase chain reaction (PCR) amplification of the *rbc*L gene sequences was carried out using a TaKaRa Dice (Takara, Shiga, Japan) or Bio-Rad I-Cycler (Hercules, CA, USA) with TaKaRa ExTaq (Takara). The total volume for the PCR amplification was 25 µl and consisted of 3.0 µl of 10× ExTaq buffer, 0.25 mM of dNTP mixture, 0.25 µM of each primer, 0.45 units of TaKaRa ExTaq, and 2.0 µl of DNA solution containing 1.0–1.5 µg of DNA. The primers used for the amplification were rbc-F0, rbc-F1, rbc-F3, rbc-F4, and rbc-R2 from Kawai & Sasaki (2004); PRB-F2, PRB-R2, and PRB-R3 from Kogame *et al.* (1999); and Ral-R952 (5′CATACGCATCCATTTACA 3′) was designed for this study. The parameters of PCR amplification were 3 min at 94°C, followed by three cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min; followed by another three cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were sequenced using a CEQ 8000 automated sequencer (Beckman Coulter, Fullerton, CA, USA).

Sequences from this study were preliminarily aligned using the CLUSTAL W program (Thompson *et al.* 1994) and subsequently manually aligned. The aligned sequences were subjected to maximum-parsimony (MP) and maximum-likelihood (ML) analyses using PAUP* 4.0b10 (Swofford 2002). The MP tree was constructed using the heuristic search option, 100 random sequences additions, tree bisection reconnection (TBR) branch swapping, and unordered and unweighted characters. Bootstrap percentage (BP) was computed with 1000 replications.

To find the least-rejected model of sequences by an Akaike Information Criterion (AIC), the program ModelTest v.3.7 (Posada & Crandall 1998) was used. The AIC indicated that the least-rejected model for the present study data set was general-time-reversible of sequence (GTR) + proportion of invariable sites (I) + variable sites (G) (a = 1.7402, b = 4.89320, c = 2.1206 d = 2.2303, e =10.5608, f = 1.0000; gamma distribution shape parameter = 0.8235; proportion of invariable sites = 0.4048). The ML was performed using TBR swapping with 10 random sequence additions in PAUP using the estimated parameters from the ModelTest. BP was computed with 100 replications.

Table 1. List of specimens that were used for the molecular phylogeny analysis. DDBJ accession number in **boldface** shows new sequences published in the present study.

Name of taxa	Origin (published year/collector/ specimen no.)	DDBJ accession number for *rbc*L
Phaeophyceae		
Discosporangiales		
Choristocarpus tenellus (Kützing) Zanardini	Draisma *et al.* (2001)	AJ287861
Choristocarpus tenellus (Kützing) Zanardini	Draisma *et al.* (2001)	AJ287862
Desmarestiales		
Desmarestia tabacoides Okamura	Kawai & Sasaki (2000)	AB037140
Himantothallus grandifolius (A. Gepp et E.S. Gepp) Zinova	Draisma *et al.* (2001)	AJ287850
Dictyotales		
Dictyota dichotoma (Hudson) Lamouroux	Draisma *et al.* (2001)	AJ287852
Padina australis Hauck	Kawai *et al.* (2007)	AB252656
Zonaria diesingiana J. Agardh	Lee *et al.* (published only in database)	AY422682
Ectocarpales *s.l.*		
Dictyosiphon foeniculaceus (Hudson) Greville	Siemer *et al.* (1998)	AF055396
Ectocarpus siliculosus (Dillwyn) Lyngbye	Valentin & Zetsche (1990)	X52503
Scytosiphon lomentaria (Lyngbye) Link	Kogame *et al.* (1999)	AB022238
Fucales		
Ascophyllum nodosum (Linnaeus) Le Jolis	Draisma *et al.* (2001)	AJ287853
Sargassum muticum (Yendo) Fensholt	Draisma *et al.* (2001)	AJ287854
Ishigeales		
Ishige okamurae Yendo	Kawai *et al.* (2005)	AB117951
I. sinicola (Setchell et Gardner) Chihara	Kawai *et al.* (2005)	AB117952
Laminariales		
Chorda filum (Linnaeus) Stackhouse	Kawai *et al.* (2001)	AB035786
Laminaria digitata (Hudson) Lamouroux	Cho *et al.* (2004)	AY372984
Ralfsiales		
Mesosporaceae		
Mesospora sp. C	Tg. Gemoh, Johor, Malaysia (P-E Lim) (KU-d3634)	**AB250065**
Mesospora sp. D	Nakura, Ishigaki Isl., Okinawa Pref., Japan (P.-E. Lim) (KU-d3635)	**AB250066**
Mesospora sp. G	Shimoda, Shizuoka Pref., Japan (M. Sakaguchi) (KU-d2225)	**AB250069**
Ralfsiaceae		
Analipus japonicus (Harvey) Wynne	Oshoro, Hokkaido Pref., Japan (H. Kawai) (KU-883)	**AB264042**
Heteroralfsia saxicola (Okamura et Yamada) Kawai	Ohma, Aomori Pref., Japan (H. Kawai) (KU-882)	**AB250070**
Endoplura aurea Hollenberg	Inubouzaki, Chiba Pref., Japan (K. Kogame) (KU-d2273)	**AB264039**
Ralfsia fungiformis (Gunnerus) Setchell et Gardner	Akkeshi, Hokkaido Pref., Japan (K. Kogame) (KU-d2206)	**AB250071**
Ralfsia verrucosa (Areschoug) J. Agardh	Roscoff, Brittany, France (H. Kawai) (KU-d2305)	**AB250072**
Ralfsia sp. A	Akou, Hyogo Pref., Japan (S. Uwai) (KU-d2259)	**AB250073**
Ralfsia sp. B	Bergen, Norway (H. Kawai) (KU-d2315)	**AB250074**
Ralfsia sp. C	Katiki Beach, New Zealand (H. Kawai) (KU-d2201)	**AB250075**
Ralfsia sp. D (*Ralfsia expansa* related species)	Durban, South Africa (H. Kawai) (KU-2317)	**AB250076**
Ralfsia expansa (J. Agardh) J. Agardh	Hamasaki, Ishigaki Isl., Okinawa Pref., Japan (P.-E. Lim) (KU-d2132)	**AB250077**
Ralfsia expansa (J. Agardh) J. Agardh	Desaru, Johor, Malaysia (P.-E. Lim) (KU-d2317)	**AB250078**
Ralfsia expansa (J. Agardh) J. Agardh	Igumi, Hyogo Pref., Japan (H.Uchida) (KU-d2243)	**AB250079**
Diplura simplex Tanaka et Chihara	Maruyama, Hyogo Pref., Japan (P.-E. Lim) (KU-d2582)	**AB250084**
Diplura sp. B	Maruyama, Hyogo Pref., Japan (P.-E. Lim) (KU-d2574)	**AB250086**
Diplura sp. C	Teguma, Nagasaki Pref., Japan (A. Tanaka) (KU-d2247)	**AB250087**
Scytothamnales		
Scytothamnus australis (J. Agardh) Hooker et Harvey	Peters & Ramrez (2001)	AJ295833
Splachnidium rugosum (Linnaeus) Greville	Peters & Ramrez (2001)	AJ295834
Sphacelariales		
Sphacelaria cirrosa (Roth) C. Agardh	Draisma *et al.* (2001)	AJ287865

Table 1. Continued.

Name of taxa	Origin (published year/collector/ specimen no.)	DDBJ accession number for *rbc*L
Sporochnales		
Carpomitra costata (Stackhouse) Batters	Sasaki *et al.* (2001)	AB045257
Sporochnus scoparius Harvey	Kawai & Sasaki (2000)	AB250086
Syringodermatales		
Microzonia velutina (Harvey) J. Agardh	Burrowes *et al.* (2003)	AY157697
Syringoderma phinneyi Henry et Müller	Draisma *et al.* (2001)	AJ287868
Tilopteridales		
Halosiphon tomentosus (Lyngbye) Jaasund	Kawai and Sasaki (2000)	AB037136
Phyllariopsis brevipes (C. Agardh) Henry et South	Sasaki *et al.* (2001)	AB045244
Tilopteris mertensii (Turner) Kützing	Sasaki *et al.* (2001)	AB045260
Incertae sedis		
Asterocladon lobatum Müller, Parodi et Peters	Peters & Ramrez (2001)	AJ295824
Asterocladon rhodochortonoides (Børgesen) Uwai, Nagasoto, Motomura et Kogame	Peters & Ramrez (2001)	AJ295825
Phaeostrophiaceae		
Phaeostrophion irregulare Setchell et Gardner	Kawai *et al.* (2005)	AB117948
Onslowiaceae		
Onslowia endophytica Searles	Draisma *et al.* (2001)	AJ287864
Phaeothamniophyceae		
Phaeothamnion confervicola G. Lagerheim	Bailey *et al.* (1998)	AF064746
Schizocladiophyceae		
Schizocladia ischiensis Henry, Okuda et Kawai	Kawai *et al.* (2003)	AB085615

Bayesian analysis was performed using MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001), using GTR model with gamma distribution. The program was set to start with a random starting tree, analysis using four chains of Markov chain Monte Carlo iterations simultaneously for 2×10^6 generations and sampling the data every 100 generations. The likelihood scores stabilized after 200,000 generations. However, for our analyses a ''burn-in'' of 400,000 generations was used. For bootstrapping comparison purposes, we only considered nodes with Bayesian posterior probabilities (PP) greater than 95% as a validly supported clade.

RESULTS

Morphological observations

The representative morphological features of the specimens used for the present study are summarized in Table 2.

Molecular phylogenetic analyses

The aligned *rbc*L sequences consisted of 1409 sites. Both the ML and Bayesian analyses gave similar topology tree (Fig. 1). MP analysis resulted in eight equally most parsimonious trees, of which only the strict consensus tree is shown; the strict consensus MP tree was slightly different from the formal two but gave very similar results in the overall topologies and branching order of the major clades (Fig. 1). *Choristocarpus* branched first in the Phaeophyceae, followed by the clades of Ishigeales and Dictyotales. The crustose brown algal taxa were split into two major clades, I and II. Clade I formed a highly supported clade (BP for ML = 100%, BP for MP = 98%, ≥0.95 PP) and included the genera *Ralfsia, Heteroralfsia, Analipus, Endoplura* and *Mesospora* (Mesoporaceae). Clade I was divided into four

subclades, most of which were moderately to highly supported by the bootstrap values and Bayesian PP: clade Ia: *Ralfsia* spp., *Analipus japonicus, Heteroralfsia saxicola* and *Endoplura aurea* Hollenberg, formed highly supported to moderately supported clade (BP for ML = 90%, BP for MP = 76%, ≥0.95 PP); Ib: *Ralfsia expansa* (J. Agardh) J. Agardh and its related taxa were highly supported (BP for ML = 100%, BP for MP = 98%, ≥0.95 PP); Ic: *Mesospora* species with bootstrap values of 51% (MP), but less than 50% BP for ML and less than 0.95 for PP; and Id: an undescribed *Ralfsia*-like species formed a highly supported clade (BP for ML = 100%, BP for MP = 100%, ≥0.95 PP). Clade I was a sister to the clade composed of Tilopteriales and Fucales, and was moderately supported (BP for ML = 95%, BP for MP = 72%, ≥0.95 PP).

Clade II, supported by full bootstrap values in ML and MP and ≥0.95 PP, was composed of *Diplura* species and was sister to Ishigeales, and was one of the most basal taxa in the brown algae excluding Discosporangiales.

DISCUSSION

Our molecular phylogenetic analyses on the basis of *rbc*L sequences (Fig. 1) showed that the crustose brown algae are not monophyletic, but split into two major groups in the ML, Bayesian and MP analyses. Furthermore, our preliminary analyses suggested that the taxa belonging to Lithodermataceae also formed an independent clade distant from the two clades (data not shown). Comparison of the major taxonomic characters of each clade of the crustose brown algae is presented in Table 3.

Among the major clades, clade Ia consists of *R. fungiformis* (generic type), *Ralfsia verrucosa* (Areschoug) J. Agardh, *H. saxicola, A. japonicus,* and *Endoplura aurea*, and corresponds to the family Ralfsiaceae. Although the

Table 2. Comparisons of morphological features of crustose brown algae in the present study.

Taxa	Thickness of thalli (μm)	Symmetry/asymmetry of vegetative filaments	Distinct delineation of cortex and medullar layer	Paraphyses	Plurilocular zoidangia	Unilocular zoidangia	No. of plastids
Clade Ia							
Ralfsia fungiformis	265–575	bilateral; tightly adhered; curving upwardly and downwardly from a central layer	yes	present	—[1]	–	single
Ralfsia verrucosa	143–215	unilateral; tightly adhered; upwardly curved toward the surface, which arises from basal part	no	present	subterminal (position) with one single sterile terminal cell; partly biseriate	–	single
Endoplura aurea (culture strains)	390–700	unilateral; tightly adhered; curving upward from hypothallial layer	no	present	subterminal (position) with sterile terminal of three to four cells	–	several
Heteroralfsia saxicola	80–125	unilateral	no	present	–	–	single
Ralfsia sp. A	187.5–305	straight; tightly adhered	no	present	–	–	single
Analipus japonicus	20–25 cm (erect axes)	unilateral; tightly adhered	no	present	–	–	single (multiple in inner cells)
Clade Ib							
Ralfsia sp. D	457–700	bilateral; tightly adhered; curving upwardly and downwardly from a central layer	yes	present	–	–	single
Ralfsia expansa (AB250077)	138–390	bilateral; tightly adhered; curving upwardly and downwardly from a central layer	yes	present	subterminal (position) with one sterile cell	terminal (origin) with three-celled pedicels	single
Ralfsia expansa (AB250079)	110–280	bilateral; tightly adhered; curving upwardly and downwardly from a central layer	yes	present	subterminal (position) with one sterile cell	terminal (origin) with three-celled pedicels	single
Ralfsia expansa (AB250078)	141–160	bilateral; tightly adhered; curving upwardly and downwardly from a central layer	yes	present	subterminal (position) with one sterile cell	terminal (origin) with six-celled pedicels	single
Clade Ic							
Mesospora sp. C	127–187	straight and free living	no	absent	subterminal (position) with three sterile cells	terminal (position) with three-celled pedicels	single
Mesospora sp. D	180–200	straight and free living	no	absent	subterminal with two sterile cells	terminal (position) with five-celled pedicel	single
Mesospora sp. G	230–500	straight and free living	no	absent	subterminal with three sterile cells	terminal (position) with six-celled pedicel	single
Clade Id							
Ralfsia sp. B	193–256	unilateral; tightly adhered; upwardly curved toward the surface, arising from basal part	no	absent	–	–	single

Table 2. Continued.

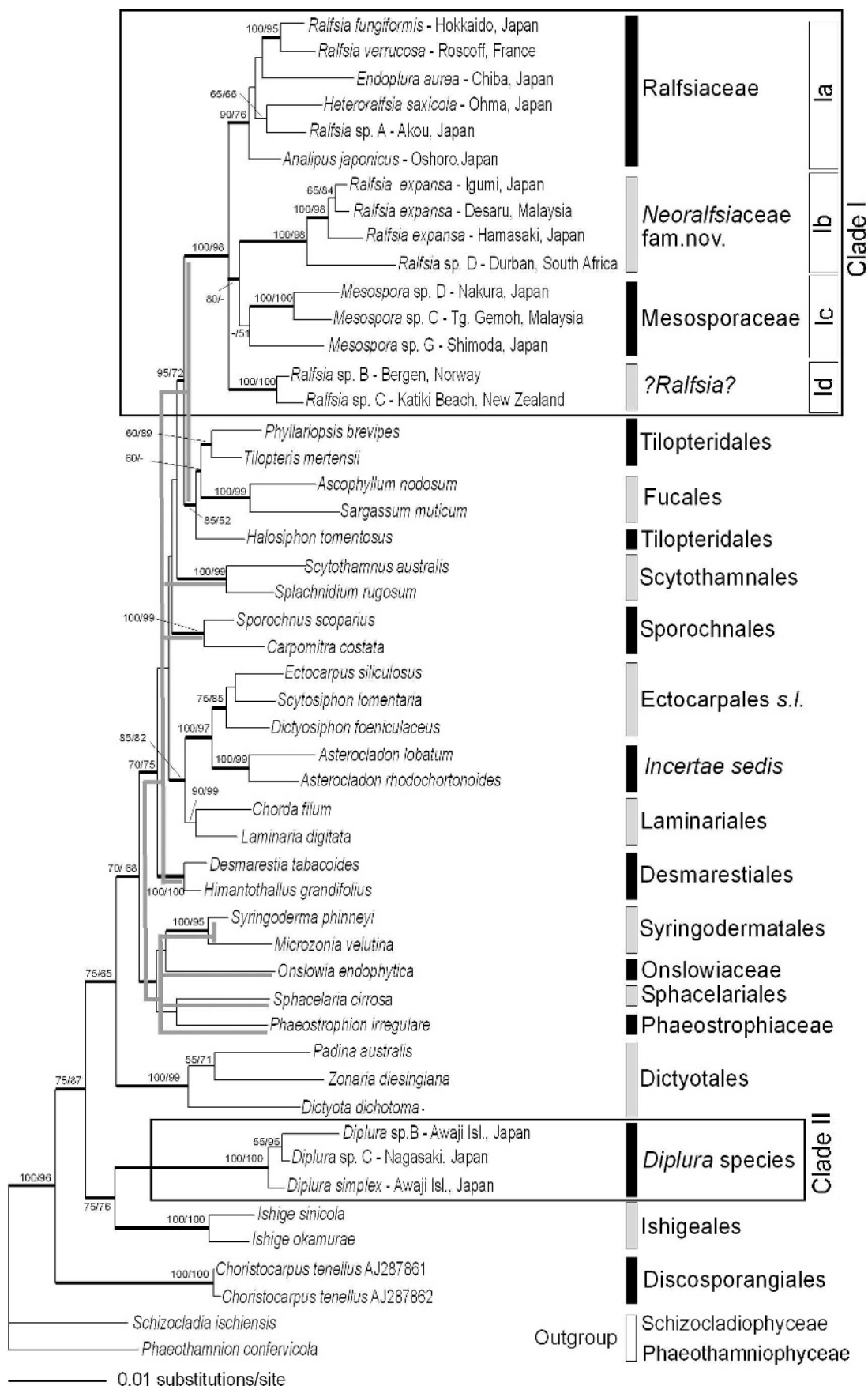| Taxa	Thickness of thalli (μm)	Symmetry/asymmetry of vegetative filaments	Distinct delineation of cortex and medullar layer	Paraphyses	Plurilocular zoidangia	Unilocular zoidangia	No. of plastids
Ralfsia sp. C	80–180	unilateral; tightly adhered; upwardly curved toward the surface, arising from basal part	no	absent	–	–	single
Clade II							
Diplura simplex	116–160	straight; tightly adhered; upwardly toward the surface, arising from basal part	no	present	subterminal (position), biseriate, single sterile cell	terminal (origin), one to two stalk cells -	several
Diplura sp. **B**	136–170	straight; tightly adhered; upwardly toward the surface, arising from basal part	no	present	subterminal (position) mostly uniseriate, single sterile cell	–	several
Diplura sp. C	152–173	straight; tightly adhered; upwardly toward the surface, arising from basal part	no	present	subterminal (position), mostly uniseriate, single sterile cell	–	several

[1] – indicates not observed.

crustose nature of the sporophytic as well as gametophytic thalli had been considered to be the characteristic feature of the family (and the order Ralfsiales, on the basis of the family), both *A. japonicus*, having an isomorphic life history alternating between erect sporophyte and erect gametophytes, as well as *H. saxicola*, having a heteromorphic life history alternating between erect sporophyte and crustose gametophyte, were confirmed to be close relatives of the crustose *Ralfsia*. Accordingly, gross morphology of the thalli (erect thalli vs crustose thalli) as well as the life history patterns (isomorphic vs heteromorphic) are not distinctive characteristics of the family and order. Comparable examples in which the life history patterns are remarkably different within a family or an order are known: In Scytosiphonales (*Ectocarpales s.l.*), *Myelophycus* and *Melanosiphon* have isomorphic life histories alternating between erect thalli (Kawai *et al.* 1994; Cho *et al.* 2003), whereas other members of the order typically show heteromorphic life histories (Wynne 1969; Tanaka & Chihara 1984). Species of *Syringoderma* (Syringodermatales) have remarkably diverse morphology among the gametophytes (Henry 1984; Kawai & Yamada 1990).

From Fig. 1, it is evident that clades Ib and Ic are more closely related to one another in comparison with clade Ia. Clades Ib and Ic shared some similarity in that the plants are solely of crustose form and have single chloroplast without pyrenoids, whereas clade Ia is very different from Ib and Ic: the thalli of clade Ia include crustose forms as well as erect forms such as *A. japanicus* (isomorphic life history with erect thalli) and *H. saxicola* (heteromorphic life history alternating erect and custose thalli; Kawai 1989). The number of chloroplasts per cell is also somewhat diverse within the family (clade Ia). *Endoplura aurea* has several to a few discoid chloroplasts in each cell (Hollenberg 1969; Tanaka & Chihara 1981b), and although *A. japonicus* basically has only one cup-shaped chloroplast in each cell, several disc-shaped chloroplasts occur in the cells of the inner part of the thallus (Kawai, unpublished data). These differences are summarized in Table 3.

Nevertheless, the independence of the Ralfsiaceae is supported by the following morphological features: unilateral or bilateral thallus construction composed of tightly packed vegetative filaments; occurrence of sessile or pedicellate (with a single-celled pedicel) unilocular zoidangia associated with multicellular parapahyses, and plurilocular zoidangia with sterile terminal cells.

We consider that clade Ib, composed of *Ralfsia expansa* and its related taxa and which formed a highly supported clade (BP for ML = 100%, BP for MP = 98%, ≥0.95 PP), should be treated as an independent taxon at the familial level. This clade is distinguished from Ralfsiaceae on the basis of the molecular data, and the genus *Ralfsia* is apparently paraphyletic if the clade is composed of *Ralfsia expansa* – Igumi, *R. expansa* – Desaru, *R. expansa* – Hamasaki and *Ralfsia* sp. D – Durban (undescribed species close to *R. expansa*). Morphologically these species are distinctive in having both unilateral and bilateral construction of the thallus within the same crust (León-Alvarez & González-González 2003) and pedicellate unilocular zoidangia with three- to six-celled pedicels. Despite the conclusion of León-Alvarez & González-González (2003)

100/95 *Ralfsia fungiformis* - Hokkaido, Japan
Ralfsia verrucosa - Roscoff, France
Endoplura aurea - Chiba, Japan
65/66
90/76 *Heteroralfsia saxicola* - Ohma, Japan
Ralfsia sp. A - Akou, Japan
Analipus japonicus - Oshoro, Japan
65/84 *Ralfsia expansa* - Igumi, Japan
100/98 *Ralfsia expansa* - Desaru, Malaysia
100/98 *Ralfsia expansa* - Hamasaki, Japan
Ralfsia sp. D - Durban, South Africa
80/- 100/100 *Mesospora* sp. D - Nakura, Japan
Mesospora sp. C - Tg. Gemoh, Malaysia
-/51 *Mesospora* sp. G - Shimoda, Japan
100/100 *Ralfsia* sp. B - Bergen, Norway
95/72 *Ralfsia* sp. C - Katiki Beach, New Zealand

Ralfsiaceae — Ia
Neoralfsiaceae fam.nov. — Ib
Mesosporaceae — Ic
?Ralfsia? — Id
Clade I

60/89 *Phyllariopsis brevipes*
60/- *Tilopteris mertensii*
100/99 *Ascophyllum nodosum*
Sargassum muticum
85/52 *Halosiphon tomentosus*
100/99 *Scytothamnus australis*
Splachnidium rugosum
100/99 *Sporochnus scoparius*
Carpomitra costata
Ectocarpus siliculosus
75/85 *Scytosiphon lomentaria*
100/97 *Dictyosiphon foeniculaceus*
85/82 100/99 *Asterocladon lobatum*
70/75 *Asterocladon rhodochortonoides*
90/99 *Chorda filum*
Laminaria digitata
70/68 *Desmarestia tabacoides*
100/100 *Himantothallus grandifolius*
100/95 *Syringoderma phinneyi*
Microzonia velutina
75/65 *Onslowia endophytica*
Sphacelaria cirrosa
Phaeostrophion irregulare
Padina australis
55/71 *Zonaria diesingiana*
100/99 *Dictyota dichotoma*
75/87

Tilopteridales
Fucales
Tilopteridales
Scytothamnales
Sporochnales
Ectocarpales *s.l.*
Incertae sedis
Laminariales
Desmarestiales
Syringodermatales
Onslowiaceae
Sphacelariales
Phaeostrophiaceae
Dictyotales

Diplura sp. B - Awaji Isl., Japan
55/95 *Diplura* sp. C - Nagasaki, Japan
100/100 *Diplura simplex* - Awaji Isl., Japan
75/76 *Ishige sinicola*
100/100 *Ishige okamurae*
100/100 *Choristocarpus tenellus* AJ287861
Choristocarpus tenellus AJ287862
100/96 *Schizocladia ischiensis*
Phaeothamnion confervicola

Diplura species — Clade II
Ishigeales
Discosporangiales
Outgroup Schizocladiophyceae
Phaeothamniophyceae

75/87

━━━━━ 0.01 substitutions/site

Table 3. Comparisons of morphological features of crustose brown algae.

	Clade I				Clade II
	Ia	Ib	Ic	Id	
Taxa/characteristics	Ralfsiaceae	*Ralfsia expansa*	Mesosporaceae	Unknown	*Diplura*
Arrangement of vegetative filaments	unilateral/bilateral; tightly packed[1,2]	unilateral to bilateral; tightly packed[1]	unilateral; loosely adhered[3]	unilateral; tightly packed	unilateral; loosely adhered[4,5]
Life history	isomorphic[6]/ heteromorphic[2]	?	direct[7]	?	?
Unilocular zoidangia	lateral/terminal (origin), sessile or with one-celled pedicel[1,4,8,9]	lateral/terminal (origin), pedicellate with three to six cells[1]	terminal (position), pedicellate[3]	?	lateral/terminal (origin), pedicellate[10]
Paraphyses	present[1,4,8]	present[1]	absent[3]	?	present
Sterile cell of plurilocular zoidangia	present (one to several)[1,5]	present (one)[1]	present (several)[3]	?	present[4,5]
Chloroplast shape and number per cell	cup-, plate-shaped, single to several[1,2,4,5]	plate-shaped; single[1]	disc-, plate-shaped, single to several[3]	plate-shaped, single	discoid, several[4,5]

[1] Tanaka & Chihara 1980b.
[2] Kawai 1989.
[3] Tanaka & Chihara 1982.
[4] Hollenberg 1969.
[5] Tanaka & Chihara 1981b.
[6] Loiseaux 1968.
[7] West & Calumpong 1996.
[8] Tanaka & Chihara 1980c.
[9] Tanaka & Chihara 1981a.
[10] First record of unilocular sporangia of the *Diplura* species having more than one stalk cell.

that *R. expansa* and *Ralfsia hancockii* Dawson are two distinct species and *R. expansa* sensu Tanaka & Chihara should be *R. hancockii*, we are of the opinion that the two share considerable similarities and could possibly be closely related species. In addition to this, the type specimens of *R. hancockii* Dawson are without plurilocular zoidangia; hence further conclusions could not be deduced. In León-Alvarez & González-González (2003), the separation of the two species as different is heavily based on unilocular zoidangia but we believe that the unilocular and plurilocular structures are equally important for taxonomic purposes. Hence the exact species status of *R. expansa* and *R. hancockii*, whether separate species or different strains of the same species, can only be deduced from molecular analysis. It is difficult to obtain *R. expansa* from the type locality because the location is very remote (González-González, personal communication). León-Alvarez & González-González (2003) noted that the available speci-

mens of *R. expansa* are very limited, with only three specimens being examined in comparison with 25 samples of *R. hancockii*. We found that our specimens are similar to the descriptions of Tanaka & Chihara (1980b) and Weber van Bosse (1913).

Regarding the number of cells of the pedicels of unilocular zoidangia in *Ralfsia* spp. (Dawson 1944; Hollenberg 1969; Tanaka & Chihara 1980a, b, c, 1981a), only *R. hancockii* Dawson (three-celled pedicels), *Ralfsia confusa* Hollenberg (one- to six-celled pedicels) and *R. expansa* (three- to six-celled pedicels) possess more than one-celled pedicels, whereas the other species, *R. fungiformis, Ralfsia hesperia* Setchell & Gardner, *Ralfsia integra* Hollenberg, *Ralfsia pacifica* Hollenberg, *R. verrucosa* and *R. endopluroides* Tanaka & Chihara are mostly sessile, with some exceptions having a single-celled pedicel (e.g. *R. verrucosa, R. hesperia* and *R. pedicellata* Tanaka & Chihara), although for *R. verrucosa* there are reports showing that it possesses multiple cells (Fletcher 1978, 1987). From our experiences, we have found that the specimens that possess unilocular ziodangia are consistently sessile and in addition to this the plurilocular zoidangia are partly biseriate, the main distinguishing feature mentioned by Tanaka & Chihara (1980b) to differentiate it from *R. pacifica*. We also consider that the comment of Hollenberg (1969) was true for *R. verrucosa*, because the thalli are usually multistriate. *Ralfsia verrucosa* can be easily distinguished from *R. expansa* because the vegetative filaments of *R. expansa* are bilateral upwardly and downwardly from the central portion, but in *R. verrucosa* the vegetative erect filaments curve upwardly from the basal stratum. This is in

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Fig. 1. *rbc*L phylogenetic trees based on ML, MP and Bayesian analyses (operational taxonomic units covering most of the phaeophycean orders). The tree indicated in black lines is based on the ML, and the difference in the strict consensus MP tree is shown in gray lines. -Ln likelihood was 19619.19990. The topology of Bayesian tree was identical to ML. For MP analysis, eight single most parsimonious trees of 4096 steps were obtained with a consistency index (CI) of 0.2839 and retention index (RI) of 0.4648. The bootstrap values (ML/MP) are shown above the branches. Bootstrap values below 50% are indicated as "–". Bootstrap values indicate percentage on the basis of 100 (ML) and 1000 (MP) replicates. Thick branches indicate Bayesian posterior probabilities ≥ 0.95.

agreement with the observation by Tanaka & Chihara (1980b). Furthermore, *R. confusa* shows a filamentous type of development in the early stages (Hollenberg, 1969), although most other *Ralfsia* species show a discoidal early development. Therefore, the taxonomic assignment of *R. confusa* needs reexamination.

Although the independence of clade Ic composed of Mesosporaceae was not clearly supported by the bootstrap values of 51% for MP and <50% for ML and ≤0.95 PP, Mesosporaceae is considered to be distinctive in having a characteristic thallus construction composed of loosely adhered erect filaments, and the occurrence of unilocular zoidangia lacking paraphyses (Tanaka and Chihara 1982). Although this family showed distinct morphological differentiation from the rest of the family, since Mesosporaceae also includes other species such as *Hapalospongidion* and *Basispora*, and because in this study we could not include the latter two species in the analysis, we reserve any conclusion on the validity of the Mesosporaceae.

Regarding clade Id, although the specimens collected from Norway and New Zealand are tentatively identified as *Ralfsia*, they are genetically rather distant from any of the other clades. However, at the moment we have very limited information about the morphology of the taxa because only silica gel-preserved specimens were available, and therefore we also reserve any taxonomic conclusions about these taxa.

Although some of the distinguishing morphological features of the order Ralfsiales defined by Nakamura (1972) were not distinctive, we consider that clade I elucidated in the present analyses constitutes a monophyletic group recognizable at the ordinal level, and corresponds to Ralfsiales. These taxa share a crustose form of thallus in at least in one of the life history stages, and the plurilocular zoidangia have sterile terminal cell(s). Most of the members normally have a single chloroplast lacking pyrenoids.

Clade II, consisting of *Diplura* species, was supported by 100% bootstrap values for ML and MP, ≥0.95 PP, was also shown to be very distant from other crustose brown algae, and showed a sister relation with Ishigeales. However, in spite of the distant molecular phylogenetic relation, we have not noticed any distinctive morphological features. However, because the life history of *Diplura* has never been reported, a future study on the life history (and perhaps cytology) may yield informative results.

In conclusion, although the original definitions of Ralfsiales by Nakamura (1972) need emendation, we consider that *Ralfsia* and its related taxa need to be treated as an independent order, and we find it reasonable to use the ordinal name Ralfsiales. The emended morphological characteristics of Ralfsiales are (1) discoid early development of the thallus; (2) one to several plate- or cup-shaped chloroplasts without pyrenoids; (3) plurilocular zoidangia with sterile terminal cell(s) and terminal unilocular zoidangia; and (4) presence of crustose gametophytic or sporophytic stage(s) in the life history, with exception for *Analipus*, in which the crustose form only exists in early stage of development.

Nemodermataceae is another family of the Ralfsiales in which the species have characteristic intercalary unilocular

and lateral plurilocular zoidangia, and several chloroplasts. The only known species is limited to the Canary Islands, Morocco, Algeria and Tunisia (Nelson 1982). Lithodermataceae is also a member of Ralfsiales that has distinct reproductive structures in which both unilocular and plurilocular zoidangia are terminal. In this study we could not include any authentic specimens of Nemodermataceae or Lithodermataceae, but on the basis of the distinct differences in the position of the unilocular and plurilocular zoidangia, we are of the opinion that the phylogenetic position of Nemodermataceae and Lithodermataceae can only be determined through molecular phylogenetic analysis but not in Ralfsiales. In addition, the study done by Cho *et al.* (2005) using the *psa*A data has shown that *Analipus* and *Nemoderma* are not closely related. The nonmonophyly of the Ralfsiaceae, Nemodermataceae, and Lithodermataceae is also presented in de Reviers *et al.* (in press, fig. 14.5). We consider that the genus *Diplura*, originally placed in Ralfsiaceae, should be separated from the true Ralfsiaceae and placed in a new family of it own in Ishigeales, but we reserve the taxonomic treatment in the present study because the relations with the related taxa are still not fully understood.

Ralfsiales Nakamura; emended by Lim and Kawai

Thallus crustosus in gradu uno vitae saltem vel in gradibus primis crescentiae, germinatione per discum; chloroplasti in quaque cellula singuli vel plures sine pyrenoidibus; zoidangium uniloculare cum vel sine paraphysibus; sporangium intercalarium pluriloculare cellulis terminalibus sterilibus singulis vel pluribus instructum. Ordines nucleotidorum in geno *rbc*L distinctae.

SPECIES TYPICA: *Ralfsia deusta* (C. Agardh) = *Ralfsia fungiformis* (Gunnerus) Setchell & Gardner.

BASIONYMUM: *Zonaria deusta* C. Agardh.

The thallus crustose in at least in one of the life history stages, or in the early stages of development, with disc-type germination; having one to several chloroplasts without pyrenoids in each cell; unilocular zoidangium with or without paraphyses; intercalary plurilocular sporangium with sterile terminal cell(s). *rbc*L gene sequences distinct.

Neoralfsiaceae fam. nov. Lim et Kawai

Cum characteribus *Neoralfsiae* Lim et Kawai.

With characteristics of *Neoralfsia* Lim et Kawai.

***Neoralfsia* gen. nov. Lim et Kawai**

Thallus crustosus expansus, per multis rhizoideis arcte affixus ad substratum et postea ad libertatem tendens, pseudoparenchymatus, e stratis manifestis corticalibus medulariisque foveis pilorum constans; cellulae medullariae unilaterales vel bilaterales; zoidangia unilocularia pedicellis paraphysibusque; zoidangia intercalaria plurilocularia cellula terminali singula sterili. Ordines nucleotidorum in geno *rbc*L distinctae.

TYPE SPECIES: *Neoralfsia expansa* (J. Agardh) Lim et Kawai comb. nov.

BASIONYM: *Myrionema expansum* J. Agardh 1848: 7.

HOMOTYPIC SYNONYM: *Ralfsia expansa* (J. Agardh) J. Agardh 1848: 63.

Thallus crustose, expanded, attached tightly to the substratum with many rhizoids and later tending to be free, pseudoparenchymatous, tightly adjoined, composed of obvious cortical and medullary layers with hair pits; medullary vegetative unilateral to bilateral; unilocular zoidangia with pedicels and paraphyses;

intercalaray plurilocular zoidangia with a single sterile terminal cell. *rbc*L gene sequences distinct.

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