PHYLOGENETIC ANALYSIS OF PSEUДОCHLORODESMIS STRAINS REVEALS CRYPTIC DIVERSITY ABOVE THE FAMILY LEVEL IN THE SIPHONOUS GREEN ALGAE (BRYOPSIDALES, CHLOROPHYTA)  

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The genus Pseudochlorodesmis (Bryopsidales) is composed of diminutive siphons of extreme morphological simplicity. The discovery of Pseudochlorodesmis-like juveniles in more complex Bryopsidales (e.g., the Halimeda microthallus stage) jeopardized the recognition of this genus. Confronted with this uncertainty, taxonomists transferred many simple siphons into a new genus, Siphonogramen. In this study, we used a multimarker approach to clarify the phylogenetic and taxonomic affinities of the Pseudochlorodesmis-Siphonogramen (PS) complex within the more morphologically complex bryopsidalean taxa. Our analyses reveal a new layer of diversity largely distinct from the lineages containing the structurally complex genera. The PS complex shows profound cryptic diversity exceeding the family level. We discuss a potential link between thallus complexity and the prevalence and profundity of cryptic diversity. For taxonomic simplicity and as a first step toward clarifying the taxonomy of these simple siphons, we propose to maintain Pseudochlorodesmis as a form genus and subsume Siphonogramen and Botryodesmis therein.

Key index words: Botryodesmis; Bryopsidales; cryptic diversity; molecular phylogenetics; Pseudochlorodesmis; Siphonogramen; thallus complexity

Abbreviations: AIC, Akaike information criterion; PS, Pseudochlorodesmis-Siphonogramen

Representatives of the marine green algal order Bryopsidales are characterized by siphonous architecture (Hillis-Colinvaux 1984). Their thallus consists of a single giant tubular cell that branches and fuses in various patterns to form a broad range of shapes upon which generic boundaries are based. The majority of the volume of the tubular cells, usually referred to as siphons, consists of the central vacuole, which is surrounded by a thin layer of cytoplasm and a cell wall (Vroom and Smith 2003). Cells are multinucleate, and cytoplasmic streaming transports organelles and nutrients throughout the thallus (Littler et al. 1988, Drew and Abel 1990).

Thallus architecture is surprisingly diverse in the Bryopsidales. In structurally simple genera, the tubular cell can be readily observed as its uniaxial branches determine the main morphological characters. Well-known examples are the genera Bryopsis and Coveresia, both common inhabitants of rocky shores worldwide. Less broadly known representatives featuring simple morphologies are the tropical sand-dwelling genus Boodleopsis, the tropical reef alga Chlorodesmis, and Dichotomosiphon, the only bryopsidalean genus to have colonized freshwater habitats (Hillis-Colinvaux 1984). Although much more sturdy and thick-walled, the genus Caulerpa has a very similar architecture consisting of branched siphons that do not form complex tissues. Pseudochlorodesmis, the subject of this study, has one of the simplest morphologies among bryopsidalean algae. Its diminutive siphon, if branched at all, does so only a few times (e.g., Meinesz 1980b). Thalli grow out of rocky (often calcareous) substrates, with which a network of constricted siphons is embedded (Kraft 2007). Thalli rarely exceed a few millimeters in length but can occur in extensive populations (Meinesz 1980b).

Several bryopsidalean lineages have evolved more complex thalli, in which individual siphons adhere or coalesce into more expansive tissues that form thick, multiaxial branches, stipes and blades, or
more amorphous structures. Such structures commonly consist of a medulla and a cortex, both formed by branches of the same tubular cell. The predominantly tropical marine genera Avrainvillaea and Udotea form stipes and blades. In the cylindrical branches of the genus Codium, cortical siphons are closely adjoined and swollen into utricles. A similar anatomy occurs in the tropical genus Halimeda, but in this case, the thallus consists of segments that are attached to one another like compressed beads on a string. Other less well-known genera with complex morphologies include Penicillus, Rhipilia, and Rhipilopsis. Several tropical bryopsidales genera with complex thallus architectures deposit calcium carbonate (aragonite) outside of their cell walls.

It is generally believed that complex bryopsidalean thalli evolved from simpler ancestors, but this hypothesis has not been tested formally. Furthermore, a phylogenetic study showed that representatives of the morphologically simple genus Chlorodesmis were nested within a clade characterized by more complex thallus architectures, suggesting that at least some simple thalli may have evolved through reduction or neoteny (Kooistra 2002).

The life cycles of most genera with simple thallus architectures have been thoroughly studied in laboratory culture (e.g., Rietema 1975, Kobara and Chihara 1984). Much less is known about the life history of anatomically complex genera. Considerable amounts of information are available for a few genera, but their life cycles have not been completed in laboratory culture (Meinesz 1980a). Several tropical genera (e.g., Caulerpa, Halimeda, Udotea) are known to engage in mass-spawning events during which male and female gametes are released into the water column and the parent thalli die (holocarpy) (Clifton 1997). Germinated zygotes have been shown to form microthalli with simple thallus architecture, but the development of more complex thalli from these microthalli has not been observed.

Since the study of the life cycle of the genus Halimeda, which reported a Pseudochlorodesmis-like life stage for the Mediterranean species H. tenua (Meinesz 1972), Pseudochlorodesmis has often been regarded as a life stage of other, more complex genera. Assuming that P. furcellata was a life stage of H. tenua, Abbott and Huisman (2004) transferred all Pseudochlorodesmis species other than P. furcellata to a new genus, Siphonogramen. Another genus of interest, Botryodesmis, was recently described based on fertile specimens with a Pseudochlorodesmis-like morphology (Kraft 2007). Thus, specimens exhibiting similar, morphologically simple architectures within the Bryopsidales have been variously assigned to the genera Pseudochlorodesmis, Siphonogramen, and Botryodesmis.

The goal of the present study is to evaluate the phylogenetic and taxonomic affinities of Pseudochlorodesmis-like specimens. Our approach consists of phylogenetic analysis of a multimarker DNA alignment of a broad range of bryopsidalean algae, including three Pseudochlorodesmis-Siphonogramen strains.

MATERIALS AND METHODS

Strains belonging to the Pseudochlorodesmis-Siphonogramen morphological complex (PS complex) were collected in Hawaii and Mediterranean Spain and grown in sterile seawater until they could be harvested for DNA extraction. DNA extraction followed a CTAB protocol modified from Doyle and Doyle (1987). The nuclear ribosomal 18S rRNA region and the plastid genes rbcL and tufA were amplified and sequenced following previously published protocols (Famá et al. 2002, Kooistra 2002, Lam and Zechman 2006).

A data set comprising 33 taxa representing all families of the suborder Halimineae of the order Bryopsidales was compiled (Table S1 in the supplementary material). Three members of the suborder Bryopsidinae were used as outgroups (Lam and Zechman 2006). The three loci were aligned separately. Alignment of the protein-coding genes rbcL and tufA was done by eye based on the corresponding amino-acid sequences. The 18S rDNA was aligned with Muscle v3.6 using standard parameters (Edgar 2004). The three alignments were concatenated prior to phylogenetic analysis. Alignments are available from TreeBase (http://www.treebase.org) and the first author’s Web site (http://www.phycoweb.net).

Selection of a model of sequence evolution for phylogenetic analysis was based on the Akaike information criterion (AIC) (Sullivan and Joyce 2005) implemented in the MrAIC.pl program (Nylander 2004). Maximum-likelihood (ML) phylogenetic analysis was carried out with PhyML (Guindon and Gascuel 2003), using the model suggested by the AIC. The tree search was started from a BioNJ tree (Gascuel 1997), and 1,000 nonparametric bootstrap replicates were carried out to assess statistical branch support (Felsenstein 1985). Bayesian phylogenetic inference was carried out with MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). Two independent runs, each consisting of four incrementally heated chains were carried out. Chains were run for 2,500,000 generations with a sample frequency of 1,000 and using default priors, heat increments, and other settings. Convergence was assessed visually in Tracer 1.4 (Rambaut and Drummond 2007), and an appropriate burn-in value was determined with the automated method proposed by Biele et al. (2006) with a sliding window of size 100. A majority-rule consensus tree was generated from the post-burn-in trees using MrBayes’ sump command.

The results obtained were compared with the hypothesis that the PS complex forms a monophyletic lineage using likelihood-based hypothesis testing (Verbruggen and Theriot 2008). To this goal, a phylogeny in which the PS complex was forced to be monophyletic was computed with Bayesian inference (analysis settings as above). A Shimodaira-Hasegawa test was carried out to evaluate whether the likelihood of this constrained phylogeny was significantly worse than that of the original tree (Shimodaira and Hasegawa 1999). The analysis was carried out in PAUP* v.4.0b10 with 10,000 RELL bootstrap replicates (Swofford 2003). The Bayes factor was also used to compare both hypotheses (Kass and Raftery 1995). It was calculated from the harmonic mean estimates of the marginal likelihoods of the hypotheses given by MrBayes’ sump command (Nylander et al. 2004).

RESULTS

The concatenated alignment consisted of 3,425 sites and 36 taxa and was 67% filled. The rbcL gene
was sampled most densely (33/36 taxa), followed by tufA (22/36 taxa) and 18S (17/36 taxa). The tufA gene failed to amplify in some taxa, particularly Udoteaceae. The AIC-based model selection procedure showed the importance of incorporating different base frequencies and allowing for different rates of change between different bases. Incorporating among-site rate heterogeneity parameters yielded strong decreases in the AIC score, showing the large improvement these parameters have on the fit of the model to the data. The GTR+I+Γ4 model received the lowest AIC score and was used for phylogenetic inference.

The burn-in of the Bayesian phylogenetic analysis was determined at 295,000 based on stationarity of the likelihood of MCMC samples (Beiko et al. 2006). The majority-rule consensus trees obtained from the Bayesian phylogenetic analysis and the ML topology were nearly identical, and we present only the ML tree with support values from both analyses (Fig. 1).

The phylogenetic tree shows that strains belonging to the *Pseudochlorodesmis-Siphonogramen* complex are widely divergent, and most cannot be readily assigned to any of the established families of bryopsidalean algae (Fig. 1). All strains were on relatively long branches and had no close relatives of higher morphological complexity. The Mediterranean *Pseudochlorodesmis furcellata* strain HV1250 (for taxonomic authors, see Table S1) was recovered as a sister lineage of the clade consisting of the families Rhipiliaceae, Pseudocodiaceae, Udoteaceae, Halimedaceae, and Caulerpaceae. A second Mediterranean *Pseudochlorodesmis* strain (HV1204) branched off near the base of the Rhipiliaceae. The third strain, *Siphonogramen abbreviata* from Hawaii (TS64), was recovered as the sister lineage of the Caulerpaceae. Even though the majority of families received strong statistical support in the tree, relationships among certain families remain unresolved.

Trees obtained from ML analysis of individual loci yielded similar results to the tree shown in

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**Fig. 1.** Phylogenetic tree showing the relationships of three strains belonging to the *Pseudochlorodesmis-Siphonogramen* complex with bryopsidalean families. The tree was inferred from a DNA matrix consisting of three loci using the maximum-likelihood criterion. Numbers at nodes represent nonparametric bootstrap values (before slash) and Bayesian posterior probabilities (after slash). Bayesian posterior probabilities are shown as percentages.
Figure 1. Although some poorly supported relationships differed, the overall signal among loci seemed to correspond. Trees of individual loci are available on TreeBase (http://www.treebase.org) and the first author’s Web site (http://www.phycoweb.net).

The hypothesis that the PS complex is monophyletic can be formally rejected. A phylogeny constrained to have a monophyletic PS lineage scored 189 log-likelihood units lower than the ML solution, sufficient for the Shimodaira-Hasegawa test to reject the null hypothesis that both phylogenies are equally likely ($P < 0.0001$). Similarly, the Bayes factor suggests that a monophyletic PS complex is highly unlikely ($2 \cdot \log_e B_{10} = 355.78$).

**DISCUSSION**

**Cryptic diversity above the family level.** Our data show that members of the PS morphological complex form distinct lineages in the bryopsidalean phylogeny. The phylogenetic distinctness of several of these newly discovered lineages would warrant recognition at the family level were one to extrapolate the typical phylodiversity of better-characterized families (Fig. 1). Due to the extremely simple morphology of the PS complex, defining species boundaries is troublesome. Currently, species boundaries are based on branching patterns, but this character varies within populations and changes when organisms are grown in different culture conditions (Meinesz 1980b; personal observation). Considering the widely divergent DNA sequences and the strong morphological similarity, one can conclude that the PS complex harbors cryptic diversity exceeding the family level.

**Cryptic diversity and thallus complexity.** Observing profound cryptic diversity in a markedly simple genus entices thinking of cryptic diversity as a direct consequence of morphological simplicity. Any effort to evaluate such a relationship requires that both elements in the equation be quantified. The morphological complexity of a higher taxon could be quantified as the number of characters used to distinguish its morphospecies. In quantifying cryptic diversity, two different aspects should be taken into account: prevalence (number of cryptic species) and profundity (phylogenetic diversity accounted for by cryptic species). From a strictly morphological point of view, it is simple to conceive that the potential prevalence of cryptic diversity within any given taxon is a function of its morphological complexity. For example, if the morphology of the members of the taxon can be scored as a set of X binary characters, and morphological species boundaries are defined by a minimum of one character difference, the maximum number of morphologically determinable species increases exponentially with the number of characters available ($N = 2^X$). In other words, for a higher taxon containing a given number of species, chances of encountering cryptic diversity increase dramatically with decreasing morphological complexity.

However, evolutionary forces can easily overthrow these elementary predictions and are also likely to account for differences between the prevalence and profundity aspects of cryptic diversity. Selection pressure can be predicted to be a key element in the amount of observed parallel, convergent, and divergent morphological evolution. It can also lead to evolutionary innovations that increase the morphological complexity of the higher taxon. Although one could imagine selective forces that favor morphological stasis and thereby promote cryptic diversity, a majority of selective regimes will cause divergent morphological evolution. In contrast, the absence of selective pressure can be predicted to lead to morphological stasis, increasing the prevalence of cryptic diversity.

Evolutionary forces will also cause differences between the prevalence and profundity aspects of cryptic diversity. Whereas forces acting over relatively short timescales can cause prevalence of cryptic diversity, profundity is also affected by selective regimes acting over longer spans of evolutionary time. It is beyond the scope of this paper to give this subject an exhaustive treatment, but it is clear that this topic deserves more attention than it is currently receiving. Comparison of observed patterns of cryptic diversity with predictions of various theoretical scenarios could lead to important new insights in the causes of cryptic diversity.

Our sampling size is insufficient to engage in such comparisons, and we will restrict ourselves to reporting a few other cases of profound cryptic diversity in morphologically simple algae. The best-known example is probably that of the coccoid microscopic alga *Chlorella*, which was determined to be a complex of look-alikes distributed across two algal classes (Huss et al. 1999). Deep divergences have also recently been reported in the marine picoplanktonic species *Micromonas pusilla* (Slapeta et al. 2006). As far as macroscopic algae are concerned, the morphologically simple acrochaetioid red algae in the genus *Audouinella* present a striking example of diversity exceeding the family level (Harper and Saunders 2002). In the green algae, the genus *Cladophora* exhibits profound cryptic diversity (Leliaert et al. 2007). Finally, in the context of this study, it is noteworthy that *Chlorodesmis*, another morphologically simple bryopsidalean genus, was shown to be polyphyletic, with different lineages being independently derived from ancestors with more complex morphologies (Kooistra 2002). Admittedly, not all these examples represent cases of cryptic diversity in the true sense. In some cases, cryptic diversity is due to incorrect taxonomy rather than true morphological identity, and investigation of previously ignored characters can lead to the identification of diagnostic features.
Phylogenetic distribution of simple thalli. Previous taxonomic and phylogenetic studies have recognized two suborders within the order Bryopsidales and concluded that the great majority of simple thalli belonged to one (Bryopsidinae), whereas the other contained the bulk of complex thallus types (Halimedinae) (Hillis-Colinvaux 1984, Lam and Zechman 2006). Whereas it was previously assumed that simple morphologies would be present in the Halimedinae as microthallus stages of more complex genera, our study demonstrates that taxa with simple thallus morphologies represent a whole new layer of diversity within the Halimedinae, largely distinct from the lineages to which the morphologically complex genera belong. Even with our current limited sampling, it appears that simple morphologies account for a considerable amount of phylogenetic diversity in the Halimedineae, and we predict that the phylodiversity of morphologically simple lineages may increase to a level comparable to that of morphologically complex lineages when more thorough, directed fieldwork is carried out and additional geographic localities are sampled.

Taxonomic consequences. Our results invite a discussion of certain aspects pertaining to the taxonomy of the PS complex. It is clear that not all members of the PS complex are juvenile forms of morphologically complex genera, an assumption that formed the foundation for the erection of the genus Siphonogramen (Abbott and Huisman 2004, p. 141). These authors argued that “the type of that genus (Pseudochlorodesmis furcellata) is now thought to be a stage in the life history of Halimeda tuna, which therefore necessitates the description of a new genus to accommodate the remaining species.” It follows directly from the distinctive nature of P. furcellata in our analyses that this argument is false and that Siphonogramen loses its justification for recognition.

Regrettably, our results do not offer an obvious taxonomic solution. On the contrary, the notion that taxa belonging to the PS complex group occur in highly divergent lineages of the bryopsidalean tree, combined with the suggestion that intraspecific morphological variability may blur taxon boundaries throughout the PS complex, implies that it will be very difficult, if not impossible, to identify diagnostic morphological characters for the different PS lineages. Hence, for the sake of taxonomic simplicity and as a temporary solution, we propose to use Pseudochlorodesmis as a form genus and subsume Siphonogramen therein.

The genus Botryodesmis, which was based on fertile specimens in close agreement with the PS complex (Kraft 2007), should be subsumed in Pseudochlorodesmis for the same reasons. Although the fact that zooilangia have been observed on the simple thallus of this genus probably precludes that it is a juvenile stage of a more complex genus and that it possibly represents a distinct lineage like the ones we have identified in this study, a proliferation of genus names is not desirable in our current state of knowledge. We are of the opinion that until the diversity of the PS complex is better understood, a simpler taxonomy is a better one. Improving our understanding of the complex could be achieved by making detailed morphological observations on sequenced specimens, ideally combined with culture experiments to evaluate morphological plasticity, and a comprehensive screening of presently recognized species using methods that do not rely on external morphology (e.g. ultrastructural, biochemical, or physiological observations and DNA sequencing).

Pseudochlorodesmis exocarpa (Kraft) Verbruggen, comb. nov.


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Supplementary Material
The following supplementary material is available for this article:

Table S1. Taxon list with GenBank accession numbers and voucher or strain numbers (in parentheses). Species authorities were taken from AlgaeBase (Guiry and Guiry 2008).

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