Taxonomic reappraisal of the freshwater brown algae *Bodanella, Ectocarpus, Heribaudiella, and Pleurocladia* (Phaeophyceae) on the basis of *rbcL* sequences and morphological characters

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This study examines the phylogenetic relations among freshwater species of brown algae using RUBISCO large subunit (*rbcL*) sequences, in conjunction with diagnostic morphological and developmental traits. We focused on the phylogenetic and taxonomic placement of five freshwater species, *Bodanella lauternborni, Ectocarpus siliculosus, Heribaudiella fluviatilis, Pleurocladia lacustris*, and *Porterinema fluviatile*, each of which has traditionally been assigned to the Ectocarpales by most authors. These freshwater species were compared with 26 marine taxa of brown algae from nine orders, with particular emphasis on families within the Ectocarpales. Results of BLASTn searches and later phylogenetic analyses placed all freshwater taxa within the Phaeophyceae, but phylogenetic inferences do not support their traditional classifications at the ordinal level. There was no support for the inclusion of *H. fluviatilis* or *B. lauternborni* in the Ectocarpales, but instead formed a well-supported clade intermediate between the Syringodermatales and Sphacelariales. Although morphologies of these two species are relatively distinct and remain stable in culture, *B. lauternborni* nested between two populations of *H. fluviatilis* (on the basis of *rbcL* data) from British Columbia and Germany, making a reclassification of these two species necessary. Molecular and morphological data clearly place two other freshwater taxa, *Pleurocladia lacustris* and (freshwater) *E. siliculosus* in the Ectocarpales, each nesting among marine members of the Ectocarpaceae. When compared with sequences from 13 species from several families within the Ectocarpales, *Pleurocladia* is most closely related to members of the Ectocarpaceae and may be a sister taxon to the Scytosiphonaceae. DNA sequences from a freshwater isolate of *E. siliculosus* from Australia were nearly identical to those from a marine isolate from Germany. Molecular data did not resolve the ordinal status of a freshwater *Porterinema fluviatile*, but indicated that this species should not be classified within the Ectocarpales.

**KEY WORDS:** Bodanella, Brown algae, Ectocarpales, Ectocarpus, Freshwater, Heribaudiella, Phaeophyceae, Phylogeny, Pleurocladia, rbcL, RUBISCO

INTRODUCTION

The Phaeophyceae is a diverse class of multicellular algae ranging in size from microscopic forms to huge kelps and occur from arctic to tropical biomes (Bold & Wynne 1978; Van den Hock et al. 1995). Members of this class exhibit a wide range of morphologies, reproduction, and ultrastructure, and comprise roughly 265 genera and between 1500 and 2000 species (Van den Hock et al. 1995; De Reviers & Rousseau 1999). Of all these species, < 1% (seven species) has been documented to occur in freshwater environments (Starmach 1977; Bourrellly 1981; Wehr 2003).

Although the study of freshwater brown algae has had a long history, with several genera known for > 100 years, their classification and phylogeny is unclear. Well-documented populations of several species are few, making thorough examinations of their morphology, reproduction, and phylogeny difficult (Wehr 2003). To date, studies have relied on morphological, reproductive, and ultrastructural characters to determine the classification of freshwater members of the class. These schemes place most or all freshwater taxa in the Ectocarpales or Sphacelariales largely on the basis of their filamentous growth forms (Starmach 1977; Bold & Wynne 1978; Bourrellly 1981; Pueschel & Stein 1983; Van den Hock et al. 1995).

Five freshwater species are most often classified within the Ectocarpales sensu lato, although placements within families vary a great deal. *Pleurocladia lacustris* A. Braun (Family: Ectocarpaceae or Chordiariace) was the first “true” freshwater brown alga described in the literature (Braun 1855). It is a creeping, infrequently branched filament (with one or rarely two parietal chloroplasts) that occasionally produces upright, centrifugal (arched), multiply branched filaments, creating small cushion-like thalli. Filaments frequently produce unilocular sporangia (plurilocular sporangia rarely reported) and hairs (under P-limitation; Wehr 2003), and grow on rocks or aquatic plants in lakes and rivers (occasionally endophytic) in Europe and North America (Waern 1952; Wilce 1966; Bourrellly 1981). The most commonly reported species, *Heribaudiella fluviatilis* (Areschoug) Svedelius (Family undetermined), is a thick-walled, crust-forming alga that colonizes rocks in streams. Cells have multiple discoid...
chloroplasts, and thalli are composed of multiply branched prostrate filaments and erect, mostly dichotomously branched filaments that bear unilocular or plurilocular sporangia (Starmach 1977; Wehr & Perrone 2003). It has been reported from several hundred (mostly stream and river) locations worldwide (Wehr & Stein 1985; Wehr 2003). Bodanella lauterborni Zimmerman (Family undetermined) is a thick-walled, prostrate (creeping) branched filament with multiple chloroplasts, and unknown reproductive structures (Zimmerman 1928; Bourrelly 1981). It has been reported to colonize rocks in a few lakes in Europe (Wehr 2003). Ectocarpus siliculosus (Dillwyn) Lyngbye (Family: Ectocarpaceae) is primarily a marine or brackish species, but one freshwater population has been described from Australia (West & Kraft 1996) and another inland population was reported from a river polluted by potassium mine wastes (Geissler 1983). This alga exhibits typical Ectocarpus morphology with infrequent branching and ribbon-like parietal or spiral chloroplasts (with pyrenoids). Plurilocular sporangia have been observed in the Australian population, but unilocular sporangia are documented only in marine populations (West & Kraft 1996).

Porterinema fluviatile (Porter) Waern is an enigmatic species that inhabits marine, estuarine, and freshwater habitats and is classified in the Ectocarpales by some authors (Waern 1952; Bourrelly 1981; Wehr 2003) and in the Ralfsiales by others (Wilce et al. 1970). It is a simple, rarely branched alga with creeping filaments (with 1–3 lobed, parietal chloroplasts) that may form loosely arranged monostromatic discs on plants or rocks, or live endophytically in some aquatic plants or other algae (Waern 1952). Plurilocular sporangia are apparently common and characterized by four-celled terminal clusters or crowns (Waern 1952; Dop 1979). In general, the classification of freshwater taxa, on the basis of at least some morphological features, agrees with the circumscription of the Ectocarpales described by De Reviers & Rousseau (1999), with the possible exception of the genus Heribaudiella, whose combined multiply branched prostrate and upright forms are more complex than is seen in most taxa placed in this order (Wehr 2003).

Two freshwater members of the Sphacelariales are Sphacelaria fluviatile Jao and S. lacustris Schloesser & Blum (Family: Sphacelariaceae). Both are characterized by apical growth and heterotrichy, having both uniseriate and multisieriate branched filaments, as is characteristic of the order (Jao 1943; Starmach 1977; Schloesser & Blum 1980). In fresh waters, both form small pale brown tufts or cushions on rocks, with filaments creeping along the substratum. Filaments contain many small discoid chloroplasts lacking pyrenoids. Freshwater species are currently distinguished by branching patterns (S. fluviatile has opposite branching; S. lacustris has alternate or irregular branching) and frequency of lateral cell divisions (Wehr 2003). Unilocular sporangia are known from S. lacustris, whereas plurilocular sporangia are not found in either. Details of the morphology, reproduction, classification (including synonymies), and ecology of all known freshwater taxa are provided elsewhere (Wehr 2003). It is worth noting that Ectocarpus, Porterinema, and Sphacelaria all have marine counterparts (species within each genus), whereas the three other genera do not.

Although no classification scheme for the Phaeophyceae has been fully accepted by all workers, recent syntheses that combine molecular and traditional characters have emerged. One such classification characterizes members of the Ectocarpales as simple, uniseriate filamentous forms whose cells have one or few large band-like chloroplasts and a stalked, pyriform pyrenoid (Rousseau & De Reviers 1999). These and other details have not been fully assessed in the freshwater taxa. Grouping of marine taxa is also strongly supported by molecular studies, which show that several taxa traditionally classified within the Ectocarpales, Chordariales, Dictyosiphonales, and Scytosiphonales form a strongly supported monophyletic group (Rousseau & De Reviers 1999; Draisma et al. 2001; Rousseau et al. 2001). De Reviers & Rousseau (1999) also excluded the crust-forming Ralfsia (classified with the Ectocarpales or Ralfsiales) from the Ectocarpales on the basis of both molecular and the absence of characteristic pyrenoids. Tan & Druel (1994) showed that Ralfsia was more closely associated with members of the Desmarestiales, Dictyotales, Fucales, Laminariales, Sphacelariales, and Syringodermatales, rather than with members of the Ectocarpales, on the basis of 18S rDNA sequence data. These latter findings with marine taxa are of importance for the present study, because most freshwater taxa have been classified within the Ectocarpales, and none of the recent classification schemes or molecular approaches has considered the freshwater genera or species (Starmach 1977; Bourrelly 1981; Wehr 2003).

Molecular studies using the 18S rDNA and RUBISCO large subunit (rbcL) genes have proven useful for inferring phylogenetic relations among orders, families, and genera of marine brown algae (e.g. Assali et al. 1990; Andersen et al. 1998; Müller et al. 1998; Peters & Ramírez 2001; Draisma et al. 2001, 2002; Burrowes et al. 2003) and should be considered in efforts to understand the evolutionary history of freshwater taxa. Determining phylogenetic relations and proper taxonomic placement of these poorly known species may also prove to be important in future biogeographic and ecological studies. Links between biogeographic and molecular analyses are especially needed with freshwater representatives of the Phaeophyceae, as many species are apparently disjunct, and their origin and modes of dispersion are poorly understood (Wehr & Stein 1985).

Our goal was to characterize the phylogenetic relations and taxonomic placement of five freshwater species (B. lauterborni, E. siliculosus, H. fluviatile, Pleurocladia lacus- tris, Porterinema fluviatile) that are currently assigned to the Phaeophyceae. Field specimens and cultures of freshwater taxa from a wide range of locations were assessed and compared with 26 marine brown algal species from nine orders whose phylogenetic positions have previously been determined. Specifically, our aims were (1) to verify, using molecular evidence, that freshwater populations of presumed phaeophyte species are truly members of the Phaeophyceae; (2) to determine the correct taxonomic placement of freshwater species within the brown algae; and
METHODS

Strategy

Brown algal specimens were obtained whenever possible from live samples, either freshly collected field material or laboratory cultures. Live material was obtained for freshwater Ectocarpus, Heribaudiella, Pleurocladia, Bodanella, and Porterinema (Table 1). Repeated efforts to obtain live samples of either freshwater species of Sphacelaria (divers sampling in western Lake Michigan [S. lacustris]; requests to the Chinese Academy of Sciences [S. fluviatilis]) were unsuccessful. Intact DNA could not be extracted from dried herbarium material of S. lacustris (National Herbarium, Smithsonian Institution). Thus the present study focused mainly on those five freshwater taxa for which we had live material. Marine sister taxa of Sphacelaria were used to assess ordinal placements of all freshwater taxa that were sampled.

Field and culture specimens

A list of source material (new specimens and cultures) is provided in Table 1. Algal crusts were collected from rocks from three freshwater locations (Ashnola River, Granite Creek, Smith Creek) in British Columbia, Canada (described in Wehr & Stein 1985). These rocks were air dried, labeled, maintained at ambient temperature, and returned to the laboratory (Louis Calder Center, Armonk, NY). Upon arrival (≤ 7 d), rocks were wetted with sterile deionized water and separate crusts were scraped into sterile 12-well plates containing approximately 1 ml of Allen’s medium (Allen 1968). Observations (inverted microscope) determined which wells contained H. fluviatilis. Plates were maintained in semicontinuous culture (media changes semimonthly) for several months in a lighted incubator (20°C; 100 μmol m⁻² s⁻¹ photons irradiance; 12:12 photoperiod) until used for DNA extraction. Pure cultures of freshwater brown algae were obtained from colleagues (freshwater: Ectocarpus, Heribaudiella, Pleurocladia, Bodanella, and Porterinema) and the University of Texas Culture Collection (freshwater: Bodanella, Porterinema; marine: Ectocarpus, Sphacelaria), and maintained in Allen’s medium (Allen 1968), Basal medium (ES), or Desmidieaean medium (Pringsheim 1946) under conditions described above. Every 1–3 months a portion of the cultures was transferred to a sterile flask with fresh medium for maintenance.

DNA extraction and amplification

Genomic DNA was extracted from field and culture material (Table 1) using the DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions with minor modifications. Algal material (c. 40 mg) was disrupted in a microcentrifuge tube with a micropestle (USA Scientific, Ocala, FL) and incubated 30 min to lyse cells. After the final elution step, DNA was diluted 5-, 10-, 20-, 50-, or 100-fold for use in polymerase chain reaction (PCR) amplification.

A dilution of extracted DNA was used to amplify roughly a 1000–1450-base pair (bp) fragment of the rbcL gene (Siemer et al. 1998; Burrowes et al. 2003). PCR was performed using the Taq PCR Core Kit (Qiagen, Valencia, CA) using 1 μl of diluted DNA template, PCR buffer (containing 1.5 mM of Mg²⁺), 200 μM of each dNTP, 0.5 μM of each primer, 2.5 units of Taq DNA polymerase,
and water to 50 μl. Amplification was performed as follows: an initial denaturation step of 94°C (5 min), followed by 40 cycles of 94°C for 45 s, 48°C for 45 s, and 72°C for 2 min, with a final 7-min extension of 72°C. Amplification primers (Table 2) were synthesized on the basis of previously reported primers designed primarily for marine brown algae (Siemer et al. 1998; Kawai et al. 2000), or designed by the authors. New primers were designed since PCR amplification was rarely successful (except using DNA isolated from freshwater and marine Ectocarpus spp.) using the following primers designed by Siemer et al. (1998): BLSrbc.L130F, BLSrbc.L1378R, BLSrbc.L1433R, BLSrbc.L1450R, BLSrbc.L1450F, BLSrbc.L1455R, BLSrbc.L1455F, BLSrbc.L1480R, and BLSrbc.L1480F. BLSrbc.L1450R and BLSrbc.L1455R were designed to amplify the rbcL gene (1450–1433 bp) region that gives positive results, using Geneclean H (Biotek, Waltham, MA) and primers listed in Table 2. PCR products were sequenced using the same primers as used for PCR amplification. Occasionally internal primers were used when these primers failed. Sequencing reactions were run on an ABI Prism 3700 automated sequencer (Applied Biosystems, Foster City, CA) at the American Museum of Natural History, NY, NY.

**Table 2. Amplification primers used for PCR or sequencing.**

<table>
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<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence (5’–3’)</th>
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<th>Reference</th>
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1 F, forward; R, reverse.

To understand the taxonomic position of freshwater species within the Phaeophyceae, sequences from a diverse range of (marine) phaeophyte taxa were obtained from NCBI. Our a priori plan was (1) to sample a few members from most orders in the Phaeophyceae and (2) to heavily sample species from within several families in the Ectocarpales, because nearly all freshwater species have traditionally been classified in that order. It was not our intention to examine the phylogeny of all groups within this class, since this broader question has been addressed by others (e.g. De Reuvers & Rousseau 1999; Draisma et al. 2001). Here, rbcL sequences from a total of 24 brown algal species from nine orders within the Phaeophyceae were used (Table 3), plus two obtained in this study (Table 1). This included 13 taxa in five or six families from within the Ectocarpales (Peters & Ramirez 2001; Rousseau et al. 2001). In addition, sequences from three species of Chrysophyceae (three orders), one from the Tribophyceae and two members of the Phaeothamniophyceae, were selected (Table 3) as outgroups (Daugbjerg & Andersen 1997; Saunders et al. 1997; Andersen et al. 1998; Bailey et al. 1998).

**BLASTn search**

A nucleotide–nucleotide BLASTn (basic local alignment search tool) was used to search the nucleotide database of the National Center for Biotechnology Information website (NCBI; www.ncbi.nlm.nih.gov/BLAST; Altschul et al. 1990) to align and compare the sequences we obtained with published sequences. From these searches the closest two matches were recorded to verify that our specimens were truly members of the Phaeophyceae and to assure that DNA used for sequencing was not a contaminant. This step was necessary because (1) some freshwater species have rarely been collected or isolated (e.g. Portierina), (2) several taxa were described 100 or more years previously, and (3) all have thus far been identified using only morphological data.

**Other sources of DNA sequences and outgroups**

To understand the taxonomic position of freshwater species within the Phaeophyceae, sequences from a diverse range of (marine) phaeophyte taxa were obtained from NCBI. Our a priori plan was (1) to sample a few members from most orders in the Phaeophyceae and (2) to heavily sample species from within several families in the Ectocarpales, because nearly all freshwater species have traditionally been classified in that order. It was not our intention to examine the phylogeny of all groups within this class, since this broader question has been addressed by others (e.g. De Reuvers & Rousseau 1999; Draisma et al. 2001). Here, rbcL sequences from a total of 24 brown algal species from nine orders within the Phaeophyceae were used (Table 3), plus two obtained in this study (Table 1). This included 13 taxa in five or six families from within the Ectocarpales (Peters & Ramirez 2001; Rousseau et al. 2001). In addition, sequences from three species of Chrysophyceae (three orders), one from the Tribophyceae and two members of the Phaeothamniophyceae, were selected (Table 3) as outgroups (Daugbjerg & Andersen 1997; Saunders et al. 1997; Andersen et al. 1998; Bailey et al. 1998).
Table 3. Sequences retrieved from the NCBI nucleotide sequence database, which served as outgroups and additional marine brown algal taxa for phylogenetic analyses (listed alphabetically by order and family; groupings within Phaeophyceae after Peters & Ramirez 2001, Rousseau et al. 2001).

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3 Now named Fucus gardneri Silva.

Sequence alignment and phylogenetic analysis

Individual sequences were imported into Sequencher™ 3.0 (Gene Codes, Ann Arbor, MI), a DNA sequence editor program, for editing and to assemble consensus sequences. Consensus sequences were next exported into MacClade 4 (Maddison & Maddison 2001), where they were aligned to existing rbcL sequences. The alignment included sequences from six freshwater phaeophytes, 26 marine species (two from this study and 24 from the NCBI database), and sequences from six outgroups (NCBI), and was 1467 bp long. Four Canadian Herbadiella consensus sequences were identical; thus only one consensus sequence was used in the alignment (to avoid biasing the phylogenetic analysis); for the same reason, only one consensus sequence was used in the analysis. The alignment was then imported into PAUP (v.4.0b10; Swofford 2002) for phylogenetic analysis.

Maximum parsimony analyses were conducted under a heuristic search using the tree bisection–reconnection branch-swapping algorithm, with 100 random additions and gaps treated as missing data. Neighbor-joining (NJ) distance analyses were also performed using a GTR+I+G model selected under the AIC criterion in Modeltest (v. 3.7; Posada & Crandall 1998). Bootstrapping (Felsenstein 1985) was completed as a measure of support using 2000 replicates under NJ and 1000 for parsimony (10 random sequence additions). Bayesian analyses were performed in Mr. Bayes (v. 3.1.2; Huelsenbeck & Ronquist 2001) with two independent trials (each with parallel runs) of inference under the GTR+I+G model (but with parameters estimated during the runs). Parallel runs of four Markov chains were completed with 1,000,000 generations and sampling every 100 generations. Data were partitioned by codon and parameters were unlinked during the analyses. Log-likelihood values for the sampled trees stabilized before 30,000 generations in all analyses and the final 750 trees (250 discarded as burn-in) were used to calculate posterior probabilities.

Reconciling morphological and sequence data

To assess the usefulness of phylogenetic analyses performed here, a collection of putatively diagnostic morphological and developmental variables (based on Waern 1952; Starmach 1977; Bourrelly 1981; Wehr 2003) were compared against the rbcL-based phylogenetic groupings. These attributes included (1) type of cell division (location of
meristem and uni- or multiseriate cell division; (2) branching pattern; (3) presence/absence of perennial system of prostrate and erect filaments; (4) cell dimensions and shape; (5) form of chloroplasts and presence/absence of pyrenoids; and (6) characteristics of the pluri- and unicellular sporangia. Such comparisons between morphological and molecular groupings have proven useful in resolving the taxonomic placement of other species within the Phaeophyceae (Burrows et al. 2003; Kawai et al. 2005). Because two freshwater Sphacelaria species (but not considered here) were clearly members of the Sphacelariales (Jao 1943; Schloesser & Blum 1980), an additional diagnostic trait was assessed in two of the freshwater species (B. lauterborni, H. fluviatilis) studied here (7): a temporary blackening of cell walls when treated with hypochlorite bleach (full strength and 20% dilution) as described by Draisma et al. (2002). These results were compared against the marine Sphacelaria sp. (UTEX 800) as a positive control (after Henry 1987).

RESULTS

Position of freshwater species using BLASTn

Our first goal was to determine if molecular data obtained from the freshwater taxa (based on morphology) placed all these organisms in the Phaeophyceae. A BLASTn search compared all newly obtained rbcL sequences with those in the NCBI database to determine which sequence they most closely matched. In all cases the freshwater sequences matched most closely to the sequences of well-recognized marine members of the Phaeophyceae. Similarities ranged from 89 to 99%, and averaged ≈ 92.2%. The greatest similarity for any freshwater specimen was obtained for the freshwater E. siliculosus collected from Australia, which was most similar in its sequences to a marine E. siliculosus collected from Europe (99%). The lowest similarities obtained from the existing NCBI database were for B. lauterborni (89% with Sphacelaria caespitula Lyngbye) and P. fluviatile (89% with Alethocladus corymbosus [Dickie] Sauvageau). BLASTn searches between these same freshwater taxa using the NCBI database with 18S rDNA data produced identical or similar matches (McCauley 2002).

Phylogenetic analysis

The analysis of the rbcL alignment resulted in the formation of three large clades, with Porterimena and Dictyota falling outside of these (Fig. 1). The first (clade 1) included members of six different orders, Tilopteridales, Laminariales, Cutleriales, Fucales, Desmarestiales, and Scytosiphonales. The second clade, which had high internal support, included all members of the Ectocarpales (16 taxa) including two of the freshwater species, E. siliculosus and Pleurocladia lacustris. The alignment revealed a slight separation between the three Ectocarpus samples considered, with Kuckuckia spinosa (Kützing) Kuckuck falling in between. The marine (European) and freshwater (Australian) E. siliculosus sequences were nearly identical, whereas K. spinosa was a sister taxon to these two strains. A third marine Ectocarpus sp. (UTEX 1433) formed a sister to K. spinosa. Freshwater P. lacustris was clearly placed within the Ectocarpales, and more closely associated with the Ectocarpus subgroup (family Ectocarpaceae) than with members of any of the other five Ectocarpalean families represented, and was positioned as a sister to this grouping. However, although more closely related to members of the Ectocarpaceae, Pleurocladia was positioned between the Ectocarpaceae and Scytosiphonaceae. Other families within the Ectocarpales (Acinetosporaceae, Adencystaceae, Chordariaceae) were more distantly related to Pleurocladia. One marine species, Chorda filum (Linnaeus) Stackhouse, grouped outside of the Ectocarpales clade, forming a sister. None of the remaining freshwater taxa proved to be closely related to the Ectocarpales.

A third large clade included two smaller subclades, with one member of the Syringodermatales as a sister taxon to the two. One subclade (termed clade 3a) included all (marine) Sphacelaria and the other (clade 3b) included the freshwater species of H. fluviatilis and B. lauterborni, with Bodanella intermediate between Heribaudiella populations from British Columbia and Germany. Although the Heribaudiella and Bodanella had much closer affinities with Sphacelaria, these subclades were fairly distinct from one another, differing by roughly 4.6% (≈ 68 bp). Furthermore, the Heribaudiella–Bodanella subclade was genetically distant from the Ectocarpales, in which these genera are currently classified, by at least 11.6% (≈ 170 bp), which is only somewhat less than the number of bases by which it differs from Fucus distichus (Linnaeus) (e.g. 14.5%, ≈ 213 bp) or from any other species in the first large clade. Similarly, the distance between either Heribaudiella population and any Ectocarpus sequence is 13.6% (≈ 200 bp). The Heribaudiella–Bodanella subclade is in fact more closely related to Syringoderma phinneyi Henry & Müller (differed by 6.5%, ≈ 96 bp), as a sister to this lower clade. The freshwater-brackish P. fluviatile did not group closely with any of the species tested or show a strong association with any of the recognized brown algal orders. However, sequencing this species proved to be more difficult than for the other taxa, given that only a portion of this sequence was determined (814 bp). Therefore, its position was not clearly established.

Reconciling molecular and morphological data

As the principal focus of the present study was to resolve the phylogenetic placement of four freshwater taxa, B. lauterborni, E. siliculosus, H. fluviatilis, and P. lacustris, the rbcL-based groupings were compared with the morphological features used in current taxonomic schemes for diagnostic keys and confirmed in ultrastructural studies. The first group (placed in clade 2, Ectocarpales) included the freshwater Ectocarpus and Pleurocladia; both species are uniseriate filaments with second or irregular branching arising (from basal or intercalary meristems) from a minimal (Ectocarpus) or loosely spreading (Pleurocladia) prostrate structure. Cells were isodiametric or somewhat swollen (Table 4). Cells were typically thin-walled and cylindrical (although dimensions were quite dissimilar in the two taxa), and had one or few large band-like chloroplasts and
a single, stalked pyrenoid. Cylindrical–elongate plurilocular sporangia occur in both, whereas only *Pleurocladia* has been observed to produce unilocular sporangia.

*Heribaudiella fluviatilis* and *B. lauterborni* (*rbcL*-based subclade 3b) each possessed apical growth and formed a dense and spreading pseudoparenchymatous basal system. In *Heribaudiella*, erect filaments originated from this prostrate system. Cells in both taxa were thick-walled, swollen or lumpy in appearance, and possessed many (10 to 30) small, disc-shaped chloroplasts. Pyrenoids were not observed in either species. Both produce large ovoid unilocular sporangia, whereas plurilocular sporangia have been observed only (but infrequently) in *Heribaudiella*. Specimens of *Heribaudiella* (Ashnola River) and *Bodanella* (UTEX 2190) when exposed to two concentrations of sodium hypochlorite (diagnostic for Sphacelariales) were both negative. A similar test with *Sphacelaria* sp. (UTEX 800) was positive (blackened cell walls).

**DISCUSSION**

**Classification of freshwater species**

The present study was able to discern the phylogenetic positions of four freshwater taxa (*B. lauterborni*, *E. siliculosus*, *H. fluviatilis*, *P. lacustris*) currently placed in the Phaeophyceae. Thus far, most classification schemes place these freshwater taxa within the order Ectocarpales on the basis of morphology, reproductive features, and ultrastructure (Bourrelly 1981; Pueschel & Stein 1983; Wehr 2003). This study was the first to use molecular data to confirm the placement of these freshwater browns into the Phaeophyceae and to infer their phylogenetic relations within this class. We compared these sequences with those of 26 marine species from the class encompassing nine orders. Our results support their inclusion at the class level, but call into question some classifications at the ordinal and
perhaps genus level. Nucleotide sequences of the \textit{rbcL} gene from freshwater taxa were consistently most similar to analogous sequences of marine brown algae rather than to sequences from other taxa in the NCBI database. Specifically, sequences from the \textit{rbcL} gene showed strong support for the inclusion of the freshwater taxa into the Phaeophyceae, as analogous sequences of marine taxa were at least 89% and up to 99% identical.

The present \textit{rbcL} sequence-based analysis clearly placed two of the freshwater taxa, \textit{P. lacustris} and (freshwater) \textit{E. siliculosus}, in the Ectocarpales (Fig. 1). Each species nested among marine members of the Ectocarpaceae, although \textit{Pleurocladia} may be a sister to Scytosiphonaceae. Wilce advised in 1966 that \textit{Pleurocladia} should remain within the Ectocarpales until further studies are conducted. Our consideration of the new molecular evidence in conjunction with existing morphological and cytological data (Table 4) further supports the retention of \textit{Pleurocladia} within the Ectocarpaceae, especially given their predominantly filamentous habit, reproductive structures, ribbon-shaped chloroplast morphology, and the presence of stalked pyrenoids. Our molecular analysis demonstrated that the recently discovered freshwater \textit{E. siliculosus} from Australia (Hopkins River Falls) is closely related to European marine conspecifics, with only five nucleotide differences in their \textit{rbcL} genes. Its identity, previously based on an elongate pluriocular sporangium (West \& Kraft 1996), seems certain based on a nearly identical \textit{rbcL} sequence (> 99%) to that in a marine population from Germany (Fig. 1).

Our analyses provide no support for the inclusion of \textit{H. fluviatilis} or \textit{B. lauterborni} within the Ectocarpales. The \textit{Heribaudiella–Bodanella} subclade 3b differs (within the \textit{rbcL} gene) from members of traditional order (Ectocarpales) and from any \textit{Ectocarpus} species by more than 13.6% (= 200 bp). Morphological characteristics also differ strongly from members of the Ectocarpales, with both \textit{Heribaudiella} and \textit{Bodanella} exhibiting apical growth originating from a dense system of perennial prostrate system of pseudoparenchymatous filaments and thick-walled cells with numerous discoid chloroplasts lacking obvious pyrenoids (Table 4). The \textit{Heribaudiella–Bodanella} subclade has strong internal bootstrap support, and its relation to other sister groups is similarly strong (Fig. 1), being more closely related to members of the Sphacelariales than the Ectocarpales. Our initial BLASTn searches found the closest matches in the NCBI database using \textit{rbcL} sequences for both species with various \textit{Sphacelaria} species (McCauley 2002). However, present molecular and morphological data suggest that classification in this order is also inappropriate. The \textit{rbcL} sequences of the \textit{Heribaudiella–Bodanella} subclade differed by 4.6% (= 68 bp) from those of three \textit{Sphacelaria} species that were included in the present analysis (similar to differences among other brown algal orders). Members of the Sphacelariales are character-

Table 4. Analytical comparison of \textit{Ectocarpus siliculosus}, \textit{Pleurocladia lacustris}, \textit{Bodanella lauterborni}, and \textit{Heribaudiella fluviatilis} on the basis of \textit{rbcL} groupings and morphological and reproductive attributes.

<table>
<thead>
<tr>
<th>\textit{rbcL} Grouping</th>
<th>Clade 2: Ectocarpales</th>
<th>Clade 3b: Bodanella + Heribaudiella</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Character</strong></td>
<td>\textit{Ectocarpus siliculosus}</td>
<td>\textit{Pleurocladia lacustris}</td>
</tr>
<tr>
<td>(1) Cell division</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meristem</td>
<td>intercalary</td>
<td>basal</td>
</tr>
<tr>
<td>Uni-/multiseriate</td>
<td>uniseriate</td>
<td>uniseriate</td>
</tr>
<tr>
<td>(2) Branching</td>
<td>sparse</td>
<td>common, spreading; secund, alternate, irregular</td>
</tr>
<tr>
<td>Frequency Pattern</td>
<td>secund or irregular</td>
<td></td>
</tr>
<tr>
<td>(3) Prostrate/erect system</td>
<td>unknown</td>
<td>common, loosely organized</td>
</tr>
<tr>
<td>Prostrate filaments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upright filaments</td>
<td>common</td>
<td></td>
</tr>
<tr>
<td>(4) Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filament width ((\mu)m)</td>
<td>20–40</td>
<td>6–12</td>
</tr>
<tr>
<td>Cell length ((\mu)m)</td>
<td>20–80</td>
<td>12–30 (40)</td>
</tr>
<tr>
<td>L:W ratio</td>
<td>1–2</td>
<td>2–4</td>
</tr>
<tr>
<td>Cell shape</td>
<td>isodiametric</td>
<td>isodiametric or slightly swollen</td>
</tr>
<tr>
<td>Cell wall thickness ((\mu)m)</td>
<td>0.1–0.2</td>
<td>0.1–0.2</td>
</tr>
<tr>
<td>(5) Chloroplasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number/shape</td>
<td>one–several; large band-like, parietal</td>
<td>one, large band-like, parietal</td>
</tr>
<tr>
<td>Pyrenoids</td>
<td>single, stalked</td>
<td>single, stalked</td>
</tr>
<tr>
<td>(6) Sporangia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pluriocular shape</td>
<td>elongate-cylindrical</td>
<td>elongate-cylindrical</td>
</tr>
<tr>
<td>Width</td>
<td>20–35</td>
<td>8–12</td>
</tr>
<tr>
<td>Length</td>
<td>70–160</td>
<td>10–12</td>
</tr>
<tr>
<td>Unilocular shape</td>
<td>unknown</td>
<td>pyriform–globose</td>
</tr>
<tr>
<td>Width</td>
<td>NA</td>
<td>(12) 20–30</td>
</tr>
<tr>
<td>Length</td>
<td>NA</td>
<td>20–40 (80)</td>
</tr>
<tr>
<td>(7) Cell wall blackening</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\) NA, not applicable; ND, not done.
ized by filaments with apical growth, which is true in both Heribaudiella and Bodanella, but also transverse division in subapical cells (leading to multiseriate axes) and blackening of cell walls after treatment with bleach. The latter two traits were negative in Heribaudiella and Bodanella (Table 4), but are regarded as basal traits for members of the order (Draisma et al. 2002). The Heribaudiella–Bodanella subclade is most likely a sister group to Sycosferugina phimeyi (differs by 6.5%, ≈ 96 bp), but both species lack dorsiventral differentiation and heteromorphic life cycle, characteristic of the Syringodermatales (Henry 1984). No other species or orders placed near this clade on the basis of rbcL sequences possess the suite of morphological, cytological, or reproductive traits exhibited in H. flaviatilis or B. lauterborni. (Table 4; Draisma et al. 2002). It is also possible that they are related to other marine crust-forming taxa in the Phaeophyceae, such as Ralfsia or Pseudolithodermia, but presently there are no rbcL sequences available for either species in the NCBI database. For these reasons both species should be removed from the Ectocarpales, and cannot be placed in either the Sphacelariales or the Syringodermatales. While it is possible that they represent a new order within the Phaeophyceae, we recommend that they remain unclassified (incertae sedis) until further work is completed on other crust-forming marine taxa, especially members of the Ralfsiales. While species of H. flaviatilis and B. lauterborni were distinct from the other brown algal species considered in our study, they themselves were closely related, differing by no more than 2.5% (≈ 36 bp). Populations of B. lauterborni consistently nested between the populations of H. flaviatilis from British Columbia and Germany, regardless of method of analysis. Future population studies on both species, perhaps using more variable gene regions (e.g. ITS region, RUBISCO spacer, or microsatellites) may resolve these two taxa.

Molecular data from our study were unable to resolve the ordinal classification of P. flaviatilis (isolate 124.79 from SAG culture collection) and lend support to earlier findings that this species is quite enigmatic (Wilce et al. 1970). Our extraction efforts yielded a sequence of only 814 bp in length, which in part may have led to ambiguous results for this species. Here, the branching distance between P. flaviatilis and the common node for the Ectocarpales clade was > 10.9% or ≈ 160 bp (Fig. 1). While the correct phylogenetic position of P. flaviatilis remains ambiguous, our data provide no support for its inclusion within the Ectocarpales (as classified by Bourrelli 1981). Moreover, the shortest distance of this species to any specific taxon was Dictyota dichotoma (Hudson) Lamouroux (13.0%, ≈ 190 bp difference), suggesting that this species was not closely related to any of the taxa included in the present study. However, if Porterinema truly belongs in the Ralfsiales (as suggested by Wilce et al. 1970), then our data strongly suggest that Heribaudiella and Bodanella cannot also be placed in this order.

Ecological and evolutionary considerations

Ecologically, nearly all populations of Pleurocladia lacastris are distinctly anticoastal in their freshwater distributions (Isaachsen, 1938; Waern 1952; Ekenstam et al. 1996), although one notable population has been collected from just above the high-tide line in the Canadian Arctic (Wilce 1966). The origin of this species from its marine relatives seems difficult to discern at this point. But the increasing number of freshwater Pleurocladia populations being reported substantially inland in Europe (e.g. Szymanska & Zakrys 1990; Kusel-Fetzmann 1996) and North America (Ekenstam et al. 1996, E.B. Young, unpublished observations [Lake Michigan]) support the suggestion by Waern (1952) that this species may be older than the postglacial history of the Northern Hemisphere. For this reason, it would be informative to compare sequences (perhaps using mtDNA) of different populations of Pleurocladia to test distance from the ocean or conductivity (salinity) as possible factors leading to its genetic divergence. Studies on this species are also of interest at the intrapopulation level because of its widespread but apparently disjunct geographic distribution (Waern 1952; Kirby et al. 1972; Ekenstam et al. 1996; Kusel-Fetzmann 1996).

The ecological and biogeographic history of freshwater Ectocarpus is likely quite different. We have observed that in culture this isolate can grow at 0 parts per thousand (ppt) and 7 ppt seawater (unpublished data), whereas West & Kraft (1996) have shown that it grows equally well in 5, 15, and 30 ppt seawater. The authors also noted that the freshwater population was able to acclimate to much wider ranges of conditions than were isolates from fully marine sites. These findings taken together suggest that this freshwater population is likely a marine invader, and may have the potential to expand its range inland in perhaps other estuarine locations. An earlier culture study of clonal (marine) populations of E. siliculosus demonstrated that many geographically distant populations have a sterility barrier, and that Australian populations were infertile with nearly all strains isolated from the Northern Hemisphere (Müller 1979). Being located only 25 km from the coast and 40 m above a waterfall, the Australian freshwater population may have very recently evolved traits that enable its tolerance to fresh water. Future studies on its method of invasion from marine habitats and possible rapid evolution (using the ITS region) vs marine Ectocarpus could prove to be informative (e.g. Müller et al. 1998). Studies are also needed to clarify the phylogenetic position of the two described freshwater species of Sphacelaria (S. flaviatilis Jao, S. lacastris Schloesser et Blum), which have been collected from only three locations worldwide.

The ecology of Heribaudiella is fairly well established, being a species apparently restricted to very clear mountain streams with rapid current velocity, slightly alkaline pH (7–8.5), moderate dissolved P (10–60 µg L⁻¹), NH₄⁺ (15–65 µg L⁻¹ N), and Ca (≈ 10–70 mg L⁻¹), but relatively high specific conductance (≈ 600–1000 µS cm⁻¹) (Wehr & Stein 1985, Wehr & Perrone 2003). Its global distribution includes a few hundred known populations, but many are hundreds or thousands of kilometres from the next nearest population (Wehr 2003). Because of its locally abundant but patchy distribution, further efforts are needed to characterise its genetic, reproductive, and morphological variation. In addition, the morphologies of H. flaviatilis and B. lauterborni, while quite distinct in field populations and laboratory cultures, are genetically quite closely
related. Nonetheless, the morphologies of each remain stable and distinct in laboratory culture (Wehr & McCauley, unpublished data), their ecological and geographic patterns (thus far) do not overlap (Bourrelly 1981, Wehr 2003), and no reports of new localities for Bodanella are known. Whether the production of upright filaments in *Heribaudiella* is a fixed trait and cannot be induced in *Bodanella* remains to be seen. These findings suggest that more extensive biogeographic and molecular studies on these two species are warranted. Like Pleurocladia, their distinctly anticoastal distributions (Wehr & Stein 1985, Wehr 2003) suggest that their freshwater origins are also quite old. Further efforts to understand their biogeographic patterns may also help to better understand their evolutionary origins.

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