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DNA SEQUENCE VARIATION IN THE RIBOSOMAL INTERNAL TRANSCRIBED SPACER REGION OF FRESHWATER *CLADOPHORA* SPECIES (CHLOROPHYTA)[1]

Jane C. Marks[2] *and Michael P. Cummings*[3]

Department of Integrative Biology, University of California, Berkeley, California 94720

ABSTRACT

Freshwater species of Cladophora *(Chlorophyta) are globally distributed and occupy an unusually wide range of ecological habitats. Delineating species is difficult because most easily observed morphological traits are highly variable and because sexual reproduction has not been clearly documented. Synthesizing ecological data on freshwater* Cladophora *species is problematic because it is unclear whether freshwater* Cladophora *species comprise many genetically distinct species or a few ecologically and morphologically variable and/or plastic species. We determined nucleotide sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron of freshwater* Cladophora *species from a wide range of habitats and geographic locations. We compared these sequences to those derived from culture collections of* C. fracta *and* C. glomerata, *the two most commonly reported freshwater* Cladophora *species.* Cladophora fracta *and* C. glomerata *had very similar ITS sequences (95.3%). All other sequences were identical to those from the* C. fracta *or* C. glomerata *culture collections with the exception of one California sample that was similar to both*

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[2] Author for reprint requests at U.S.A.I.D., G/ENV/ENR, SA18, Rm 509, Washington, D.C. 20523-1812.

[3] Present address: Department of Botany and Plant Sciences, University of California, Riverside, California 92521-0124.

C. fracta *(95.6%) and* C. glomerata *(92.4%). ITS genotypes did not correlate with morphology or geography. This analysis shows that common freshwater* Cladophora *species comprise very few (possibly one) ecologically and morphologically variable species.*

Key index words: Chlorophyta; Cladophora; *freshwater algae; genetic diversity; internal transcribed spacer*

Species of *Cladophora* are among the most widely distributed freshwater macroalgae in the world. In freshwater habitats, *Cladophora* has been reported from pristine streams, eutrophic lakes, and estuaries (Whitton 1970, Dodds and Gudder 1992, Sheath and Cole 1992; see Marks 1996). It has attracted much attention both because it is an important primary producer at the base of undisturbed aquatic food webs and because it can cause nuisance blooms in eutrophic habitats (see Dodds and Gudder 1992 for review). Despite comprehensive studies of *Cladophora* ecology, many questions remain about the ecological requirements and impacts of different species or morphotypes. A potential problem in synthesizing ecological data is that *Cladophora* taxonomy is confusing, and it is unclear whether researchers are comparing one or many genetically distinct species.

Cladophora is in the Siphonocladales/Cladophorales complex. The complex is defined by morphology and cell ultrastructure (van den Hoek 1984) and is supported as monophyletic with molecular data (Zechman et al. 1990, Bakker et al. 1995b). The genus *Cladophora*, the largest in the complex, is less clearly defined and comprises over 38 described freshwater and marine species divided into 12 architectural types (van den Hoek 1984). Taxa with "further specializations" (i.e. synapomorphies) are placed in separate genera within the order (e.g. *Rhizoclonium, Pithophora, Chaetomorpha*) (van den Hoek 1984). Thus, the genus *Cladophora* is partially based on shared primitive characters (pleisiomorphies) and is clearly not monophyletic. This problem was acknowledged by van den Hoek (1963, 1984), who argued that creating one very large genus would result in an "amorphous mass genus," whereas creating 12 or more smaller genera would be difficult because the sections are not clearly delineated. Bakker et al. (1994) confirmed with molecular data that *Cladophora* is paraphyletic but also argued that it is impractical at this time to dissolve the genus.

Taxonomic identification within the genus has challenged phycologists for decades because most morphological characters used to define species (branching pattern, color, cell diameter, cell length : width, zoospore formation) overlap between described species and vary with plant age and environment. Delineating species boundaries is particularly problematic in freshwater taxa because sexual reproduction is rare or undocumented in many species and populations. Most identified freshwater samples are reported as *C. glomerata* or *C. fracta*, but many researchers refrain from keying it to species and either assume their samples are *C. glomerata* or report them as *Cladophora* sp.

Cladophora fracta var. *fracta* and *C. glomerata* can potentially be distinguished by cell diameters, cell length : width, branching patterns, and akinete size (van den Hoek 1963), although they are difficult to distinguish due to overlap in all morphological characters. *Cladophora glomerata* produces biflagellate zoospores, whereas zoospore formation has not been documented in *C. fracta* var. *fracta*. Because zoospore formation in the field is rare, its absence cannot be used to identify *C. fracta* var. *fracta*. Ecologically, *C. glomerata* is described as primarily attached, whereas *C. fracta* var. *fracta* is reported to be primarily loose-lying or free-floating (Prescott 1951, van den Hoek 1963). This may be why attached *Cladophora* from running water is most often reported as *C. glomerata*. A third taxon, *C. fracta* var. *intricata*, is described as primarily attached and as forming biflagellate zoospores (van den Hoek 1963). Thus, a sample with a "*fracta*-like" morphology and a "*glomerata*-like" ecology and life history should technically be keyed to *C. fracta* var. *intricata*, although this taxon is rarely reported.

The widespread distribution of freshwater *Cladophora* and the difficulty of taxonomic identification motivated our use of molecular techniques to assess genetic variation and systematic relationships of freshwater *Cladophora* species. It is essential to develop a workable taxonomy of common freshwater *Cladophora* species to address fundamental questions on the evolution and ecology of this taxon. By using molecular data to describe the genetic structure of common freshwater *Cladophora* species, we will be better able to differentiate ecological plasticity from genetic divergence and thus understand how *Cladophora* occupies such a wide variety of habitats. Molecular data have been used to determine discrete biogeographic groups of marine *Cladophora* and *Cladophoropsis* species, a closely related genus (Bot et al. 1989, Bakker et al. 1992, Kooistra et al. 1992), but have not yet been used to analyze genetic diversity in freshwater Cladophorales.

We sequenced the internal transcribed spacers (ITS 1 and ITS 2) and the intervening 5.8S ribosomal DNA of the nuclear ribosomal cistron from freshwater *Cladophora* taken from a wide range of ecological and geographical habitats. The ITS has been used extensively in plants (Baldwin 1992) and fungi (Gardes et al. 1991), primarily either to analyze systematic relationships among closely related species or to differentiate genetically distinct populations within a species. In general, ITS sequences are either identical or very similar within species but show enough divergence between closely related species to be phylogenetically informative (but see Anderson and Stasovski 1992). The ITS region is beginning to be employed in a range of phycological

TABLE 1. *Field collections of* Cladophora *spp. and their corresponding ITS types. All collection sites were freshwater except for the intertidal collection from Big Sur, California. Type 1 =* C. fracta *culture collection type, Type 2 =* C. glomerata *culture collection type, see Table 2. See Figure 1 for sequences of the three freshwater ITS types. See Figure 2 for phylogenetic relationships among the three types.*

Watershed and location	Collection sites and comments	ITS type
Big Creek watershed, Big Sur, California (Landels Hill–Big Creek Reserve)	3 sites in Big Creek (approximately 0.5 mi apart); 3 sites in the main tributary, Devils Creek (approximately 0.5 mi apart); 1 site below the confluence of Big Creek and Devils Creek; 1 site at the mouth of Big Creek	Type 1
Eel River, California (Northern California Coast Range Preserve)	1 site in South Fork Eel River, 1 site in Elder Creek, a tributary of the South Fork Eel River	Type 3
Emerald Riffle, South Fork Eel River watershed, California	Macroscopically different than *Cladophora* at other sites in the Eel River watershed; see text for details	Type 1
Putah Creek, Davis, California	1 site	Type 1
Strawberry Creek, U.C. Berkeley Campus, Berkeley, California	1 site	Type 2
Lake Huron, Straits of Mackinaw, Michigan	1 site	Type 1
Maple River, Pellston, Michigan	1 site	Type 1
Crocodile River, Transvaal, South Africa	1 site	Type 2

studies. It has been effective for distinguishing biogeographic groups of marine Cladophorales (Bakker et al. 1992, Kooistra et al. 1992), determining interspecific relationships and population boundaries in centric diatoms (Zechman et al. 1994), identifying syngens in freshwater Volvocales (Coleman et al. 1994), delineating species of red algal agarophytes (Goff et al. 1994), and differentiating species and populations of dinoflagellates (Adachi et al. 1994).

The goals of this study were to 1) assess the utility of the ITS and the 5.8S ribosomal DNA in differentiating freshwater *Cladophora* species and populations, 2) use the ITS to estimate the amount of genetic variation in freshwater *Cladophora* species within and between watersheds and between *C. glomerata* and *C. fracta*, and 3) compare genetic data with morphological data to test taxonomic hypotheses. To meet these goals, we compared ITS sequences of *Cladophora* collected from a wide range of ecological and geographic habitats with *C. glomerata* and *C. fracta* from culture collections. We also compared sequence variation found in our samples with variation found among populations of *C. albida*, a widespread marine species (Bakker et al. 1992). We tested whether genetic variation in our freshwater field samples correlated with either geography or morphology. To gain insights into the relationships between freshwater and marine *Cladophora* species, we used the more conservative 5.8S rRNA gene to develop a preliminary phylogeny of these taxa.

MATERIALS AND METHODS

We acquired collections of freshwater *Cladophora* from throughout the United States, one sample from South Africa, and one marine species, *C. columbiana*, from the California coast (Table 1). All freshwater samples were attached to substrates, and most samples would probably be reported by field biologists as *C. glomerata*. To compare variation within a population with variation among populations, we sampled the Big Creek, California, watershed at three sites on each of its main two tributaries (Big Creek and Devils Creek) and at two sites below the confluence of Big Creek and Devils Creek. We sampled all other watersheds only once with the exception of the Eel River, California, watershed. Two distinct morphotypes grow in this watershed. The common morphotype was collected in the South Fork Eel River and in Elder Creek, a tributary of the South Fork Eel River. We have observed the second morphotype primarily in one riffle of the South Fork Eel River that we have named "Emerald Riffle." This morphotype is a brighter green color and is more branched than the common morphotype and would probably be keyed to *C. fracta*. Emerald Riffle is an unusual riffle because it is directly downstream from a large shallow backwater pool that is very productive.

We compared field-collected samples with UTEX (Starr and Zeikus 1993) culture collection samples of *C. glomerata*, *C. fracta*, and *C. fracta* var. *fracta* (Table 2). Because the *C. glomerata* and *C. fracta* culture collections were deposited by C. van den Hoek, we assume that they conform to his delineation of the two species (van den Hoek 1963).

DNA extraction and polymerase chain reaction amplification. Field samples were microscopically scanned for cells that were not epiphytized. These cells were isolated to form our sample. Approximately 5 g fresh algae per sample were placed in a 15-mL plastic centrifuge tube and frozen in liquid nitrogen. Samples were lyophilized overnight and stored at −20° C, and freeze-dried tissue was ground to a fine powder. DNA extractions followed a modified version of the CTAB extraction protocol (Rogers et al. 1989). We added 500 μL of extraction buffer containing 2× CTAB, 100 mM Tris, 1.4 M, 20 mM EDTA NaCl, and 5 μL of 1% β-mercaptoethanol to approximately 0.1 g of ground algae. Samples were heated at 65° C for 1 h at which time an equal volume of 1:1 cholorform/phenol was added. Samples were centrifuged for 15 min, and the aqueous layer was retained. DNA was precipitated by adding 500 μL of cold isoproponal to each sample. Samples were kept on ice for at least 1 h and then centrifuged for 15 min. The pellet was retained. Pellets were washed in 95% ethanol and then in 70% ethanol and resuspended in 50–100 μL TE buffer. DNA extracts were diluted 1000-fold in H_2O (doubly distilled and filter-sterilized). The ITS region was amplified with universal primers, ITS4 and ITS5 (White et al. 1990). The 25-mL reactions contained 12 mL extracted/diluted DNA, 7.0 μL H_2O, 0.25 μL of 50 μM each primer, 0.125 μL of Taq polymerase (Perkin-Elmer Corp., Branchburg, New Jersey) and 2.5 μL buffer containing 0.1 M Tris (pH 8.8), 20 mM $MgCl_2$, 0.1 M β-mercaptoethanol, and 0.1% gelatin. Samples were amplified for 35 cycles using the following temperature profile: 95° C denaturation, 55° C annealing, 72° C extension. Times for denaturation, annealing, and extension were 35, 55, and 45 s, respectively, for the first 13 cycles; extension time was extended to

TABLE 2. *Culture collections of* Cladophora *spp. used in DNA analysis and their corresponding ITS types. All cultures were obtained from The Culture Collection of Algae at the University of Texas at Austin. ITS types are presented in Figure 1.*

Species	Culture number	Collection site and date	Collector	ITS type
C. fracta (Müll. *ex* Vahl) Kütz.	LB 1487	North Breveland, The Netherlands, 1960	C. van den Hoek	Type 1
C. fracta var. *fracta* Van den Hoek	LB 474	River Cam, Grantchester, England, 1948	E. A. George	Type 1
C. glomerata (L.) Kütz.	LB 1486	Leiden, The Netherlands, 1963	C. van den Hoek	Type 2

2 min for the next 13 cycles and was extended to 3 min for the final 9 cycles. Samples were then maintained at 72° C for 10 min and were stored at <5° C. Polymerase chain reaction (PCR) product was purified by running 20 μL on a 1.5% low-melt agarose gel that was stained with ethidium bromide. Illuminated bands were cut out of the gel, melted in 250 μL H_2O at 65° C for 15 min, and diluted 1000-fold in H_2O for reamplification. Double-stranded product was reamplified for cycle sequencing in 50-μL reactions using the preceding reagents and primers in the same ratios. This PCR product was purified by filtration or by adhesion to glass powder (Vogelstein and Gillespie 1979).

Cycle sequencing. We used an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer Corp., Applied Biosystems Division, Foster City, California) to determine sequences. Because the ITS (including the 5.8 S region) was over 900 basepairs, we sequenced ITS 1 and ITS 2 separately. ITS 1 was sequenced in both directions with the universal primer ITS5 (White et al. 1990) and J21 (a *Cladophora*-specific primer that we designed; sequence: CGATGATTCACGGAATTCTGC). ITS 2 was sequenced in both directions with universal primers ITS3 and ITS4 (White et al. 1990). We prepared cycle sequencing reactions with PRISM Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kits (Perkin-Elmer Corp., Applied Biosystems Division, Foster City, California) following the vendor's protocol and the following amplification profile for 25 cycles: 96° C, 30 s; 50° C, 15 s; 60° C, 4 min). Unincorporated dye product was removed, the samples vacuum-concentrated, and 5 μL of loading buffer (150 μL formamide, 30 μL EDTA with blue dextrin) was added to samples prior to loading. Samples were loaded onto a 10% acrylamide sequencing gel and were run for 10 h at 2500 V, 40 mA, and 25 W.

Sequence data analysis. We evaluated phylogenetic relationships among the three freshwater genotypes by comparing their ITS sequences (including the 5.8S rDNA gene) using *C. columbiana* as an outgroup. We separately analyzed ITS sequences (including the 5.8S region) of species and populations of marine *Cladophora* species using the same parameters as the freshwater analysis so that we could directly compare sequence divergence among freshwater samples with sequence divergence within and among marine species. The marine sequences included *C. columbiana*, *C. vagabunda*, and four populations of *C. albida* (Huds.), a species with a global distribution. Sequences for *C. albida* and *C. vagabunda* were acquired from Bakker et al. (1992) and GenBank/Entrez (URL http://www.ncbi.nlm.nih.gov/search/index.html), respectively.

To assess phylogenetic relationships across the broad range of freshwater and marine *Cladophora* species, we compared the relatively conserved 5.8S rDNA regions of our taxa with *C. vagabunda*, a marine species that is morphologically similar to *C. glomerata* and *C. fracta* and is in the "Section Glomeratae" (sensu van den Hoek 1963), and with *C. albida*, a morphologically distinct species in the "Section Rupestres" (sensu van den Hoek 1963). We restricted this analysis to the 5.8S coding region because the ITS regions were too variable across the full set of species to produce an alignment we deemed appropriate.

Sequence alignments were done using the program CLUSTAL W (Thompson et al. 1994). Phylogenetic relationships were determined by maximum likelihood analyses (Felsenstein 1981) using the program fastDNAml (Olsen et al. 1994). Ten separate analyses were conducted using random sequence addition (jumble) with a transition:transversion ratio of 2:1, empirical base frequencies, one rate class, and global branch swapping. Alternative topologies were compared with a log-likelihood test (Kishino and Hasegawa 1989) using the alpha test version of DNAML from PHYLIP 4.0 (Felsenstein 1995).

Morphology. We quantified apical cell size by measuring cell lengths and widths in 20 random cells in each sample using an Olympus CH microscope at 400×. To estimate overall cell size, we measured cell lengths and widths of 20 random, nonapical cells, which we refer to as "regular" cells. We recorded the presence of intercalary growth, apical growth, dichotomous branching, and psuedodichotomous branching in each sample.

RESULTS

DNA sequence data. Cladophora fracta and *C. glomerata* from culture collections had distinct but similar ITS sequences (Fig. 1, Table 2). Three distinct ITS sequences were identified from field collections; two were identical to either *C. fracta* (Type 1) or *C. glomerata* (Type 2). The third (Type 3) was collected from the Eel River, California, watershed (Table 1). ITS sequences among these three groups were very similar and primarily differed in one region that exhibited a series of insertions and deletions (Fig. 1). ITS sequences types did not correlate with geography (Table 1). Samples from Europe, the United States, and South Africa had identical ITS sequences while all three genotypes were found in California, and two of the three genotypes were found in the Eel River, California.

Pairwise comparisons of *C. fracta* and *C. glomerata* showed that variation between these two described species was equivalent to variation among populations within oceanic groups of *C. albida* and that there was much less similarity between disjunct populations of *C. albida* than between the two described freshwater species (Table 3). Similarity between the Eel River samples and either the *C. fracta* or *C. glomerata* genotypes was only slightly lower than similarity among closely related populations of *C. albida*. The maximum likelihood tree yielded similar results and showed that branches connecting the Pacific and Atlantic isolates of *C. albida* populations are longer than branches connecting the two described freshwater species, *C. fracta* and *C. glomerata* (Fig. 2).

The alignment of the 5.8S ribosomal gene was unambiguous with only a single nucleotide gap. The maximum likelihood tree indicated that the freshwater taxa were monophyletic and were most closely related to *C. columbiana* (Fig. 2). Contrary to our expectations, *C. vagabunda* was not the sister taxon

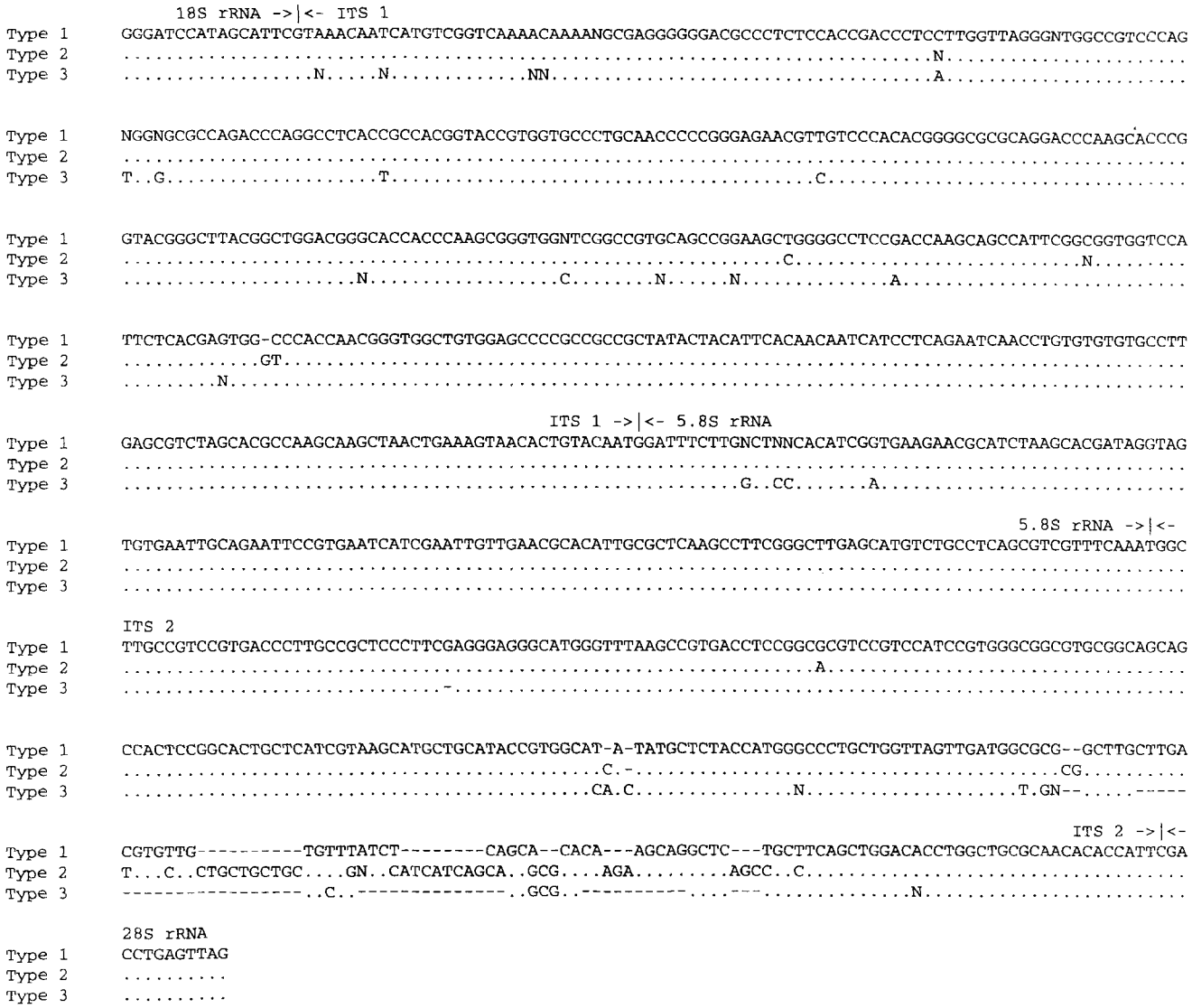

FIG. 1. Sequence alignment of the three distinct ITS 1-5.8S-ITS 2 sequences found in the freshwater samples. All freshwater field collections had one of the preceding sequences (see Table 1). Type 1 = *Cladophora fracta* culture collection type, Type 2 = *C. glomerata* culture collection type. "." indicates basepair is the same as Type 1 sequence; "-" indicates an insertion/deletion; N indicates that the base identity was ambiguous.

to the *C. fracta* / *C. glomerata* complex. However, there was no significant difference in the log-likelihood values (Kishino and Hasegawa 1989) between a tree that placed *C. vagabunda* as a sister taxon to the *C. fracta* / *C. glomerata* complex and the tree shown in Figure 2.

Morphological data. Neither apical nor regular cell lengths, widths, or length : width correlated with ITS genotype (Fig. 3). There was as much variation in apical cell size and shape within the Big Creek, California, watershed samples as there was between samples with different ITS genotypes (Fig. 3). Regular cells tended to be narrower in Type 1 genotypes than in Type 2 genotypes, although there was overlap between genotypes. The sample with the Type 3 genotype (Eel River) had wider cells than the other genotypes (Fig. 3). Because we only had one watershed containing this genotype, it was impossible to evaluate morphological variation and/or plasticity of this genotype. All samples showed both intercalary and apical growth and both dichotomous and pseudodichotomous branching. Although samples differed in the amount of branching and the relative frequency of branch types, these differences did not correlate with ITS genotype. Samples representing the two genotypes found in the Eel River watershed differed dramatically in morphology. Emerald Riffle *Cladophora* (Type 1 genotype) had higher length : width and was much more branched than common Eel River *Cladophora* (Type 3 genotype).

DISCUSSION

Freshwater *Cladophora* species exhibit little sequence variation within the ITS region. Although *C. fracta* and *C. glomerata* have distinct ITS sequences, there is very little sequence divergence between

TABLE 3. *Pairwise proportion of observed identical sites for ITS sequences, including the 5.8S gene, among the three freshwater genotypes and among Pacific and Atlantic isolate of* Cladophora albida. *Freshwater and marine groups were analyzed separately using analogous sequence positions and identical alignment parameters. See Table 1 for geographic distributions of the three freshwater genotypes. Sequences of the freshwater genotypes are presented in Figure 1.* Cladophora albida *sequences are from Bakker et al. (1992).*

Comparisons of freshwater genotypes		
Genotype	Type 1	Type 2
Type 1	—	
Type 2	0.953	—
Type 3	0.957	0.923

Comparisons of populations of marine *C. albida*[a]			
C. albida isolate	U.S.A. (Atlantic)	France (Atlantic)	Australia (Pacific)
U.S.A. (Atlantic)	—		
France (Atlantic)	0.988	—	
Australia (Pacific)	0.673	0.672	—
Japan (Pacific)	0.677	0.675	0.996

[a] Proportion sequence divergences for the *Cladophora albida* populations were lower than that reported in Bakker et al. (1992) because we only included ITS and 5.8S sequences. We omitted sequences from the 18S and 26S coding regions to allow for direct comparison of the two data sets.

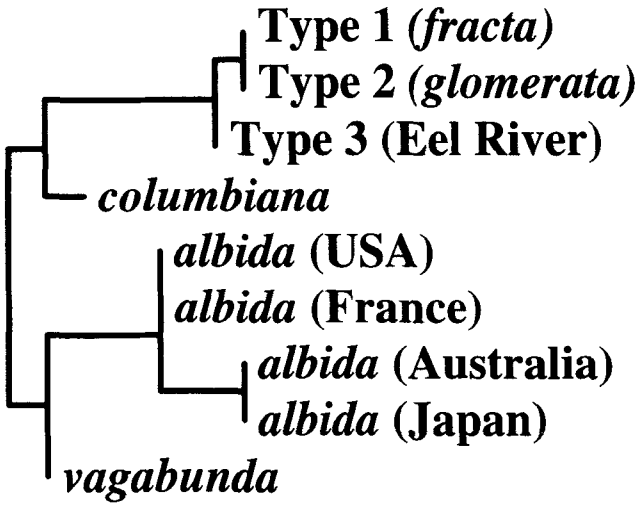

FIG. 2. Phylogenetic tree for freshwater and marine species of *Cladophora* determined by maximum likelihood, based on the 5.8S sequence, using default gap weighting for the alignment. Branch lengths are proportional to the expected number of nucleotide changes. The tree is midpoint rooted. The log-likelihood value for this tree is −318.29.

the two species or among all field-collected samples. The three ITS genotypes did not correlate with either geography or morphology. Our data conclusively show that identical ITS genotypes of freshwater *Cladophora* species have wide biogeographic distributions, occupy diverse habitats, and display a range of morphological variation. Contrary to our expectations, most field-collected samples were genetically identical to *C. fracta*, even though they were all attached to substrates and were mostly collected from lotic habitats, features that have been associated with *C. glomerata*.

Marine *Cladophora* species exhibit discrete ITS types that are roughly species related and contain little variation within types (Bakker et al. 1995). Variation among their ITS types is much higher than among the three types described in the present study. The variation among our freshwater sequences is more similar to that found within marine types and is considerably less than that found between disjunct populations of *C. albida* (Bakker et al. 1992, 1995). Recent work indicates that the two populations (Pacific and Atlantic Oceans) of *C. albida* may not be monophyletic (Bakker et al. 1995), so perhaps the most appropriate comparison to our results is the intraoceanic variation for *C. albida*.

Our freshwater *Cladophora* species/genotypes are as similar as potentially interbreeding populations of *Gonium pectorale* Mueller (~95%) and potentially interbreeding syngens of *Pandorina morum* Bory (Coleman et al. 1994). These are freshwater Volvocales that are globally distributed, form common gene pools (based on mating studies), and hence correspond to "biological species" (*sensu* Mayr 1948). Thus, the amount of variation that we observed among described freshwater *Cladophora* species is similar to that found within "biological species" of freshwater Volvocales.

Whereas molecular data provide insight into genetic variation, delineating species boundaries remains difficult and arbitrary. The problem is more acute in asexual taxa for which species concepts are unresolved. One option includes considering all three genotypes as one species and each genotype as a subspecies/strain. This would be easy to implement but ignores the potentially discrete identity of *C. glomerata* and *C. fracta*. ITS sequences of closely related fungal species are not always different (Anderson and Stasovski 1992) and may not correlate with mating types (Kasuga et al. 1993, T. Bruns, pers. commun.), pointing to limitations of using ITS data to delineate species.

Alternatively, we could maintain the identities of *C. glomerata* and *C. fracta*. Because the Eel River genotype is equally divergent from the *C. fracta* and *C. glomerata* genotypes, it is difficult to assign it to one over the other, based on the ITS genotype. It is helpful to compare the common Eel River morphotype (Type 3 genotype) to the Emerald Riffle sample that was also collected from the Eel River watershed but has the Type 2 genotype. Emerald Riffle *Cladophora* has narrower cells and is more branched than the common morphotype ("Eel River" genotype). The common morphotype ("Eel River genotype") also produces biflagellate zoospores (J. Marks, pers. observ.). Based on morphology and life history, the Eel River morphotype would be keyed to *C. glomerata* and the Emerald Riffle to *C. fracta*. The morphological differences between these two genotypes, in this habitat, corresponds well with

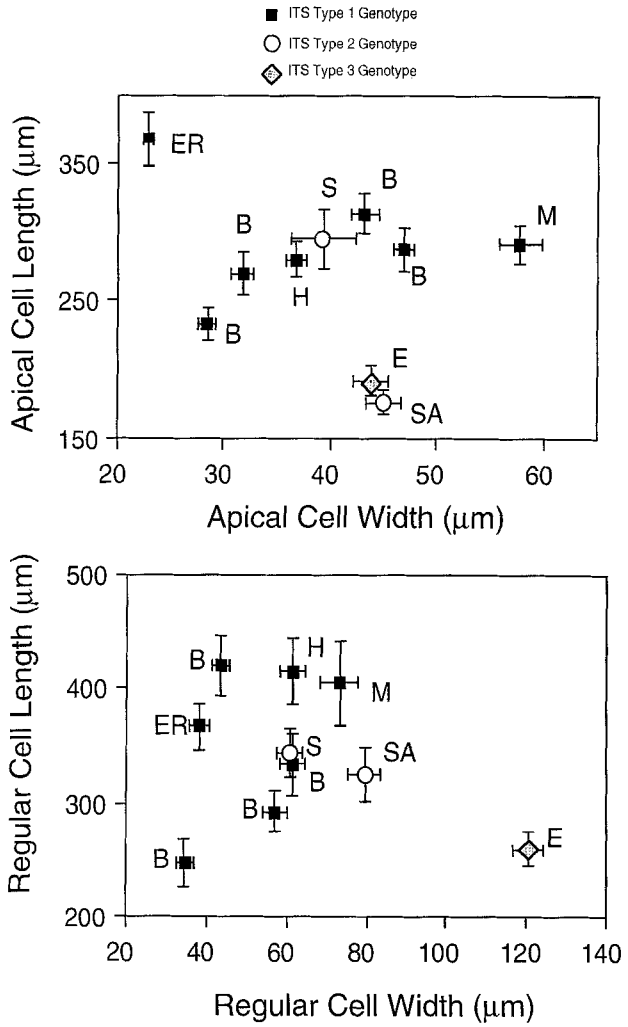

FIG. 3. Mean apical and regular cell lengths and widths (means ± 1 SE) for freshwater *Cladophora* samples identified by ITS genotype and collection site. B = Big Creek, California, watershed; E = Eel River, California (dominant morphotype); ER = Emerald Riffle (in the Eel River, California, watershed); H = Lake Huron, Michigan; M = Maple River, Michigan; S = Strawberry Creek, California; SA = Crocodile River, South Africa. See Table 1 for descriptions of collection sites; see Figure 1 for the three ITS sequences. N = 20 cells per sample.

van den Hoek's (1963) separation of *C. glomerata* and *C. fracta* and supports maintaining the two as separate species. Identifying other samples from other habitats, however, as either *C. fracta* or *C. glomerata* is much more difficult due to overlap in morphological traits. Common garden experiments may yield information on the ranges of morphological plasticity of the three genotypes.

It will be useful to expand our ITS database to determine how ITS patterns correlate with geography, morphology, ecology, and life history over a broader range of samples. Although it is possible that a detailed morphological analysis of many samples of each genotype may reveal distinct "multivariate morphological" groups that correlate with genotype, it is clear that there is overlap in traditionally measured morphological traits, making it difficult to develop a reliable method of distinguishing species and ITS genotypes based on morphology. We are cautious to recommend that ITS Types 1 and 2 be used to distinguish *C. fracta* and *C. glomerata* because the two types are so similar. This sequence similarity indicates recent divergence of these taxa, as sequence differences accumulate at a rate that is proportional to time, independent of sexual or asexual life histories. The present distribution of the ITS types can be explained by dispersal. If there was ancestral polymorphism within the group prior to speciation, then there is the possibility that more than one ITS type could be present in either or both species. While this is frustrating to researchers who want to unequivocally indentify all samples, it also indicates that this group can provide a case study of speciation and genetic structure of asexual freshwater taxa. Further genetic data from additional loci will complement the ITS database and will provide independent estimates of the relationships of ITS genotypes.

Relationships among a broader range of *Cladophora* species based on the 5.8S rDNA suggest that freshwater *Cladophora* species are monophyletic, but this needs to be confirmed with a larger database. *Cladophora vagabunda* L. was not the sister taxon to the freshwater species, as was expected based on morphology, again indicating the limitations of morphology in understanding relationships within this group. This result needs further examination because the amount of information in the 5.8S sequence data is insufficient to rigorously determine the position of *C. vagabunda* relative to the other species. A productive direction for future research might be to sequence the more conserved 18S rDNA region of a wide range of freshwater *Cladophora* species and closely related genera (*Rhizoclonium, Pithophora, Basicladia*) to integrate into the 18S rDNA phylogeny of marine *Cladophora* species (Bakker et al. 1994). This will allow us to test whether freshwater Cladophorales are monophyletic or have evolved multiple times from different marine species.

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