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A Molecular Phylogenetic Assessment of Pseudendoclonium

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A MOLECULAR PHYLOGENETIC ASSESSMENT OF
PSEUDENDOCLONIUM

A Thesis Presented

by

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From my perspective, Susan Capistran is the most dedicated program director on this campus. Thank you so much for your assistance, patience and understanding.

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ABSTRACT

A MOLECULAR PHYLOGENETIC ASSESSMENT OF *PSEUDENDOCLONIUM*

SEPTEMBER 2007

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Pseudendoclonium was established in 1900 by N. Wille to include a crust-forming green microalga occurring near the high water line on jetties in Drobak, Norway. Ordinal and familial affiliation of the genus have remained uncertain due to a lack of distinguishing morphological characteristics and because molecular phylogenetic data have not been generated for the type species. Ribosomal SSU rDNA sequence data for *Pseudendoclonium submarinum,* the type species, are presented. Phylogenetic analysis of these data place *Pseudendoclonium* within the Ulvales. SSU rDNA sequence data from three additional species, *Pseudendoclonium basiliense, Pseudendoclonium akinetum* and *Pseudendoclonium fucicola* are included in the analyses and clearly support the hypothesis that *Pseudendoclonium* is polyphyletic. Based on the sequence data, *P. submarinum* and *P. fucicola* share ulvalean lineage, but these algae are not congeneric and *P. fucicola* must be removed from *Pseudendoclonium.* Sequence data support the classification of *P. basiliense* and *P. akinetum* as distinct species of a single genus. The close affiliation of these two species with *Ulothrix* and other Ulotrichalean genera, however, reveals their ordinal separation from *P. submarinum. P. basiliense* and *P. akinetum* must also be removed from *Pseudendoclonium* and require generic reassignment within the Ulotrichales.

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CHAPTER 1

THE GENUS *PSEUDENDOCLONIUM*

1.1 Introduction

Pseudendoclonium was established by N. Wille (1900) to accommodate a crust-forming green alga that he recognized near the high water line on jetties in Drobak, Norway. This microalgal species was described as having short, prostrate and erect irregularly branched filaments, and cells containing a single, parietal chloroplast with one pyrenoid. Wille also described rhizoid-like cells of diminished chlorophyll content presumed to assist attachment of the prostrate basal disc. Wille noticed that sections of the alga easily separated into what he termed *Pleuroccocus*-like fragments and considered this a distinguishing feature. *Pseudendoclonium submarinum* Wille remains the type species of the genus.

Other details of vegetative and reproductive morphology are presented in Wille's original manuscript. Asexual reproduction is accomplished either by the production of ovoid, quadriflagellate zoospores or by akinetes. Akinetes are described as being of two types: thick-walled resting cells and thin-walled cells that germinate without a dormant period. Sexual reproduction was not observed by Wille or subsequent investigators of the species.

In liquid culture the mature thallus appears as a tuft of highly-branched short filaments arising from the basal disc (Fig.1.1, Fig. 1.2). Tufts are thus roughly circular in outline when viewed from above. They are more or less hemispherical in vertical section as longer and more highly branched filaments arise from the center of the

thallus. Branching is highly irregular and without any distinct pattern or apparent

organization. Margins are uneven due to the haphazard protrusion of filaments. The alga

attaches to any firm substratum. It eventually forms a continuous crust as new tufts arise

from zoospores or akinetes and develop between or upon the older thalli. Individual

mature tufts measure 0.5 to 1.0 mm in diameter. Organisms several years in age attained

a maximum diameter of 1.6 mm.

During the past century eight additional species have been referred to

Pseudendoclonium on the basis of morphological and reproductive features. A complete

list of species is given (Table 1.1).

The following systematic history of *Pseudendoclonium* illustrates the

uncertainty encountered in the use of classical morphological characteristics to

reconstruct phylogeny.

1.2 Taxonomic History

Familial association of *Pseudendoclonium* with the Chaetophoraceae originated

with Wille. The new alga was placed in this family primarily because of its much-

branched heterotrichous growth habit. Wille considered *P. submarinum* to be a highly

reduced and adapted form descended from the upright and highly branched

Stigeoclonium and *Endocladium* (Wille 1900).

Collins (1909) was first to describe the occurrence of *Pseudendoclonium*

submarinum in North America from the coasts of three New England states and retained

its familial placement in the Chaetophoraceae. In a broad study of green algae, Collins

proposed a system of six orders within the class Chlorophyceae. His very broadly

circumscribed order Ulotrichales included virtually all branched and unbranched

filamentous green algae from freshwater and marine habitats. In the Ulotrichales he placed the Chaetophoraceae including *Pseudendoclonium*, the Ulotrichaceae, the Ulvaceae and six other green algal families.

Fritsch (1935) acknowledged simularities between Ulotrichaceae and the Chaetophoraceae, but removed the Chaetophoraceae from Ulotrichales and elevated the family to ordinal status. Heterotrichy, the differentiation of the vegetative body into both a prostrate and an erect system of branching filaments, distinguished taxa of the new order, Chaetophorales. Fritsch considered this vegetative differentiation to be taxonomically fundamental and on this basis retained unbranched filamentous forms in the Ulotrichales. He assigned two families, the Chaetophoraceae and the Trentepohliaceae to the Chaetophorales. In the former were included the "more reduced" forms, or those taxa whose erect systems were diminished or absent. Forms placed in the Trentepohliaceae were the presumably "more primitive" examples that had retained a robust erect system. Fritsch placed *Pseudendoclonium* in the Trentepohliaceae presuming a close alliance with *Gongrosira.*

During the two decades following the compilation by Fritsch, systematics of filamentous green algae based on general morphology became increasingly confused and controversial. Smith(1950), Printz, (1964), and Bourrelly, (1966) each proposed taxonomic systems that differed significantly at the familial and ordinal levels. It was Bourelly's system that would be generally accepted and included the more restrictive orders Ulotrichales, Ulvales and Chaetophorales. Bourelly assigned six families to the Chaetophorales and divided the Chaetophoraceae into three sub-families. He placed

Pseudendoclonium in the sub-family Leptosiroideae with approximately thirty-five

other genera based on general morphology (Bourelly, 1966).

During the 1970's, ultrastructural studies of mitosis, cytokinesis and the flagellar

apparatus led to the erection of the class Ulvophyceae to accommodate *Ulva* and related

genera that shared a number of ultrastructural characteristics. A counter-clockwise

orientation of basal bodies in motile cells and the absence of a phycoplast during

vegetative cell division were the definitive characters of the class (Stewart, Mattox and

Floyd, 1973, Stewart and Mattox, 1978, Mattox and Stewart, 1984, O'Kelly and Floyd,

1984a). A summary of all available ultrastructural information included data from

Pseudendoclonium submarinum, P. basiliense and *P. akinetum* (O'Kelly and Floyd,

1984b). These observations supported inclusion of *Pseudendoclonium* in the Ulotricales

and therefore the Ulvophyceae, but also revealed differences between the three species

that cast doubt on the monophyly of the genus. *P. basiliense* and *P. akinetum* possess

minute, diamond-shaped body scales on the surface of zoospores. Such scales are absent

from zoospores of *P. submarinum.* Also, in *P. submarinum* and *P. basiliense* basal

bodies are nearly perpendicular to the long axis of the zoospore while in *P. akinetum*,

like *Ulothrix zonata,* a V-shaped configuration is evident.

The preceeding account cites significant developments in the effort to

reconstruct a natural phylogeny of filamentous green algae relying primarily on light

and electron microscopy. More recently, use of molecular sequence data has both

supported and expanded our hypotheses regarding these phylogenetic concepts. DNA

sequence data from the nuclear-encoded small subunit (SSU) ribosomal RNA gene (18s

rDNA) and others, including genes from the chloroplast, are routinely used along with

morphological and ultrastructural data to infer phylogenetic relationships among the green algae (Friedl 1996, Hayden and Waaland 2002, O'Kelly *et al.* 2004).

Circumscription of the Ulvophyceae remains controversial as elevation of some orders to class rank has been proposed by several investigators (van den Hoek *et al*, 1995). There has also been debate over the inclusion of the Trentepohliales in the Ulvophyceae (López-Bautista and Chapman, 2003). There is consensus, however, that the Ulvales and Ulotricales form a monophyletic group within the Ulvophyceae, are closely related, and represent early divergent forms of the class (Zechman *et al* 1989, Watanabe *et al,* 2001). Available data suggest that species of *Pseudendoclonium* are closely related to other taxa of the Ulotrichales and Ulvales.

Previous phylogenetic studies based on gene sequence data include many genera of the Ulotricales and Ulvales (Friedl, 1996, Hayden and Waaland, 2002, O'Kelly *et al* 2004a & b, Lindstrom and Hanic, 2005). At the species level, SSU rDNA sequence data are available for *Pseudedoclonium basiliense* and *P. akinetum.* Partial SSU rDNA sequence data are available for *P. fucicola.*

In the current study, SSU rDNA sequence data for the type species of *Pseudendoclonium, P. submarinum* are presented. These data are utilized along with previously published data in phylogenetic analyses designed to establish ordinal affiliation of *Pseudendoclonium* and test monophyly of the genus from a molecular phylogenetic perspective.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation and Culture

Specimens of *Pseudendoclonium submarinum* from Drobak, Norway were provided by R. Nielsen (Copenhagen) and J. Rueness (Oslo) collected independently in 1999 at Wille's type locality. Unialgal cultures were developed from this inoculum.

Culture of *Pseudendoclonium submarinum* began with the preparation of von Stosch's enriched seawater medium as previously described (von Stosch 1964).

Salinity of the initial seawater medium at 35 ppt was lethal to all specimens in culture. Many experimental dilutions of seawater media confirmed that optimal growth conditions were realized when salinity was reduced to the natural level found in brackish estuaries. Growth also improved with reduction of the concentrations of added nutrients. The final medium contained seawater diluted by 50% with sterile, distilled water and a 50% reduction of the concentration of added nutrient salts. This modified von Stosch's enriched seawater medium measured 16 ppt dissolved salts and was utilized exclusively in the algal cultures with the addition of one or more antibiotics. Culture medium was renewed at least once per month.

Unialgal cultures were grown in 20 X 100 mm glass Petri dishes under fluorescent and incandescent light at an intensity of 5-20 μmol photons $\cdot$ cm^{-2} $\cdot$ s^{-1} and a photoperiod of 12 hours darkness / 12 hours light at an average temperature of 16° C.

Contamination of cultures by unwanted organisms was eliminated by the use of antibiotics and serial dilution. Diatoms were controlled by addition to the medium of 6

mg/liter germanium dioxide to inhibit frustule development. GeO_2 is not readily soluble in water; the germanium solution was heated to 70° C with constant stirring to facilitate dissolution. Penicillin or ampicillin and streptomycin were added for control of cyanobacteria and were effective against all but a single strain of a coccoid cyanobacterium. Field collected samples of *Pseudendoclonium submarinum* were invariably contaminated with a fungus, identified as a species of *Cladosporium* that thrived in the unialgal cultures of this organism. The anti-fungal amphotericin-B proved to be effective for control of this fungus. This compound is also insoluble in water necessitating pre-dissolution in dimethyl sulfoxide before addition to the medium solution. Elimination of the fungus in cultures with heavy contamination required the use of amphotericin-B at 10 mg/L, or four times the recommended concentration. In new cultures, and cultures from which the fungus had been eliminated, the standard concentration of 2.5 mg/L effectively prevented fungal growth. The concentrations of nutrients, vitamins and antibiotics are given (Table 2.1).

2.2 Microscopy

Cultures were monitored using a Nikon SMZ800 dissection microscope equipped with an Excel Technologies F0-150 illumination system (Excel Technologies, Enfield, CT, USA). Cellular morphology was observed using an Olympus Model BH-2 compound microscope. Digital images from both microscopes were captured utilizing a Model 18.2 Color Mosaic digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.3 DNA Extraction

200 mg samples were scraped from Petri dishes of pure algal cultures using a stainless steel razor blade after draining of the liquid medium and partial air drying. Material of the species from several culture dishes were typically harvested in order to obtain sufficient material for DNA extraction. Samples were air-dried for several hours to a weight of approximately 75 mg. The moisture content of the harvested material proved to be a critical factor in the disruption process and extensive air-drying of the material was required. The samples were next placed in a 2 ml microcentrifuge tube containing 8-10 2.3 mm glass beads and frozen in liquid nitrogen for 30 seconds. Cells were disrupted using a Mini Bead Beater (Biospec Products, Bartlesville, OK, USA) at maximum speed (46,000 Hz) for one or two 20-second cycles with an additional cooling step between cycles of 30 seconds in liquid nitrogen. After bead mill processing, the material was again frozen in liquid nitrogen for 30 seconds at which time lysis buffer was immediately added. Genomic DNA was then extracted and purified using the DNeasy Mini Plant Kit (Qiagen Inc., Valencia, CA, USA) or the Phytopure DNA Purification Kit (Tepnel Life Sciences, Manchester, UK) according to the manufacturer's directions. The Phytopure kit consistently yielded genomic fragments of greater length than did the Qiagen kit and the use of template DNA from the former kit increased PCR efficiency significantly.

2.4 DNA Amplification

The nuclear encoded small-subunit ribosomal DNA (18S rDNA) gene was amplified using two separate polymerase chain reaction series that generated fragments of approximately 800 and 1000 base pairs in length. This strategy was utilized after

realization that PCR product from the smaller fragments had a much higher DNA concentration and purity than product obtained by amplifying the gene as a single fragment.

A 200 µL polymerase chain reaction master mix was prepared containing 129 µL sterile distilled water, 20 µL Amplitaq PCR BufferII (Applied Biosystems, Foster City, CA, USA), 16 µL 25mM $MgCl_2$, 4 µL of each 0.2mM dNTP (New England Biolabs Inc., Ipswich, MA, USA), and 8 µL of each oligonucleotide primer (Integrated DNA Technologies Inc., Coralville, IA, USA) at a concentration of 10µM. Immediately prior to each reaction 1.0 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 2 µL of genomic DNA were added and the mix divided into four 50 µL reactions in 0.5 ml reaction tubes. Reactions were run in a Mini Cycler (MJ Research Inc., Watertown, MA, USA) with a reaction profile of 3 min initial denaturing and enzyme activation at 95° C, and 35 cycles of 45 seconds denaturing at 94° C, 45 seconds annealing at 50° C and 90 seconds extension at 72°C with a final extension of 5 min. at 72° C. Four 50 µl reactions were subsequently pooled for analysis.

2.4.1 PCR Primers

The two regions of the SSU gene that were separately amplified were designated 18sA and 18sB. The 18sA fragment includes positions 2 – 811 and was amplified with a forward primer from a previously published study (O'Kelly *et al* 2004). A compatible reverse primer was chosen from conserved regions of the previously published sequence of *Pseudendoclonium basiliense* (Genbank accession Z47996) with the aid of the IDT website tools (www.idt.com). 18sB includes positions 788 – 1771 and in this

case both primers were designed for this study. A complete list of primers used in this study is given (Table 2.2).

2.5 Detection and Analysis

PCR products were visualized by gel electrophoresis in 1%, 50 ml agarose gels stained with 1.5 μg ethidium bromide solution (Sigma, St. Louis, MO) and examined in ultraviolet light. 3 μL of PCR product were typically run for 90 min. at 100 volts DC. DNA concentrations were estimated by comparison of band intensity with known concentrations present in a 1kb DNA ladder (New England BioLabs, Ipswich, MA).

2.5.1 Purification

PCR products were first prepared for sequencing using the QIAquick PCR Purification Kit (Qiagen) but products purified in this manner resulted in ambiguous and unreliable sequence data. Subsequent purification utilizing the QIAquick Gel Extraction Kit (Qiagen) yielded PCR product of sufficient purity to generate reliable sequence. For the gel extraction protocol, 35-50 μL of PCR product were loaded into each of two adjacent lanes of a 100 ml agarose gel along with 10 μL of loading dye. After electrophoresis, the resulting bands were excised from the gel and purified according to the manufacturer's instructions.

2.5.2 Sequencing

Purified products were sequenced utilizing the dideoxy chain termination method (Sanger *et al.* 1977) in an ABI automated sequencer at the University of Maine, Orono sequencing facility. Sequencing was performed primarily in the forward direction utilizing sequencing primers spaced approximately every 400 bp over the

length of the gene. Reverse sequencing primers at approximately 300 bp from the beginning of each fragment and overlap of the forward sequences provided verification.

2.5.3 Sequencing Primers

Sequencing primers were chosen from previously published work or designed for this study in the above fashion as noted (Table 2.2). PCR primers seldom yielded acceptable results in the sequencing reactions. Sequencing primers that included the latter end of the PCR primer sequence and extended beyond it, however, returned excellent results in most cases.

2.5.4 Sequence Editing

Editing was effected by alignment of the raw chromatogram data with previously published sequences of the closely related species *Ulothrix zonata* and *Pseudendoclonium basiliense* in Clustal X (Higgins and Sharp 1988; Thompson *et al.* 1997; Thompson *et al.* 1994). Each site of the newly generated sequence data was then evaluated with respect to the integrity of the chromatogram signal, its alignment with the known sequence data and its alignment with overlapping data. Editing was performed in BioEdit (Hall 1999). Each edited data file was sequentially added to a master alignment in Clustal X until the complete sequence of the two regions was realized.

2.6 Phylogenetic Analysis

Molecular phylogenetic analyses presented in this study are modelled, to a large extent, after that of a previously published study of the Ulvophyceae (Hayden and Waaland 2002). Hayden and Waaland investigated the systematics of the orders Ulvales and Ulotrichales using partial SSU rDNA sequence data and sequence data from the large subunit of the chloroplast encoded RUBISCO gene. Phylogenetic analyses were presented for twenty taxa first using separate sequence data from each of the genes and a third analysis using a combined data matrix from both genes. Tree topologies were nearly identical in all analyses and clearly recovered the two orders as well-supported monophyletic groups. Data from *Pseudendoclonium fucicola* included in their study clearly placed this species within the Ulvales clade. This brought into question the monophyly of the genus since a previous molecular phylogenetic investigation had shown a close affiliation of *Pseudendoclonium basiliense* with the Ulotricales (Friedl 1996).

The suggestion of Hayden and Waaland that *Pseudendoclonium* is polyphyletic is tested in the current study by implementation of two analyses similar in scope to theirs, but also including data from *P. submarinum, P. basiliense* and *P. akinetum.* Importantly, and a primary objective of this study, these analyses will infer an ordinal alliance of the type species of *Pseudendoclonium* (*P. submarinum*) which is unresolved. Resolution of the phylogenetic position of the type species must preclude any discussion of the systematics of the genus.

The partial 18s sequences generated by Hayden and Waaland represent a 753 bp fragment spanning the variable 4 region of the gene and represent only 42 percent of the

gene. Since this partial sequence data is the only data available for *P. fucicola* and the closely related genera *Tellamia* and *Kornmannia*, two separate analyses of 18s rDNA sequence data are presented in this study. For most genera in the Hayden and Waaland analysis, full 18s sequence data is available for species other than those for which these authors generated partial sequences. This allows presentation in this study of two similar analyses, one using only partial sequence data including that of *P. fucicola,* and another utilizing complete SSU sequence data that excludes this species, *Kornmannia leptoderma* and *Tellamia contorta.*

Sequences were aligned in Clustal X for both analyses and edited by eye using BioEdit. Sequences were truncated to equal lengths. Gap opening and gap extension penalties were set to the default values. Alignment files were exported in the NEXUS format for use by PAUP*.

Heuristic searches were first conducted under the MP criterion in PAUP* for both analyses with tree bisection-reconnection and MULTREES options in effect. Character state changes and nucleotide positions were unweighted and introduced gaps were coded as missing data. Ten replicate searches with randomized taxon input were conducted using the random stepwise-addition method. Branch support was estimated by nonparametric bootstrap analysis (Felsenstein 1985) with 2000 replicates.

Appropriate evolutionary models for the maximum likelihood analyses were determined by hierarchical likelihood ratio tests of each data matrix using Modeltest v.3.7 (Posada and Crandall 1998). From the full-length sequence data set the chosen model was a Tamura-Nei 1993 model with equal base frequencies, an estimated proportion of invariable sites ($I = 0.5815$), and gamma distributed rate variation among

sites (α = 0.6095). The substitution rate matrix is as follows: A-C = 1.0000, A-G = 2.5585, A-T = 1.0000, C-G = 1.0000, C-T = 4.3987, G-T = 1.0000.

In the Modeltest analysis of the partial sequence data set, the chosen model was the TIMef + I + G model known as the Transversion model with equal base frequencies, an estimated proportion of invariable sites (I = 0.6337), and gamma distributed rate variation among sites (α = 0.5914). The substitution rate matrix is: A-C = 1.0000, A-G = 3.7630, A-T = 2.2750, C-G = 2.2750, C-T = 8.5392, G-T = 1.0000. Heuristic searches were conducted with the same options in effect as in the MP searches. Bootstrap analysis with 100 replications under the ML criterion was used to estimate branch support (Felsenstein 1985).

CHAPTER 3

RESULTS

3.1 General

The complete nucleotide sequence of the 18s SSU rDNA gene for *Pseudendoclonium submarinum* Wille from Drobak, Norway is identified (Table 3.1). Sequence data has been deposited in Genbank accession number EF591129.

Alignment of the ingroup taxa for both the partial and full sequence analyses was straightforward; few gaps were introduced. The Trebouxiophytes, *Chlorella vulgaris* and *Myrmecia astigmatica* were included as outgroup taxa following Hayden and Waaland (2002). Sequences for these taxa introduced the shortest branches of all available outgroups from the Ulvophyceae. Insertions in the outgroup taxa were removed at several sites to prevent resulting gaps in the entire alignment.

Maximum likelihood trees inferred from both partial and full-length SSU data recovered the orders Ulvales and Ulotricales as monophyletic sister groups. This assessment must be qualified by the following two facts. The first is that bootstrap support for the branch leading to the Ulotricales in both analyses under the ML criterion is weak. Secondly, O'Kelly *et al.* (2004a) show that when certain coccoid ulotricalean genera (not included here) are included in an analysis spanning both of these orders, Ulvales are recovered as a monophyletic group within a paraphyletic order Ulotricales. Support for monophyly of the Ulvales was robust in these analyses. Relationships among taxa closely allied with *Ulothrix zonata* are not resolved in either analysis due to the high degree of conservation in the 18s gene. Among these taxa, putative species may be separated by less than 10 substitutions in the entire gene.

3.2 Partial-Sequence Analysis

Pseudendoclonium fucicola, Tellamia contorta and *Kornmannia leptoderma* are excluded from the full-length SSU sequence analysis. Only partial SSU sequence data are available for these taxa. They are included in an analysis of partial SSU sequence data, along with partial-sequence data from all taxa included in the full-length sequence analysis. It has previously been shown that *P. fucicola, T. contorta* and *K. leptoderma* group together with *Blidingia* to form a distinct clade within the Ulvales (Hayden and Waaland 2002). This clade is recovered as sister to the clade containing the Ulvaceae and *Acrochaete.* These clades represent separate lineages within the Ulvales. The partial sequence analysis indicates that *P. submarinum* groups within the clade containing *P. fucicola, T. contorta, K. leptoderma, P. salina* and *Blidingia dawsonii.* Inclusion of taxa known to group closely with *P. fucicola* is useful in the evaluation of the evolutionary relationship between *P. submarinum* and *P. fucicola.*

Alignment of the partial-sequence data consisted of 749 total characters of which 611 were constant, 138 were variable and 102 were parsimony-informative. Heuristic searches under the MP criterion returned 32 most parsimonious trees of 281 steps in length. Three trees shared topology with the maximum-likelihood tree (Fig. 3.1). The most significant difference relative to this discussion between the MP and ML trees is the placement of *Pirula salina* with respect to *Pseudendoclonium submarinum.* In one half of the MP trees, *P. salina* is basal with regard to *P. submarinum,* while in the other sixteen MP trees these species are placed sister to each other as they are in the ML tree. The remaining two instances of noncongruity among MP trees involve relationship between *Percursaria* and *Ulvaria* and that between *Acrosiphonia* and

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Gloeotilopsis. These inconsistencies do not directly impact the present discussion of *Pseudendoclonium.*

The likelihood score of the ML tree from partial-sequence data is substantially lower (-ln L = 2541.69159) than that of the full-sequence tree (-ln L = 5817.70501) and reflects the lack of phylogenetic signal in the partial sequence data. The high degree of conservation within the SSU gene among taxa that group closely with *Ulothrix* precludes resolution of the generic relationships among *Pseudendoclonium basiliense, P. akinetum, Trichosarcina,* and *Ulothrix*. The data support only the conclusion that *P. basiliense* and *P. akinetum* group within the ulotricalean lineage and are closely related to *Ulothrix zonata* and *Trichosarcina mucosa.*

On the other hand, the ulvalean clade containing the type species, *P. submarinum* and *P. fucicola* is well supported under both the MP and ML criteria. Evolutionary relationships among *Tellamia, Blidingia, Kornmania, Pirula* and the two species of *Pseudendoclonium* are well resolved. *P. submarinum* and *Pirula salina* form a subclade that is sister to the remainder of the group, though these branches are weakly supported in the bootstrap analyses. *Kornmania* and *Blidingia* occupy positions basal to a terminal subclade formed by *P. fucicola* and *Tellamia contorta.* Bootstrap support for the latter two branches is robust.

Although the sequences of *P. submarinum* and *P. fucicola* are recovered as part of this clade, their phylogenetic positions within in the clade are distant. Comparison of the 749 bp partial SSU sequence data of these two species reveals that their sequences differ by 36 base pairs. The difference would be significantly greater if all variable

regions of the *P. fucicola* gene were considered, and greater than distances that typically

separate genera within these two orders.

3.3 Full-Sequence Analysis

Alignment of the full sequence SSU data for the 22 taxa included in this study

was also straightforward. There were 1683 total characters of which 1344 were

constant, 339 were variable and 232 were parsimony-informative. Heuristic searches

under the MP criterion returned 2 most parsimonious trees of 642 steps in length. One

of these trees shared topology within the Ulotricales and the ulvalean clade containing

P. submarinum with the maximum-likelihood tree shown (Fig. 3.2). This tree differed

from the ML tree in placement of *Enteromorpha, Ulva, Percursaria* and *Ulvaria.*

Relationships within the Ulvaceae are not well resolved in this analysis. However

bootstrap support for monophyly of the Ulvales and for the Ulvaceae is robust.

The full-sequence analysis recovers *P. submarinum* and therefore the genus as

part of the Ulvalean lineage. *P. submarinum* and *Pirula salina* form a terminal subclade

in a monophyletic group that includes *Blidingia* and *Bolbocoeleon. Blidingia dawsonii*

occupies a position sister to the *P. submarinum* and *P. salina* clade and bootstrap

support for the branch leading to these three taxa is robust. Tree topology differs

between the partial and full sequence analyses with respect to the position of

Bolbocoeleon piliferum. In the partial sequence analysis this species occupied a position

basal to the entire clade that includes *P. submarinum.* Here, *Bolbocoeleon* is placed in a

position sister to the remainder of the clade and boostrap support for the entire clade is

poor. Support for the clade excluding *Bolbocoeleon* in the partial sequence tree was

robust. Placement of *Bolbocoeleon* within this clade seems equivocal. Excepting the enigmatic position of *Bolbocoeleon*, relationships within the clade are well resolved.

The Ulotrichales were again recovered as a monophyletic group in the full-sequence analysis. Bootstrap support for the ulotrichalean clade was robust under the MP criterion but poor in the ML analysis. Two subclades containing *Acrosiphonia* and *Gloeotilopsis* received robust bootstrap support under both criteria. Generic relationships among *Pseudendoclonium basiliense, P. akinetum*, *Monostroma grevillei, Trichosarcina mucosa* and *Ulothrix zonata* were not clearly resolved due to low sequence variation among these taxa. The polytomy including *P. basiliense, U. zonata, M. grevillei,* and *T. mucosa* recovered in the partial sequence analysis is better resolved utilizing the full sequences. However, bootstrap support for the topology of the tree in this region is poor. The sequence data support a close affinity of *P. basiliense* and *P. akinetum* with *Ulothrix* and therefore their inclusion within the Ulotricales.

CHAPTER 4

DISCUSSION

4.1 Generic

The objectives of this study were: (1) determination of the taxonomic position of *Pseudendoclonium* inferred from analysis of SSU rDNA sequence data obtained from isolates of *P. submarinum,* the type species and (2) molecular phylogenetic evaluation of the monophyly of the genus with respect to three species currently assigned to *Pseudendoclonium* for which SSU rDNA sequence data are available.

Phylogenies reconstructed in the present analyses clearly support the hypothesis that *Pseudendoclonium,* as it is currently circumscribed, is polyphyletic. These analyses are based on SSU rDNA sequences that include data from the type species of the genus. These data clearly indicate that the genus requires redefinition. The three species currently assigned to *Pseudendoclonium* discussed in this study must be removed from the genus. *P. submarinum* and *P. fucicola* are part of the ulvalean lineage. Based on the molecular sequence data generated and reviewed during this study, these two species are not congeneric. From a genetic perspective, *P. basiliense* and *P. akinetum* are clearly ulotrichalean, and group closely with *Ulothrix zonata* despite significant morphological differences. On the basis of molecular analysis, *P. basiliense* and *P. akinetum* should be placed in a single genus as they are certainly closely related. Unquestionably, *P. basiliense* and *P. akinetum* lack affinity with *Pseudendoclonium* and require an alternate generic identity.

Pseudendoclonium submarinum, P. fucicola, P. basiliense and *P. akinetum* are remarkably similar in vegetative and reproductive morphology. All are minute forms

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with cells 5 to 10 μm in length and filaments of only 5 to 20 cells in length. They share a heterotrichous growth habit having irregularly branching vertical filaments arising from a prostrate basal disc of cells some of which are rhizoid-like in appearance and function. All possess a single, parietal chloroplast containing one distinctive pyrenoid. Species have been separated based on general morphological and reproductive characteristics. These characteristics include overall size, proportion of basal and vertical filaments, extent of branching and akinete characteristics. Morphological characteristics such as these may be subject to environmental variability; their usefulness as taxonomic markers is questionable.

P. submarinum and *P. fucicola* are primarily marine species, though *P. submarinum* occurs in brackish estuaries. *P. basiliense* and *P. akinetum* are freshwater epiphytes of a number of aquatic angiosperms (Vischer 1926, Tupa 1974).

4.2 Specific

Pseudendoclonium remained monospecific until Vischer (1926) described *P. basiliense.* Vischer isolated the alga from a small pool in a garden stream at Bâle (Basel) Switzerland. Vischer's cultures have been lost, however Tupa (1974) described an epiphytic alga from various freshwater locations in Texas that she believed to be *P. basiliense*. Details of vegetative and reproductive morphology of *P. basiliense* are nearly identical to those of *P. submarinum* and *P. fucicola*. Mature, erect filaments in all three species become biseriate or pleurococcoid in appearance. This is a distinguishing feature that Wille noted in *P. submarinum.* Filaments of *P. basiliense*, both prostrate and erect, are several cells longer than those of *P. submarinum* and *P. fucicola* so that the thallus is generally larger (Tupa 1974).

Zoospore germination is bipolar in *P. basiliense* in contrast to the unipolar germination observed in *P. submarinum.* Zoospores of *P. basiliense* are quadriflagellate; no biflagellate motile cells have been described (Tupa 1974). Ultrastructural studies reveal that zoospores of *P. basiliense* are covered with distinct body scales, a ulotricalean characteristic absent from zoospores of *P. submarinum* (Mattox and Stewart 1973, Floyd and O'Kelly 1984).

Tupa (1974) also collected and described two new species which she assigned to *Pseudendoclonium. P. akinetum* and *P. prostratum* are freshwater epiphytes collected from a number of aquatic hosts in widely separated localities in Texas and Illinois. *P. prostratum* is so named because of the virtual absence of upright filaments. Its thallus is described as "a richly branched attached prostrate filament, a single layered, pseudo-parenchymatous disc with or without erect branches" (Tupa 1974). Lack of sequence data from this species precludes any further discussion in the present study.

Pseudendoclonium akinetum (Tupa 1974) is distinguished from *P. submarinum, P. fucicola* and *P. basiliense* by its production of characteristic akinetes, and the spherical shape of its cells. It is further distinguished by the paucity of erect filaments, the shorter length of filaments and the lack of biseriate or "pleurococcus-like" mature filaments. Akinetes are thick-walled and often surrounded with a granular, flaky, dark reddish-brown material. These akinetes measured up to 40 μm diameter and are much larger than any described for the other species (Tupa 1974, Yarish 1975). Like *P.basiliense,* the zoospores of *P. akinetum* possess tiny, organic body scales (Floyd and O'Kelly 1984).

SSU rDNA sequence data support the hypothesis that *P. basiliense* and *P. akinetum* are closely related, but distinct species. These sequences differ by seven substitutions of 1683 positions considered. This difference is comparable to interspecific variation within other genera of the Ulotricales for which sequence data are available. However, the SSU gene is highly conserved within the ulotricalean lineage. The single gene is not sufficiently variable to reliably recover phylogeny at the generic and specific levels in all instances. SSU rDNA sequences of *P. akinetum* and *Ulothrix zonata,* for example, differ at only 9 positions, a difference that corresponds to a specific difference within several other putative genera of the lineage. Sequence data support only the conclusion that *P. basiliense, P. akinetum* and *U. zonata* are very closely related organisms within the taxonomic concept of the Ulotrichales. Thus the contention that *P. basiliense* and *P. akinetum* be considered congeneric taxa relies heavily on vegetative and reproductive morphological evidence.

Ulvella fucicola was described by Rosenvinge (1893), a marine epiphyte of *Fucus inflatus* (= *F. evanescens*) collected from the west coast of Greenland at Egesdesminde. Wille (1909) transferred *U. fucicola* and a second species, *U. confluens*, to *Pseudopringsheimia* separating both from *Ulvella* and *Pseudulvella* based on "the presence (in *Pseudopringsheimia*) of rhizoidal outgrowths from the base penetrating the host" (Setchell and Gardner 1920). It is noteworthy that Wille did not consider transferring *U. fucicola* to *Pseudendoclonium* at this time since he had described *P. submarinum* nine years previously. Nielsen (1980) examined several isolates of *Pseudopringsheimia fucicola* in culture for comparison of this alga with *P. submarinum*. She distinguished the two species by the position and shape of the

sporangia. Sporangia of *P. fucicola* are described as being conical in shape and developing only from the uppermost cells of the vertical filaments. Several authors who previously studied *P. submarinum* including Wille, noted that its sporangia develop from any vegetative cell of the upright filaments. Citing numerous morphological similarieties between the two species, Nielsen transferred *Pseudopringsheimia fucicola* to *Pseudendoclonium* but made little note of species distinction.

Nielsen was the first author to observe both quadriflagellate and biflagellate motile cells in both organisms. In one isolate of *P. submarinum* she observed that biflagellate swarmers were of two different sizes and were released from larger sporangia than were the quadriflagellate zoospores. Nielsen did not determine whether biflagellate and quadriflagellate swarmers arose from separate thalli. Biflagellate motile cells observed in cultures of *P. fucicola* were of equal size and were released from separate, morphologically similar thalli. This observation supports isomorphic alternation. However, copulation between biflagellate swarmers was not observed for either species.

Comparison of SSU rDNA sequence data from *Pseudendoclonium submarinum* and *P. fucicola* reveal a level of sequence variation that is comparable to or greater than variation between putative genera of this lineage. Based on these data, *P. submarinum* and *P. fucicola* cannot be considered congeneric. *P. fucicola* must therefore be removed from *Pseudendoclonium.*

The principal morphological features that distinguish the Ulotricales and Ulvales are present only in sexually reproductive forms. A *Codiolum*-type zygotic stage in the life cycle typifies the life history of members of the Ulotricales. This club-shaped

zygote contains the only diploid cell of the life history of these species. In contrast, sexually reproductive algae of the Ulvales, where known exhibit an isomorphic, diplohaplontic life cycle in which haploid gametophytes alternate with morphologically identical diploid sporophytes. However, sexual reproduction has not been described for any of the four taxa considered in this study and considerable knowledge of life histories is lacking. Morphological features that might be used to support the molecular data regarding ordinal affiliation of these taxa are therefore few. Following is a summary of these characters.

The occurrence of minute, organic body scales on the surface of zoospores is one feature that supports ordinal separation of *Pseudendoclonium basiliense* and *P. akinetum* from *P. submarinum.* These scales are very similar to the inner body scales of the Prasinophyceae, a green algal class of unicellular organisms considered ancestral with respect to the Ulvophyceae. The scales are thus considered a vestigal character (van den Hoek *et al.* 1995). The scales are present on zoospores of *P. basiliense, P. akinetum* and *Ulothrix zonata.* They are not present on zoospores of *P. submarinum* and have not been reported to occur on those of *P. fucicola* or any other algae of ulvalean lineage. The presence of the scales supports the close relationship of *P. basiliense, P. akinetum* and *Ulothrix* indicated by the molecular data. The absence of scales from *P. submarinum*, and *P. fucicola* separates these taxa from *P. basiliense* and *P. akinetum.* This is congruent with the view that the Ulvales are the more derived lineage of the two orders.

Both biflagellate and quadriflagellate zoospores have been observed in cultures of *P. submarinum* and *P. fucicola*, but only quadriflagellate swarmers have been noted in *P. basiliense* and *P. akinetum* (Vischer 1926, Tupa 1974, Neilsen 1980).

Zoospore germination is unipolar in *P. submarinum* and *P. fucicola* while bipolar germination has been described for both *P. basiliense* and *P. akinetum* (Vischer 1933, Tupa 1974, Neilsen 1980).

Two morphological differences support generic separation of *Pseudendoclonium submarinum* and *P. fucicola.* The zoosporangia of *P. fucicola* develop only in the most distal cells of the upright filaments (Nielsen 1980). *P. fucicola* shares this feature with species of *Pseudopringsheimia,* from which it was transferred by Nielsen (1980). Zoosporangial development in *P. submarinum,* on the other hand, may occur in any cell of the mature, upright filaments. *P. submarinum* is further distinguished from *P. fucicola* by the size of biflagellate motile cells. Both organisms produce biflagellate and quadri-flagellate motile cells. The biflagellate cells of *P. submarinum* are of two distinct sizes while those of *P. fucicola* are of equal size (Nielsen 1980).

CHAPTER 5

CONCLUSION

5.1 Evolutionary Considerations

Molecular phylogenies recovered in this study are inconsistent with long-standing and widely-accepted taxonomy regarding the circumscription of *Pseudendoclonium*. These results were unexpected. Phylogenies recovered using molecular sequence data are typically consistent with traditional phylogenies inferred from morphological diversity. This is evident in previous molecular phylogenetic investigations of the Ulotrichales and Ulvales (O'Kelly *et al* 2004a, Friedl 1996, Zechman *et al* 1990). *Gloeotilopsis, Achrocheate, Phaeophila* and *Acrosiphonia* are examples of genera that were first circumscribed on the basis of morphological criteria. Subsequent molecular-based phylogenies clearly recover these genera as monophyletic groups. Morphological similarity among the "species" of *Pseudendoclonium* discussed in this study is sufficient to warrant their inclusion in a single genus. Here, molecular evidence does not support morphological phylogeny. Possible explanations for this dichotomy are difficult to test experimentally and must remain, at least presently, somewhat speculative.

The ulotrichalean and ulvalean lineages are widely considered to be early-diverging lineages among the green algae. Fritsch (1935) hypothesized that the unbranched filamentous form of *Ulothrix* represents a single evolutionary step beyond the motile, unicellular condition characteristic of the ancestral Prasinophyceae. The heterotrichous condition, characteristic of the organisms discussed in this study, is a simple elaboration of a subsequent innovation in the evolution of green algae; the

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branched filament. These ancient organisms or their immediate ancestors were among the first eukaryotic, multicellular autotrophs. It is likely that these rudimentary green algae predate the appearance of vascular plants by several hundred million years.

The heterotrichous growth habit is a familiar and evolutionarily convergent form evident in several major lineages of algae. The simplicity and efficiency of a prostrate system of filaments for substratum attachment and vertical filaments extending toward insolation has evolved many times. Considering the antiquity of the ulotrichalean and ulvalean lineages, there has been ample evolutionary time for any number of organisms to converge upon this common form, persist or become extinct and for others to subsequently evolve as well. This has likely occurred several times and separately within both the ulotrichalean and ulvalean lineages. The significant number of synapomorphies in cytological structure among the organisms considered in this investigation support this view.

5.2 Specific Assignment

Pseudendoclonium is typified in this study by elucidation of the SSU rDNA gene sequence for *Pseudendoclonium submarinum* Wille. Unialgal cultures of this organism were established with innoculum collected at the type locality in Drobak, Norway. Genomic DNA extracted from the unialgal cultures was used to generate sequence data. Phylogenetic analyses place *P. submarinum* within the Ulvales as part of a clade representing a lineage within the Ulvales that is separate from that containing the *Ulvaceae.* Based on the sequence data, *Pseudendoclonium* shares a close relationship with *Pirula, Kornmannia* and *Blidingia* within this clade.

Based on partial SSU sequence data, *Pseudendoclonium fucicola* groups within the same Ulvalean clade as *P. submarinum* but is separated from the type species by several other genera in the phylogenetic analysis. Therefore, *P. fucicola* cannot be retained in *Pseudendoclonium* and requires generic reassignment. *P. fucicola* forms a terminal subclade in the partial-sequence tree with *Tellamia contorta.* A BLAST search of the nucleotide database in GenBank confirms that *T. contorta* is the most closely related alga to *P. fucicola* among organisms for which sequence data are available. However, the two sequences differ at 14 of the 753 sites in the partial sequences. The distance would likely be considered generic were the full SSU sequences evaluated. Therefore, no generic assignment for *Pseudendoclonium fucicola* is suggested here.

The freshwater ulotrichalean species currently known as *Pseudendoclonium basiliense* and *P. akinetum* are separated from *P. submarinum* at the ordinal level and therefore require generic reassignment. *P. basiliense* and *P. akinetum* share a high degree of SSU sequence similarity with *Hazenia mirabilis* and *Trichosarcina mucosa.* SSU sequences of these four species are identical at all but 14 of 1683 sites. As previously noted, the SSU rDNA gene is highly conserved among "core" ulotrichalean genera and is not sufficiently variable to resolve relationship at the generic level in many instances. The sequence of *P. basiliense* for example, differs from that of *P. akinetum* at 7 sites but differs from *H. miribilis* at only six. Resolution of generic relationships among these taxa requires further genetic analysis with more variable genes.

From a morphological perspective, *Pseudendoclonium basiliense* and *P. akinetum* share higher similarity with *Trichosarcina mucosa* than either does with

Hazenia miribilis. Hazenia is a soil alga with branched filaments enclosed in a tube-like sheath of mucilage with terminal conical cells. Sexual reproduction occurs via isogamous biflagellate gametes and zoospores are unknown. No ultrastructual data is available for *H. mirabilis.*

Trichosarcina has been considered by several authors to be a close relative of *Pseudendoclonium basiliense. Trichosarcina* differs from *P. basiliense* in its production of a single zoospore per cell, a lack of heterotrichy and its multiseriate condition. Like *P. basiliense* and *P. akinetum,* reproduction occurs via quadriflagellate zoospores and sexual reproduction is unknown. In a comparative study of cell division, Mattox and Stewart (1974) found that the details of mitosis and cytokinesis are so alike in *T. mucosa* and *P. basiliense* that "a single text shall suffice for both." Based on genetic, ultrastructural and morphological simularities, *P. basiliense* and *P. akinetum* are tentatively refferred to *Trichosarcina* as *Trichosarcina basiliense* and *Trichosarcina akinetum.*

Collins (1909) cited the first record of *Pseudendoclonium submarinum* in North America from the coasts of three New England states. This alga was collected during 2005 for purposes of this study from Crane's Salt Marsh in Ipswich, Massachusetts. Unialgal cultures of this North American organism were morphologically indistinguishable from the culture material established with innoculum from Drobak, Norway. Analysis of SSU rDNA sequence data, however, does not support Collins' suggestion that the North American alga he identified is *P. submarinum.* Additionally, the sequence data do not support assignment of this alga to *Pseudendoclonium.* Moreover, these data suggest that the North American alga currently recognized as *P.*

submarinum is distinct, at the generic level, from *P. fucicola, P. basiliense, P. akinetum* and *P. submarinum.*

Resolution of the identity of the North American alga, repeatedly cited as *Pseudendoclonium submarinum* Wille from Norway, represented in this study by material from Ipswich, Massachusetts, requires taxonomic separation from the Wille alga. The alga appears Ulvalean and separated from *Pseudendoclonium submarinum* and *Pseudendoclonium fucicola* on the basis of SSU rDNA sequence data.

Table 1.1 The genus *Pseudendoclonium.*

_____

1	Pseudendoclonium akinetum Tupa
2	Pseudendoclonium basiliense Vischer
3	Pseudendoclonium dynamenae Nielsen
4	Pseudendoclonium fucicola (Rosenvinge) R. Nielsen
5	Pseudendoclonium informe P. Dangeard
6	Pseudendoclonium laxum John & Johnson
7	Pseudendoclonium murale Brand
8	Pseudendoclonium prostratum Tupa
9	Pseudendoclonium submarinum Wille

Source: Index Nominum Algarum

_____

Table 2.1 Composition of modified von Stosch's Enriched Seawater Medium

_____

SALTS		CONCENTRATION / LITER
	$NaNO_3$	21.25 mg
	$Na_2HPO_4 \cdot 12H_2O$	5.375 mg
	$FeSO_4 \cdot 7H_2O$	139.0 µg
	$MnCl_2 \cdot 4H_2O$	9.90 µg
	$Na_2EDTA \cdot 2H_2O$	1.86 mg
Vitamins		
	Thiamine-HCl	0.20 mg
	Biotin	1.00 µg
	B_{12}	1.00 µg
Antibiotics		
	Penicillin or Ampicillin	100,000 units
	Streptomycin	100.0 mg
	GeO_2	6.0 mg
	Amphotericin B	2.5 mg

_____

Table 2.2 Oligonucleotide primers used in this study.

Region Reference	Primer	Direction	Annealing position	Sequence 5'→ 3'	
PCR primers					
SSU					
	Pseud A1	F	2 – 19	CTGGTTGATTCTGCCAGT	b
	R 813	R	787 – 811	AGTCCTGTCGTGTTATCCCATGCT	a
	F 790	F	788 – 813	AGCATGGGATAACACGACAGGACT	a
	R 1773	R	1747 – 1771	CAGGTTCACCTACGGAAACCTTGT	a
Sequencing primers					
SSU					
	NS 1	F	18 – 36	GTAGTCATATGCTTGTCTC	c
	18s 1b	F	359 – 379	GGAGAATTAGGGTTCGATTCC	a
	Rseq 295	R	276 – 299	AGTTGAATGAAACATCGCCGGCAC	a
	Seq 3.6	F	825 – 848	TCTGGCCTATCGTGTTGGTCTGTA	a
	Seq 4	F	1276 – 1298	TGAATGGCCGTTCTTAGTTGGT	a
	NS7m	F	1422 – 1438	GGCAATAACAGGTCTGT	c
	Rseq 1035	R	1024 – 1047	TCCCTAGTCGGCATCGTTTATGGT	a

References:

    a: primer designed for this study; b: O'Kelly *et al* 2004; c: Friedl 1996;
Numbering based on the 18s SSU sequence of *Ulothrix zonata,* GenBank accession number Z47999

Table 3.1 SSU rDNA sequence of *Pseudendoclonium submarinum.*

```
ORIGIN

      1 tctggttgat tctgccagta gtcatatgct tgtcttaaag attaagccat gcatgtctaa
     61 gtataaactg cttatacggc gaaactgcga atggctcatt aaatcagtta gagtttattt
    121 gatggtacct tactactcgg ataaccgtag taaagctaca gctaatacgt gcgcagatcc
    181 cgactcacga agggacgtat ttattagatt caagaccgac cgtgcttgca cgctcttggt
    241 gaatcatggt aacttcacga atcgcacggc ctcgtgccgg cgatgtttca ttcaactttc
    301 tgccctatca actttcgacg gtagtataga ggactaccgt ggtagtaacg ggtgacggag
    361 aattagggtt cgattccgga gagggagcct gagaaacggc taccacatcc aaggaaggca
    421 gcaggcgcgc aaattaccca atcctgacac agggaggtag tgacaaaaaa tatcaatact
    481 gggcctcatg gtccggtaat tggaatgagt acaatctaaa tccgttaacg aggatccatt
    541 ggagggcaag tctggtgcca gcagccgcgg taattccagc tccaatagcg tatatttaag
    601 ttgttgcagt taaaaagctc gtagttggat ttcgggtggg ctgcgccggt ctcctaacgg
    661 ttgtactggc gacgcctgcc ttgctgccgg ggacgggctc ctgccttaac tgtcgggacc
    721 cggaatcggc gacgttactt tgagtaaatt agagtgttca aagcaagcct acgctctgaa
    781 tataatagca tgggataaca cgacaggact ctggcctatc gtgttggtct gtaggaccgg
    841 agtaatgatt aagagggaca gtcgggggca ttcgtattcc gttgtcagag gtgaaattct
    901 tggatttacg gaagacgaac atctgcgaaa gcatttgcca aggatgtttt cattgatcaa
    961 gaacgaaagt tggggggctcg aagacgatta gataccgtcg tagtctcaac cataaacgat
   1021 gccgactagg gattggcgga agttcttttg atgactccgc cagcacctca tgagaaatca
   1081 aagtttttgg gttccggggg gagtatggtc gcaaggctga aacttaaagg aattgacgga
   1141 agggcaccac caggcgtgga gcctgcggct taatttgact caacacggga aaacttacca
   1201 ggtccagaca tgcgaaggat tgacagattg aaagctcttt cttgattgta tgggtggtgg
   1261 tgcatggccg ttcttagttg gtgggttgcc ttgtcaggtt gattccggta acgaacgaga
   1321 cctcagcctg ctaaataggt tcgtctgctc cggcagtcga ctatcttctt agagggactg
   1381 ttggcgtcta gccaatggaa gtatgaggca ataacaggtc tgtgatgccc ttagatgttc
   1441 tgggccgcac gcgcgctaca ctgacacgtt caacaagttc cttgtccgaa aggtctgggt
   1501 aatctttgaa accgtgtcgt gatggggata gaacattgca attattgttc ttcaacgagg
   1561 aatgcctagt aagcgcgagt catcatctcg cgttgattac gtccctgccc tttgtacaca
   1621 ccgcccgtcg ctcctaccga ttgaacgtgc tggtgaagcg ttaggactgg actgttggct
   1681 aggtttcctg gtcggcagtt cgggaatttc gttaaaccct cccgtttaga ggaaggagaa
   1741 gtcgtaacaa ggtttccgta ggtgaacctg
```
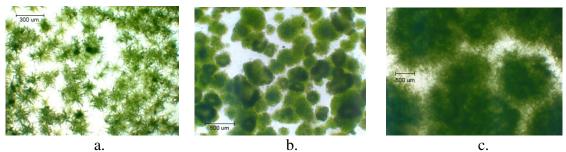
Fig. 1.1 Growth habit of *P. submarinum* in liquid culture. (a) germlings at 2-3 months (b) mature tufts at 6-8 months (c) mature tufts after 2 years.
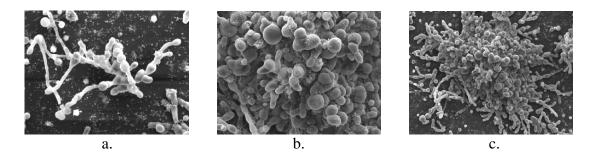

Fig. 1.2 Scanning electron micrographs of *Pseudendoclonium submarinum.* (a) germling (b) upright filaments (c) individual tuft
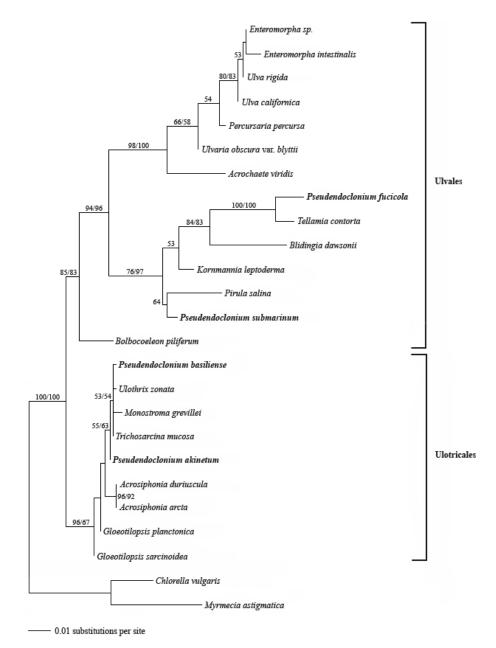
Fig. 3.1 Maximum likelihood phylogram inferred from partial 18s rDNA sequences of 25 green algal taxa illustrating ordinal affiliation of four species of *Pseudendoclonium* as the genus is currently circumscribed. Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above branches or adjacent to nodes reflect bootstrap support based on 2000 and 100 replications under MP and ML criteria respectively. Single numbers are shown where bootstrap support was less than 50 % under the MP criterion. Unnumbered branches received less than 50 % bootstrap support under both MP and ML optimality criteria.
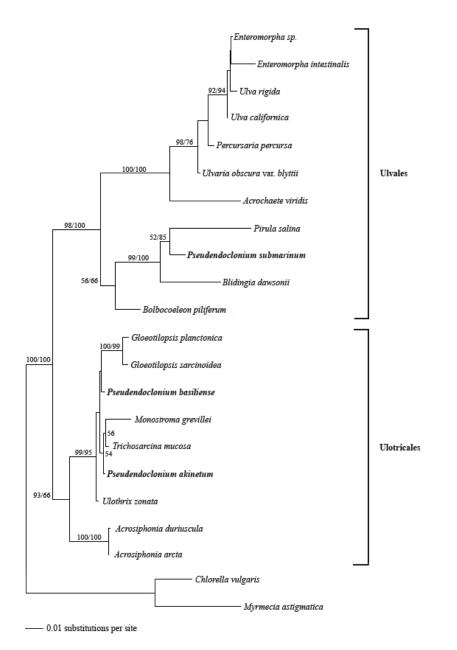
Fig. 3.2 Maximum likelihood phylogram inferred from 18S rDNA sequences of 22 green algal taxa illustrating ordinal affiliation of three species of *Pseudendoclonium* as the genus is currently circumscribed. Horizontal branch lengths are proportional to the mean number of nucleotide substitions per site. Numbers above branches or adjacent to nodes reflect bootstrap support based on 2000 and 100 replications under the MP and ML criteria respectively. Single numbers are shown where bootstrap support was less than 50 % under the MP criterion. Unnumbered branches received less than 50 % bootstrap support under both MP and ML optimality criteria.

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