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The diversity of *Scenedesmus* and *Desmodesmus* (Chlorophyceae) in Itasca State Park, Minnesota, USA

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Species of *Scenedesmus* and *Desmodesmus* are ubiquitous in freshwater ecosystems. However, phenotypic plasticity coupled with the minute variations between named species make the identification of taxa by light microscopy extremely difficult, especially from field samples. As a result, the actual level of diversity and distributions of these genera are not known. In this study, the diversity of *Scenedesmus* and *Desmodesmus* from Itasca State Park (ISP), Minnesota, USA was studied using culture techniques followed by light microscopy and internal transcribed spacer (ITS-2) rDNA sequence analysis. From 100 characterized isolates of *Scenedesmus, Desmodesmus* and allied isolates, 34 different sequence types were detected, 24 of which are different enough from each other to correspond to distinct taxa. This diversity greatly exceeds that described for these genera in previous studies of ISP algae using light microscopy alone. The sequences of five ISP isolates identically matched those of previously described species or isolates, and sequences of six ISP isolates differed by eight bases or less from those of previously described species. The isolation of *Desmodesmus* species from Itasca State Park with sequences identical to those from isolates from highly disjunct locations around the world suggests that these species are widely distributed. Results from this study also indicate that isolates with very similar (< 8 substitutions) ITS-2 sequences tend to have only very slight morphological variation. We conclude that culturing and ITS-2 sequence analysis is an effective technique for investigating species level diversity in *Scenedesmus* and *Desmodesmus.*

KEY WORDS: Chlorophyceae, *Desmodesmus,* Distribution, Diversity, Internal transcribed spacer, *Scenedesmus*

INTRODUCTION

Scenedesmus Meyen and *Desmodesmus* (Chodat) An, Friedl & Hegewald are common green algal genera in freshwater habitats, soil (Hilton & Trainor 1963) and microbiotic crusts (Lewis & Flechtner 2004). Typically, these algae are found as colonial forms, most commonly with 4-celled coenobia. However, single-celled individuals, as well as 8-, 16-, and even 32-celled coenobia frequently are observed. Those species that bear wall ornamentation recently were described as the genus *Desmodesmus,* separate from *Scenedesmus* (An *et al.* 1999). Molecular phylogenetic analyses have placed these genera in the family Scenedesmaceae and the order Sphaeropleales of the class Chlorophyceae (Buchheim *et al.* 2001).

Scenedesmus and *Desmodesmus* are thought to include some species that are perhaps more widely distributed than any other freshwater algae (Prescott 1962). Floristic studies from around the world often identify the same few species, particularly *S. obliquus* (Turpin) Kützing, and *D. communis* (Hegewald) Hegewald [often as *S. quadricauda* (Turpin) de Brébisson], from many varied habitats. Despite the few taxa reported in most floristic studies, over 1300 species and subspecific taxa of *Scenedesmus* (including *Desmodesmus*) have been described (Hegewald & Silva 1988). These descriptions were based on light microscopy (LM) observations of morphological characteristics, such as size and shape of cells and presence of wall ornamentation. For these descriptions, it generally was assumed that morphology was a stable feature. Therefore, when subtle differences in morphology were observed, new species were erected without the knowledge of whether these characters were stable or not (Norton *et al.* 1996). Several studies, however, have shown that many of the characters used to describe taxa of these two genera, in fact, are highly plastic (summarized in Trainor 1998). Nutrient concentrations, light intensity, temperature, and the presence of herbivores all have been shown to affect phenotype. Phenotypic plasticity makes identification of species from field samples very difficult, especially if information on the environmental conditions from collection sites is unavailable (Trainor 1992). In order to understand the actual level of diversity, phenotypic plasticity and cyclomorphosis within each species should be studied (Trainor *et al.* 1976). Studies that considered phenotypic plasticity have suggested that the true number of *Scenedesmus* and *Desmodesmus* species may be about 30 (Trainor 1998).

However, morphology still can be valuable for distinguishing taxa at higher levels. Hegewald (1978) used electron microscopy (EM) to study the cell wall composition and ultrastructure of *Scenedesmus* (including *Desmodesmus*) isolates. His observations prompted him to divide the genus *Scenedesmus* into the subgenera *Scenedesmus, Acutodesmus* Tsarenko, and *Desmodesmus.* Electron microscopy, especially scanning electron microscopy (SEM), ultimately could be useful for identifying stable morphological characteristics for distinguishing species. Recent analyses have suggested that certain morphological characters may well be stable enough for species determinations in *Desmodesmus* (e.g. Hegewald *et al.* 2005, Leon & Hegewald 2006).

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Molecular characters also show promise for studying diversity. Molecular characters are useful because they show a more uniform rate of evolution than morphological or physiological characters (Li 1997). Whereas morphological characters can be influenced by the environment, DNA sequences are stable heritable entities, subject only to rare mutation events. Kessler *et al.* (1997) used sequence analysis of the 18S rDNA to support the split into the two subgenera *Scenedesmus* and *Desmodesmus* that previously had been suggested by Hegewald (1978). However, the 18S rDNA was too conserved to distinguish closely related species. The 5.8S-26S ribosomal DNA internal transcribed spacer region (ITS-2), which is much more variable than the 18S rDNA, was used by An *et al.* (1999) to split the genus *Scenedesmus* into two distinct genera, *Scenedesmus* (including *Acutodesmus*) and *Desmodesmus.* The ITS-2 region has proven useful for phylogenetic analysis at the species level in *Desmodesmus* and *Scenedesmus* (Marks & Cummings 1996; Cerbah *et al.* 1998; An *et al.* 1999; Hegewald 2000; Van Hannen *et al.* 2002; Lewis & Flechtner 2004). The stability of this molecular marker, ease of molecular techniques and availability of sequence data make the use of the ITS-2 region practical for describing diversity of *Scenedesmus* and *Desmodesmus.*

Van Hannen *et al.* (2000) found that *Scenedesmus* species differed by 10% to 30% in the ITS-2 sequence and that strains of the same species differed by a much smaller amount, 2% to 10%. Lewis & Flechtner (2004) used this idea when studying cryptic species isolated from desert microbiotic crusts. They found that two desert species with the morphology of *S. obliquus* differed in ITS-2 sequence from each other by 2.0% to 2.6%, translating into pair-wise differences ranging from 14 to 18 substitutions. The ITS-2 sequence differences within each species were much smaller, one to six differences. New species were erected on the basis of this small substitution difference coupled with habitat differences, despite their morphological similarity.

The goal of this study is to assess the levels of diversity and distribution of *Scenedesmus* and *Desmodesmus* using culturing, ITS sequence analysis and LM, and to compare the results to previous investigations that relied solely on LM from field samples. This comparison also will permit an evaluation of the reliability of the ITS-2 region for taxon identification. For this study, 100 isolates from six different lakes and ponds in Itasca State Park, Minnesota, USA, were evaluated. This broad sampling of new isolates also was used to evaluate the phylogeny of the Scenedesmaceae.

MATERIAL AND METHODS

Site description

Water samples were collected from six different sites in Itasca State Park, Minnesota (ISP). These sites include Lake Itasca, Mary Lake, West Twin Lake, North Deming Pond, Tower Pond and Picnic Pond (Phillips & Fawley 2000), all located between 47°10′ and 46°16′N latitude. Lake Itasca and Mary Lake are both mesotrophic and moderately deep, exceeding 10 m in depth. Lake Itasca was not stratified at the sampling site, whereas Mary Lake had a well-developed thermocline during the summer and early fall. West Twin Lake, North Deming Pond, and Tower Pond are all dystrophic, and Picnic Pond is moderately eutrophic. Physical characteristics for all sites are given in Fawley *et al.* (2004).

Isolation and culture methods

Samples were collected seasonally from each site, during the months of September 2000 and February, June and August of 2001 (Fawley *et al.* 2004). Samples also were collected in October of 2003 and June of 2004 with a focus on Tower Pond, although a few isolations were made from other sites as well. Plankton samples were obtained using a Kemmerer or Van Dorn sampler (Phillips & Fawley 2000). These samples were collected from the surface water at all sites, as well as from mid-depth and near the bottom at Lake Itasca, and from above and below the thermocline, when present, in Mary Lake. Tychoplankton samples were obtained by squeezing a vegetation sample. Individual organisms were isolated from live samples using a spread-plate method (Phillips & Fawley 2000; Fawley *et al.* 2004). The plates were incubated for approximately 2 weeks, and individual colonies were picked from the plates. Cultures then were maintained on agar slants.

Individual organisms also were isolated from samples collected in October 2003 and June 2004 using a modification of the micropipette technique of Hoshaw & Rosowski (1973). A glass micropipette was used to pick coenobia of *Scenedesmus* and *Desmodesmus,* but unlike the normal micropipette method, the coenobia were not passed through several sterile rinses. Instead, multiple coenobia were placed directly in 100 μl of medium, which then was spread on agar plates. Plates were incubated for approximately 2 weeks and individual colonies were picked from the plates.

Additional unicellular isolates from ISP that are allied with the Scenedesmaceae based on analysis of 18S rDNA (Fawley *et al.* 2004) also were included in this study. The ITS sequence also was generated for an ISP isolate of *Coelastrum* sp. (DQ417572).

Molecular characterization

For the molecular characterization, genomic DNA was isolated from liquid cultures (Fawley & Fawley 2004). The ITS-1, 5.8S rDNA and ITS-2 regions were amplified by polymerase chain reaction (PCR) using the primers ITS-AF (5′-CGTTTCCGTAGGTGAACCTGC-3′) and ITS-BR (5′-CATATGCTTAAGTTCAGCGGG T-3′), which are truncated versions of the A and B primers of Coleman *et al.* (1994). PCR conditions were those given in Fawley & Fawley (2004). All isolates initially were characterized by PCR-Restriction Fragment Length Polymorphism (RFLP) analysis (Phillips & Fawley 2000) of the amplicon using the restriction enzymes *Alu*I and *Msp*I. The RFLP patterns generated were used to distinguish the isolates as different types based on shared fragment patterns. The PCR products of at least two isolates from each type were sequenced, except for those types that were represented by only one isolate. In some cases, sequence analysis revealed further diversity in the ITS-2 regions. In these cases, the additional restriction enzymes *Mse*I and *Hae*III were used to discriminate between the isolates.

For sequencing, two 25-μl PCR reactions were pooled and purified using the GeneClean Turbo for PCR kit (MP Biomedicals, Irvine, California), or the Montage PCR centrifugal

filter devices (Millipore Corp., Billerica, Massachusetts). Sequences were generated using a Beckman CEQ 2000 automated sequencer and Quick Start sequencing kits (Beckman Coulter, Fullerton, California), or commercially by Northwoods DNA (Solway, Minnesota). Primers used for sequencing were ITS-AF and ITS-BR (previously described), and ITS3 from White *et al.* (1990). The sequences generated included the complete ITS-1, 5.8S and ITS-2 regions, with complete double-stranded reads for ITS-2. Sequences were assembled and evaluated using the Staden Package (Staden 1996). Isolates used for sequence analysis, along with the GenBank accession numbers of the sequences, are listed in Table 1.

Diversity analysis

The ITS-2 sequences from 61 of our isolates representing the 34 different ITS types were aligned with published ITS-2 sequences from additional isolates of the Scenedesmaceae available from GenBank. All GenBank sequences from UTEX strains used here are named according to Hegewald (1989) and differ in some cases from the names given in GenBank. Two separate analyses were performed, one for *Desmodesmus* isolates and one for *Scenedesmus* and related isolates. In each case, the ITS-2 sequence from *Neodesmus danubialis* was used to root the dendrogram. Sequences were aligned with Multalin (Corpet 1988) and then manually corrected using GeneDoc (Nicholas *et al.* 1997) and McClade 4 (Maddison & Maddison 2000). A total of 291 characters were used. Some regions of the alignment could not be unambiguously aligned over the entire data set; however, these regions were unambiguously aligned within groups of sequences from closely related isolates (Fawley *et al.* 2004). Neighbor-joining (NJ) analyses were done using phylogenetic analysis using parsimony (PAUP*) 4.06 10 (Swofford 2000), with a distance matrix that employed the total nucleotide differences with missing sites ignored. The intent of these analyses is to depict graphically the sequence variations among isolates, not to perform a phylogenetic analysis. Thus, no bootstrapping or other assessment of node support was performed, and the resulting dendrogram should not be used to infer evolutionary relationships.

Phylogenetic analyses

For phylogenetic analysis of the Scenedesmaceae, the alignment included only unique sequences from the diversity analyses, plus additional sequences from GenBank. In addition, four sequences from the Hydrodictyaceae were included as an outgroup. The resulting alignment included 112 different ITS-2 sequences. Small regions that could not be unambiguously aligned were excluded, resulting in a total of 216 characters used in the phylogenetic analysis, with 168 variable characters and 121 parsimony-informative characters. Neighbor-joining (NJ) and maximum parsimony (MP) analyses were performed. For NJ analysis, the HKY85 model (Hasegawa *et al.* 1985) was used to construct the distance matrix, a model previously used in phylogenetic analyses of *Scenedesmus* and *Desmodesmus* (An *et al.* 1999). For the MP analysis, a heuristic search was used with the tree bisection and reconstruction (TBR) branch-swapping method, and addition was random. Characters were given equal weight and were unordered. Gaps were treated as missing. Bootstrapping of both NJ and MP were performed with 1000 replicates. Stepwise addition was used for bootstrapping the MP analysis.

For a more discriminating phylogentic analysis, the set of unique sequences was aligned using the program MARNA (Siebert & Backofen 2005), which seeks to align sequences based on a combination of primary and secondary structures. Because of software limitations, two separate analyses were performed for *Desmosdesmus* and *Scenedesmus* lineages. *Neodesmus danubialis* was used as the outgroup for both analyses. Neighbor-joining and MP analyses were performed as described above, except that MP analysis employed all data, with gaps treated as a fifth state. For the analysis of *Desmodesmus* sequence data, the alignment derived from MARNA included 71 sequences with 306 characters of which 167 were parsimony informative. The *Scenedesmus* alignment derived from MARNA included 36 sequences, with 302 characters and 138 parsimony informative characters.

Microscopy

Light microscopy was performed using a Nikon E-600 microscope equipped with differential interference contrast optics (Nikon, Melville, New York). Digital images were acquired using a Pixera 150ES digital camera (Pixera Corp., Los Gatos, California). For light microscopy analysis, algae were grown in liquid cultures of WH+ medium (Fawley *et al.* 1990) for approximately 10 to 13 days. The number of cells per coenobium, cell shape, cell arrangement, as well as the presence of any cell wall ornamentation, were documented. Placement of ornamentation on the individual cells also was noted. Those cells with cell wall ornamentation were identified as *Desmodesmus.* Cells with no apparent cell wall ornamentation and a spindle-like shape were identified as *Scenedesmus.*

RESULTS

Scenedesmus and *Desmodesmus* isolates were found in both tychoplankton and phytoplankton samples, with the majority isolated from tychoplankton. The ITS regions, including the 5.8S rDNA, was amplified for 100 total isolates. PCR-RFLP analysis revealed 34 different PCR-RFLP types. Of the 34 different PCR-RFLP types, 21 types were represented by more than one isolate, whereas 13 types were represented by only one isolate (Table 1). Some sequence types with multiple isolates were represented by both tychoplankton and phytoplankton isolates, but a few were exclusively tychoplankton or phytoplankton. Individual types, the total number of isolates for each sequence type and their distribution among the sample sites are listed in Table 1. The ITS-1 region could not be aligned unambiguously across genera or even across different lineages of *Desmodesmus.* In addition, many published sequences lack the ITS-1 region. Therefore, sequence analyses were limited to ITS-2.

Diversity analysis

A distance analysis based on ITS-2 sequences from ISP isolates, as well as published sequences, revealed that five ISP PCR-RFLP types possessed ITS-2 sequences identical to previously published sequences of *Desmodesmus* species. An additional 11 ISP sequences were similar (< 7 nucleotide dif-

Table 1. Characterization of *Desmodesmus* and *Scenedesmus* ITS sequence types from Itasca State Park. Nearest relatives for the sequence isolates are given, when sequences were less than 20 nucleotides different. The Morphology column refers to morphological identity to the nearest relative as determined by ITS sequence analysis.

ITS type	Number of isolates (nucleotide difference) and locations[1]	Sequenced isolate(s)	GenBank accession number for ITS sequence	Nearest relative (nucleotide difference)	Morphology
A	2 (0); I, P	Itas 6/3 T-10w	DQ417520	*D. armatus* var. *subalternans* (5)	yes
		Pic 8/18 T-11w	DQ417521		
B	4 (1); T, WT	WTwin 8/18 P-2d	DQ417522	*D. cuneatus* (12)	no
		Tow 8/18 P-4w	DQ417523		
C	1; T	Tow 8/18 P-14w	DQ417524	*D. serratus* (17)	yes
D	1; M	Mary 8/18 T-1w	DQ417525	*D. multivariabilis* var. *turskensis* (0)	yes
E	3 (0); ND, T	NDem 6/3 P-3d	DQ417526	*D. hystrix* (9)	smaller, lack "teeth"
		Tow 6/16 T-10w	DQ417527		
F	4 (0); T	Tow 8/18 T-5w	DQ417528	*D. denticulatus* (12)	no, similar to *S. bijuga*
		Tow 8/18 T-23w	DQ417529		
G	1; T	Tow 8/18 P-3w	DQ417530	*D. opoliensis* (8)	somewhat
H	2 (0); I, T	Itas 6/3 T-2d	DQ417531	*Desmodesmus* sp. NIOO-MV5 (0),	somewhat
		Tow 6/16 T-16w	DQ417532	*D. opoliensis* (8)	
I	2; ND, T	NDem9/21T-10w	DQ417533	none	
J	14 (1); I, M, ND, T, WT	Itas 8/18 D-7w	DQ417534	*D. arthrodesmiformis* (6)	yes
		Mary 6/3 T-3d	DQ417535		
		Tow 8/18 P-2w	DQ417536		
		WTwin 6/16T-1w	DQ417537		
K	4 (0); T	Tow 6/3 T-11d	DQ417538	none	
		Tow 6/16 T-8w	DQ417539		
		Tow 8/18 P-1d	DQ417540		
		Tow 8/18 P-13w	DQ417541		
L	2 (0); T	Tow 6/3 T-9w	DQ417542	none	
		Tow 8/18 P-20w	DQ417543		
M	2 (0); T	Tow 8/18 P-25w	DQ417544	*D. denticulatus* (15)	no, similar to *S. bijuga*
		Tow 8/18 T-25w	DQ417545		
N	4 (1); T	Tow 6/16 T-6w	DQ417546	*D. armatus* var. *subalternans* (4–5)	somewhat
		Tow 6/16 T-30w	DQ417547		
O	1; T	Tow 6/16T-31w	DQ417548	*D. komarekii* (10)	somewhat
P	1; T	Tow 9/21 P-12w	DQ417549	*D. asymmetricus* (2)	yes
Q	1; ND	NDem 8/18 T-11w	DQ417550	none	
R	1; ND	NDem 9/21 T-9w	DQ417551	*D. hystrix* (0)	nearly, lack "teeth"
S	2 (2); M, T	Mary 6/3 T-2d	DQ417552	*D. asymmetricus* (17)	no
		Tow 10/11 T-2w	DQ417553		
T	12 (0); T	Tow 6/16 T-17w	DQ417554	none	
		Tow 8/18 T-10w	DQ417555		
		Tow 10/11 T-12w	DQ417556		
U	1; ND	NDem 6/3 T-13w	DQ417557	*D. pirkollei* (0)	yes
V	1; ND	NDem 9/21 T-17d	DQ417558	*D. bicellularis* (0)	yes
W	9 (1); T	Tow 6/16 T-35w	DQ417559	none	
		Tow 10/11 T-1w	DQ417560		
		Tow 10/11 T-17w	DQ417561		
X	1; T	Tow 10/11 T-16w	DQ417562	*D. komarekii* (2)	yes
Y	4 (0); T	Tow 6/16 T-9w	DQ417563	*D. opoliensis* (8)	somewhat
		Tow 6/16 T-15w	DQ417564		
		Tow 10/11 T-8w	DQ417565		
Z	3 (0); T	Tow 6/16 T-2w	DQ417566	*D. cuneatus* (2)	somewhat
		Tow 8/18 P-10d	DQ417567		
AA	1; T	Tow 9/21 P-1w	DQ417568	*S. obliquus* (2–3)	yes
BB	2 (0); M	Mary 9/21 BT-16w	DQ417569	*S. acutiformis* (19)	no
		Mary 9/21 BT-19w	DQ417570		
CC	1; P	Pic 6/16 T-1w	DQ417571	none	
EE	1; T	Tow 6/16 T-26w	DQ417573	*D. serratus* (19)	yes
FF	2; T	Tow 10/11 T-3w	DQ417574	none	
		Tow 10/11 T-6w	DQ417575		
GG	2 (0); T	Tow 6/16 T-1w	DQ417576	*D. asymmetricus* (0)	yes
		Tow 6/16 T-7w	DQ417577		
HH	3 (1); I	Itas 2/24 S-1d	DQ417578	none	
		Itas 6/3 T-2w	DQ417579		
		Itas 8/18 S-6d	DQ417580		
II	4; T	Tow 6/16 T-32w	DQ417581	none	

[1] Locations are I: Lake Itasca; P: Picnic Pond; T: Tower Pond; M: Mary Lake; WT: West Twin Lake; and ND: North Deming Pond.

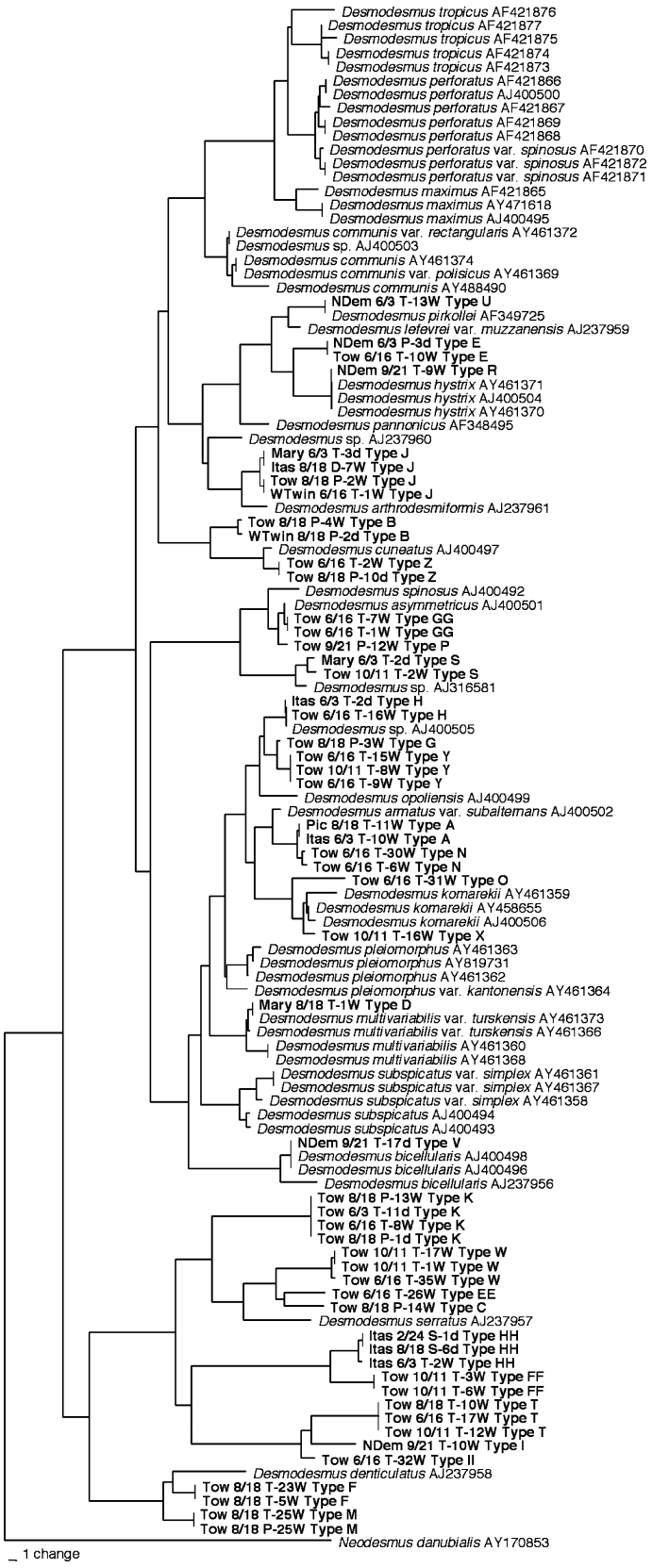


Fig. 1. Dendrogram derived from neighbor-joining analysis of internal transcribed spacer (ITS-2) rDNA sequences from 111 *Desmodesmus* isolates. The dendrogram was rooted using *Neodesmus danubialis.* Branch lengths are proportional to absolute differences.

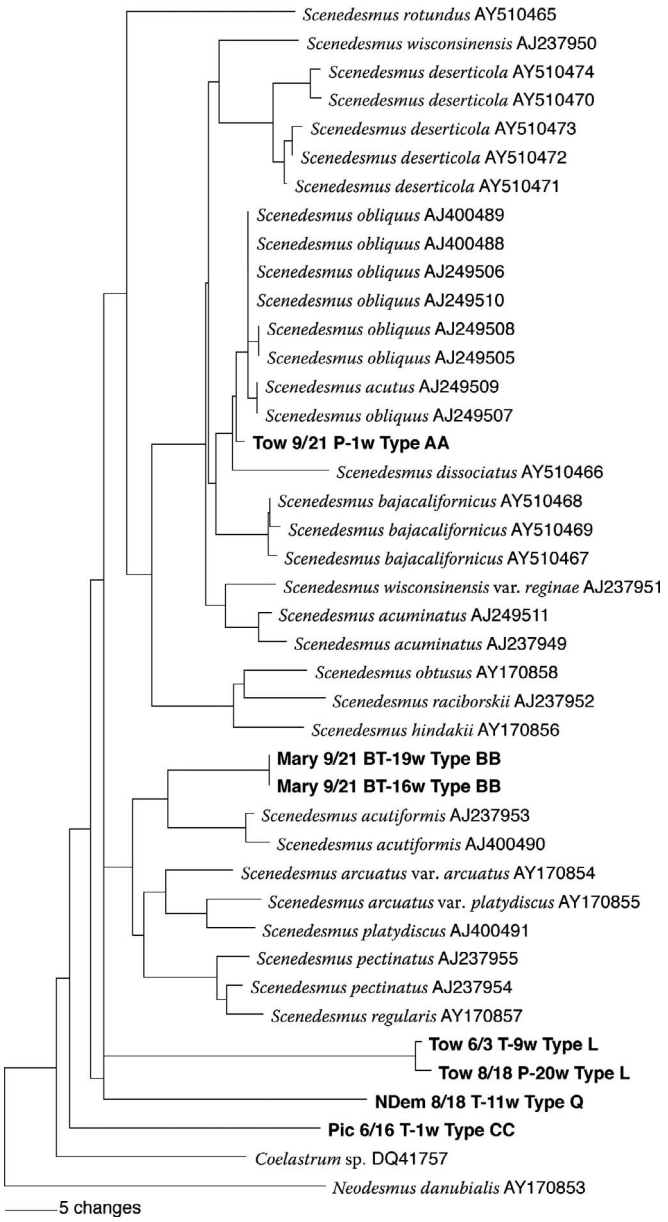


Fig. 2. Dendrogram derived from neighbor-joining analysis of internal transcribed spacer (ITS-2) rDNA sequences from 41 *Scenedesmus* and related isolates. The dendrogram was rooted using *Neodesmus danubialis.* Branch lengths are proportional to absolute differences.

ference) to scenedesmacean sequences already published in GenBank (Figs 1, 2). The remaining 18 types differed by at least eight nucleotides from the most similar sequences. For 12 of the ISP ITS-2 sequence types, duplicates of the same PCR-RFLP type produced identical ITS-2 sequences; however, ITS-2 sequences from duplicates of the remaining eight PCR-RFLP types differed by 1 to 2 bases. The sequences of seven of the ISP ITS-2 PCR-RFLP types differed from the sequences of other ISP types by six or fewer nucleotides (Fig. 1).

Morphology of ITS-2 sequence types

For LM, morphologies of isolates were compared to descriptions of known taxa with similar ITS-2 sequences, when avail-

able. These relationships were chosen based on the diversity analysis. Detailed descriptions are presented only for those isolates that were not closely related to any sequenced-described species, or that differed in morphology from closely related described species. No attempt was made to identify isolates to the species level based solely on morphology. Additional ITS studies are underway (Thomas Friedl, personal communication; Eberhard Hegewald, personal communication) employing a larger set of authentic named isolates. These sequences ultimately will be used to help name our unidentified isolates.

Desmodesmus

The ITS-2 sequence of type U, represented by a single isolate, was identical to the published sequence from *D. pirkollei* Hegewald (Fig. 1). The morphology of type U (Fig. 3) also matched the published description of *D. pirkollei* (Hegewald *et al.* 2001).

Sequence type R, also represented by a single isolate, produced an ITS-2 sequence identical to that of *D. hystrix* (Lagerheim) Hegewald (Fig. 1). ITS-2 sequences from three type E isolates were identical to each other and only differed by nine nucleotides from the ITS-2 sequences of type R and *D. hystrix* (Fig. 1). The morphologies of types E and R were very similar, except that the cells of type R were much smaller (2.7–4.4 μm wide and 8.4–13.6 μm long), whereas cells of type E isolates varied from 4.9 to 7.4 μm in width and from 13.2 to 18.1 μm in length (Figs 4, 5). *Desmodesmus hystrix* is characterized by oblong cells, and the cell wall is covered with numerous uniform short sharp teeth (Hegewald & Silva 1988; Dillard 1989). Although type R otherwise strongly resembled *D. hystrix,* the cell walls of this isolate were not covered in sharp teeth (Fig. 5).

Fourteen ISP isolates have sequence type J, and only a single substitution difference was present among the four ITS-2 sequences for this type (Fig. 1). These ITS-2 sequences differed by six nucleotides from the published ITS-2 sequence for *D. arthrodesmiformis* (Schröder) An, Friedl & Hegewald. The morphologies of all four type J isolates were the same, and the cell size, shape and arrangement were consistent with the description of *D. arthrodesmiformis* (Hegewald *et al.* 1990) (Fig. 6). Some coenobia of *D. arthrodesmiformis* have ribs or fragments of ribs on the terminal cells; this feature was not noticed on the ISP isolates.

ITS-2 sequences for two type B ISP isolates differed from each other by one nucleotide, by 11 nucleotides from ISP sequence type Z and by 12 nucleotides from the ITS-2 sequence listed in GenBank as *S. aldavei* Hegewald [*D. cuneatus* (Skabičevskij) Hegewald in Fig. 1, as per Hegewald 2000]. Cells of type B were oval and varied from 2.7 to 5 μm in width and 7.2 to 10.4 μm in length (Fig. 7). Only 4-celled coenobia were found in cultures. These isolates did not have any wall ornamentation visible with LM. The morphology of type B isolates was distinct from that of *S. aldavei* (Hegewald & Silva 1988) and ISP type Z.

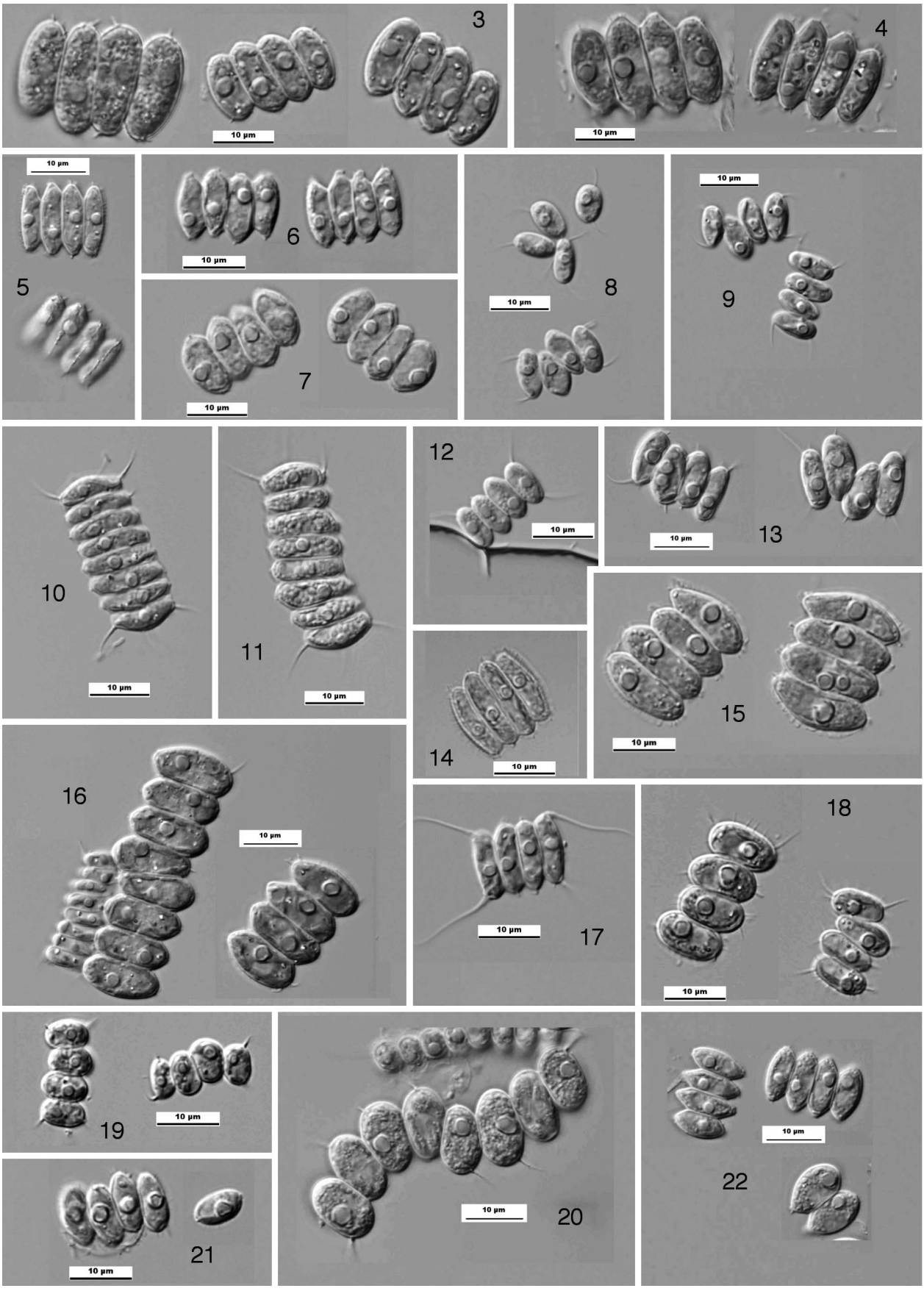
Three ISP isolates were found with ITS-2 sequence type Z. The two type Z sequences generated were identical and differed by 3 bases from the ITS-2 sequence listed in GenBank as *S. aldavei* (*D. cuneatus* in Fig. 1). Cell shape and arrangement of the type Z isolates matched the published description of *S. aldavei*; but they have more spines and the cells were larger than those of *S. aldavei.* Cells of the type Z isolates varied from 3.5 to 5.5 μm in width and 6.8 to 9.7 μm in length. Unicells had 2 spines, one at each pole (Fig. 8), although 4-celled coenobia had long spines at the apices of terminal cells (Fig. 9). Some 4-celled coenobia of the type Z isolate Tow 8/18 P-10d had shorter spines at the apices of some of the intermediate cells, but this varied greatly within the culture. Some 8-celled coenobia also were found in the culture of Tow 8/18 P-10d. These coenobia had spines at the apices of all terminal cells, some shorter spines at the apices of intermediate cells, and also some incomplete ridges on intermediate cells. *S. aldavei* recently has been reduced to synonymy with *D. cuneatus* (Hegewald 2000). Type Z isolates resemble the description of *S. aldavei* more closely than the description of *D. cuneatus.*

The ITS-2 sequence of type GG differed by two nucleotides from the sequence of type P and by two indels from the sequence of *D. asymmetricus* (Schröder) Hegewald (Fig. 1). The sequence of type P differed by two nucleotides from the sequence of *D. asymmetricus.* The morphologies of both ISP types (Figs 10, 11) match the description of *D. asymmetricus.* The oval cells of the type P isolate were more elongate, varying from 2.8 to 5.7 μm in width and from 10.3 to 17.8 μm in length, than cells of type GG, which varied from 2.4 to 4.6 μm in width and from 9.2 to 13 μm in length.

Type S was represented by two ISP isolates whose ITS-2 sequences differed by two nucleotides from each other, by four nucleotides from *Desmodesmus* sp. CL1 and by 17 nucleotides from the ITS-2 sequences of *D. asymmetricus,* type P and type GG (Fig. 1). Cells of the type S isolates were much smaller than *D. asymmetricus,* (3–6.1 μm wide and 7.7–10.9 μm long) and also were more ovoid shaped (Fig. 12). Both type S isolates had spines at the apices of terminal cells, but lacked the spines that line the middle of intermediate cells, as is typical for *D. asymmetricus.*

Sequence type H also was represented by two isolates with identical ITS-2 sequences. The type H sequence differed by four nucleotides from the ITS-2 sequence for type G and by six nucleotides from type Y (Fig. 1). Coenobia of 4- and 8-cells were found in cultures of type H (Fig. 13). Cells were arranged linearly, occasionally with a displaced central cell. Cells varied in width from 3.5 to 6.2 μm and in length from 7.6 to 12.8 μm and were ovoid with long spines at the apices of terminal cells. There also were spines at the apices of some intermediate cells, but this feature was quite variable. The ITS-2 sequence of type H was identical to the published sequence for the unnamed ''*Scenedesmus* sp. NIOO-MV5'' (GenBank AJ400505), which is referred to as *Desmodesmus* sp. in Fig. 1.

ITS-2 sequences of three type Y isolates were all identical and differed from the sequence of type G by only two nucleotides (Fig. 1). Cells of type Y and G had similar shape and arrangement. Coenobia were 4- or 8-celled, with cells linearly arranged. Cells were oval and slightly elongate (Figs 14, 15). Ridges were found on the end of terminal cells and the middle of intermediate cells for both type Y and G. Cells of the type Y and G isolates were comparable in size. Cells of type Y varied in width from 4.8 to 6.3 μm and in length from 12.4 to 17.2 μm, whereas type G varied in width from 3.5 to 6.9 μm and in length from 14.2 to 20.1 μm.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ITS-2 sequences of all six ISP isolates representing sequence types H, Y, and G differed from ITS-2 sequences of *D. opoliensis* (P. Richter) Hegewald by eight nucleotides (Fig. 1). Cells of *D. opoliensis* are arranged linearly, but intermediate cells occasionally can be displaced (Hindák 1990). Characteristics of the species are long spines at the apices of terminal cells and shorter tooth-like spines at the apices of some intermediate cells. Ribs can be found on some coenobia, but vary greatly in number and placement (Hindák 1990). Isolates of type H matched this description quite well, but lacked the ribs (Fig. 13). The other four ISP isolates (types G and Y) lacked the characteristic spines of *D. opoliensis* (Figs 14, 15), but resembled some varieties of this species in other characteristics (Hegewald & Silva 1988).

Type A was represented by two isolates with identical ITS-2 sequences, and this sequence differed from the sequence of *D. armatus* var. *subalternans* (G.M. Smith) Hegewald by 5 bases (Fig. 1). Morphologies of type A isolates matched the description for *D. armatus* var. *subalternans* (Fig. 16). The ITS-2 sequences of two type N isolates differed by one nucleotide from each other, and by one or two nucleotides from type A. Sequences of type N differ by four or five nucleotides from *D. armatus* var. *subalternans* (Fig. 1). The morphologies of the type N isolates were very similar, but not identical, to type A and *D. armatus* var. *subalternans.* Very long spines were found at the apices of terminal cells of type N, longer than those for type A, and ridges were readily noticeable on intermediate cells (Fig. 17). Cells of type N were smaller than type A, varying in width from 2.8 to 3.9 m and in length from 11 to 12.5 μm.

The ITS-2 sequence of the single type O isolate differed by 10 nucleotides from type X, and 10 nucleotides from *D. komarekii* (Hegewald) Hegewald (Fig. 1). Coenobia of 4- and 8-cells were found in culture (Fig. 18). Cells of type O were oval and ranged from 3.5 to 6.8 μm in width and from 7.2 to 12.1 μm in length. The morphology of this isolate is similar to that of *D. komarekii,* with long spines at the apices of all cells. However, shorter spines were found lining the edges of terminal cells and in rows on intermediate cells, a characteristic not described for *D. komarekii.*

The ITS-2 sequence of type X differed by two nucleotides from the ITS-2 sequence of *D. komarekii* (Fig. 1). The morphology of the ISP isolate matches the written description of *D. komarekii* (Hegewald 1989) (Fig. 19). Cells varied in size from 3.3 to 5.4 μm in width and from 5.8 to 7.8 μm in length.

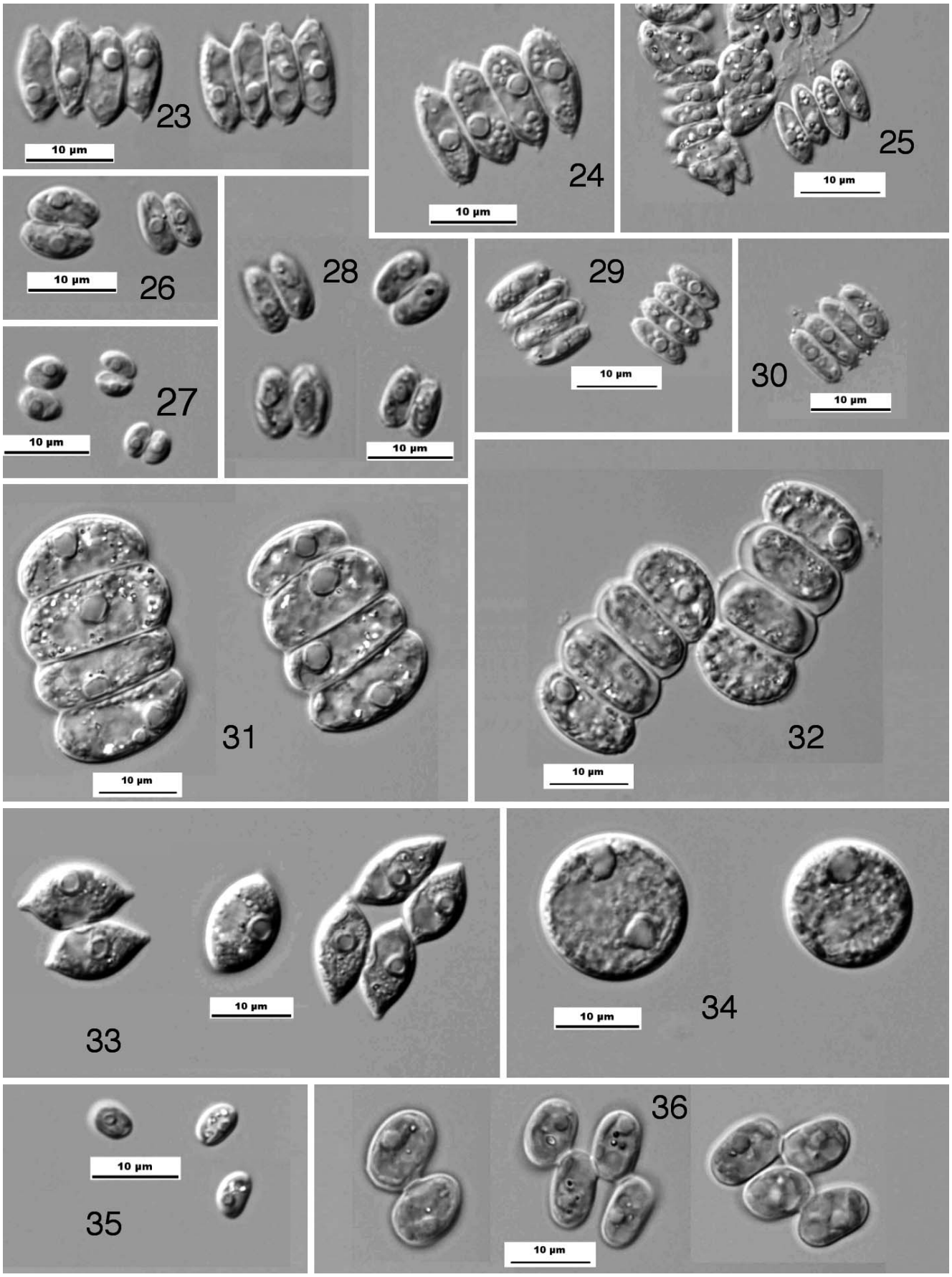
The ITS-2 sequence of the type D exactly matched the ITS-2 sequence of *D. multivariabilis* var. *turskensis* (Tsarenko *et al.* 2005) Hegewald (Fig. 1). Coenobia of 4- and 8-cells were found in cultures of type D, with cells linearly arranged (Fig. 20). Coenobia were curved, forming an S-shape. Cells were broadly oval, ranging from 2.7 to 8 μm in width and from 6.7 to 15.3 μm in length. Spines were located at the apices of all terminal cells, and at one apex of each intermediate cell. Overall, this description matches quite well with that of *D. multivariabilis* var. *turskensis* (Tsarenko *et al.* 2005), except that our isolate is somewhat more variable in size than the published description, and typically intermediate cells possess only a single spine.

The ITS-2 sequence of type V exactly matched the ITS-2 sequences of *D. bicellularis* (Chodat) An, Friedl, & Hegewald (Fig. 1). Coenobia of 4- and 8-cells were observed in the culture of type V, as well as a few unicells (Fig. 21). The morphology of the ISP isolate was similar to the description of *D. bicellularis,* except that the ISP isolate was larger and had spine-like appendages lining the edge of terminal cells and the middle of intermediate cells. Cells of the ISP isolate varied from 3.6 to 5 μm in width and from 7.7 to 13 μm in length. The spine-like appendages on the ISP isolate could be short fragments of ridges, as ridges of *D. bicellularis* can be reduced to spine-like fragments (Hegewald 1989).

Nine ISP types (C, I, K, T, W, EE, FF, HH and II) had ITS-2 sequences somewhat similar to that of *D. serratus* (Fig. 1), but the sequences of seven of these types (I, K, T, W, FF, HH and II) were different from the *D. serratus* sequence by greater than 20 substitutions. The four ITS-2 sequences representing type K were identical to each other (Fig. 1). Coenobia of 2-, 4-, and 8-cells, with cells linearly arranged, were found for all the isolates (Fig. 22). Cells were oval with pointed apices and short spiny teeth at the apices. Sequence type W is represented by three isolates that differed among them-

←

Figs 3–22. Light micrographs of *Desmodesmus* from Itasca State Park.
Fig. 3. Type U isolate NDem 6/3 T-13W.
Fig. 4. Type E isolate NDem 6/3 P-3d.
Fig. 5. Type R isolate NDem 9/21 T-9W.
Fig. 6. Type J isolate WTwin 6/16 T-1W.
Fig. 7. Type B isolate WTwin 8/18 P-2d.
Fig. 8. Type Z isolate Tow 6/16 T-2W.
Fig. 9. Type Z isolate Tow 8/18 P-10d.
Fig. 10. Type GG isolate Tow 6/16 T-1W.
Fig. 11. Type P isolate Tow 9/21 P-12W.
Fig. 12. Type S isolate Mary 6/3 T-2d.
Fig. 13. Type H isolate Itas 6/3 T-2d.
Fig. 14. Type G isolate Tow 8/18 P-3W.
Fig. 15. Type Y isolate Tow 10/11 T-8W.
Fig. 16. Type A isolate Pic 8/18 T-11W.
Fig. 17. Type N isolate Tow 6/16 T-30W.
Fig. 18. Type O isolate Tow 6/16 T-31W.
Fig. 19. Type X isolate Tow 10/11 T-16W.
Fig. 20. Type D isolate Mary 8/18 T-1W.
Fig. 21. Type V isolate NDem 9/21 T-17d.
Fig. 22. Type K isolate Tow 8/18 P-1d.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selves by one nucleotide (Fig.1). Coenobia were 4- or 8-celled (Fig. 23) with oval, elongate cells with pointed apices, varying from 3.4 to 5.7 μm in width and from 9.1 to 12.1 μm in length. Cells had short spiny teeth at the apices. Isolates representing types K and W only had the spiny teeth at cell apices in common with the description of *D. serratus* (Figs 22, 23).

The ITS-2 sequences of type C and type EE differed by seven nucleotides and by 17 and 19 nucleotides, respectively, from the sequence from *D. serratus* (Fig. 1). Coenobia of 4- and 8-cells, with cells linearly arranged, ranging from 3.5 to 5.3 μm in width and from 10.7 to 13.6 μm in length, were found for the type EE isolate (Fig. 24). Coenobia of 2-, 4-, and 8-cells, with cells linearly arranged, ranging from 2.8 to 4.9 μm in width and from 8.3 to 10.2 μm in length, were found for the type C isolate (Fig. 25). Cells of both isolates were oval, slightly elongate, with pointed apices and short spiny teeth at the apices. Spiky appendages also were found lining the edge of cells of both isolates. The ISP isolates of types C and EE had a similar morphology to the description of *D. serratus* (Figs 24, 25). *Desmodesmus serratus* is characterized by one longitudinal row of small teeth on terminal cells and two longitudinal rows on intermediate cells, as well as small teeth at all apices (Dillard 1989).

Sequence type HH is represented by three isolates that differ among themselves by one nucleotide (Fig. 1). All isolates were small, 2-celled coenobia with no visible wall ornamentation, ranging from 2.4 to 4.8 μm in width and from 5.3 to 8.7 μm in length (Fig. 26). The ITS-2 sequence of type FF differed by nine to 10 nucleotides from sequence type HH (Fig. 1). Unicells and some small 2-celled coenobia were found in cultures of type FF (Fig. 27). Cells of type FF were more rounded in shape compared to type HH. Cells of type FF ranged from 2.4 to 4 μm in width and from 3.8 to 5 μm in length. No wall ornamentation was visible on these isolates using LM. These isolates did not resemble *D. serratus.*

The ITS-2 sequences of three type T isolates were identical (Fig. 1). Coenobia of 2-, 4-, and 8-cells were found for the isolate Tow 6/16 T-17W, with cells ranging from 2.4 to 3.8 μm in width and from 6.8 to 9.8 μm in length. Coenobia of Tow 8/18 T-10W (Fig. 28) were 2- and 4-celled, with cells ranging from 2.5 to 4.6 μm in width and from 5.9 to 8.4 μm in length. Cells of both isolates were linearly arranged, oval, with rounded apices, and short blunt appendages at cell apices.

The ITS-2 sequences of type I and type II differed by 9 bases (Fig. 1). Coenobia of 4- and 8-cells, with cells linearly arranged, were found for both isolates (Figs 29, 30). Cells were oval with rounded apices. Short spiny teeth were found at the apices of cells of type I and cells ranged from 2.5 to 3.7 μm in width and from 7.9 to 10.4 μm in length (Fig. 29). Short spiny teeth were not found on cells of type II, but large warts were found covering the cell walls; cells ranged from 2.2 to 3.5 μm in width and from 8.1 to 9.9 μm in length (Fig. 30).

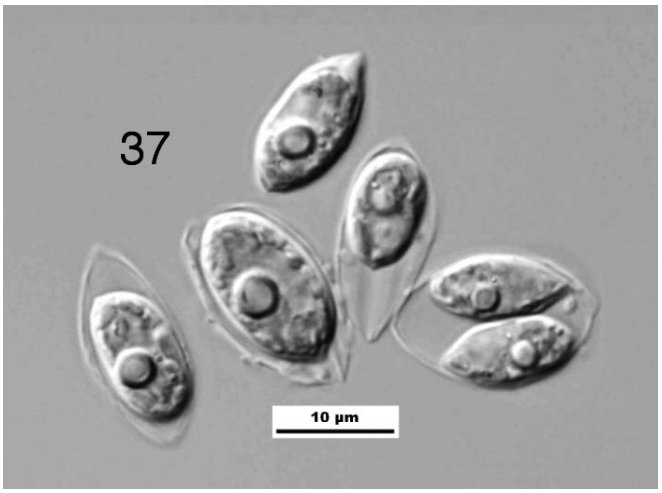

Fig. 37. Light micrograph of type CC isolate Pic 6/16 T-1W from Itasca State Park allied with *Scenedesmus*.

Four isolates, representing types F and M, were allied with *D. denticulatus* (Lagerheim) An, Friedl & Hegewald (Fig. 1). The ITS-2 sequences of the two type F isolates were identical to each other, as were the ITS-2 sequences of the type M isolates. ITS-2 sequences for types F and M differed by seven nucleotides from each other. ITS-2 sequences of type F and type M differed by 12 and 15 nucleotides, respectively, from the ITS-2 sequence of *D. denticulatus.* Coenobia of 4-cells were found for both type F and type M isolates (Figs 31, 32). Cells were broadly ovoid and arranged in linear rows, with the cells appressed over most of their length. Cells of type F ranged from 4.2 to 9.2 μm in width and from 12.2 to 21.1 μm in length (Fig. 31). Cells of type M ranged from 4.8 to 8.2 μm in width and from 11.4 to 15 μm in length (Fig. 32).

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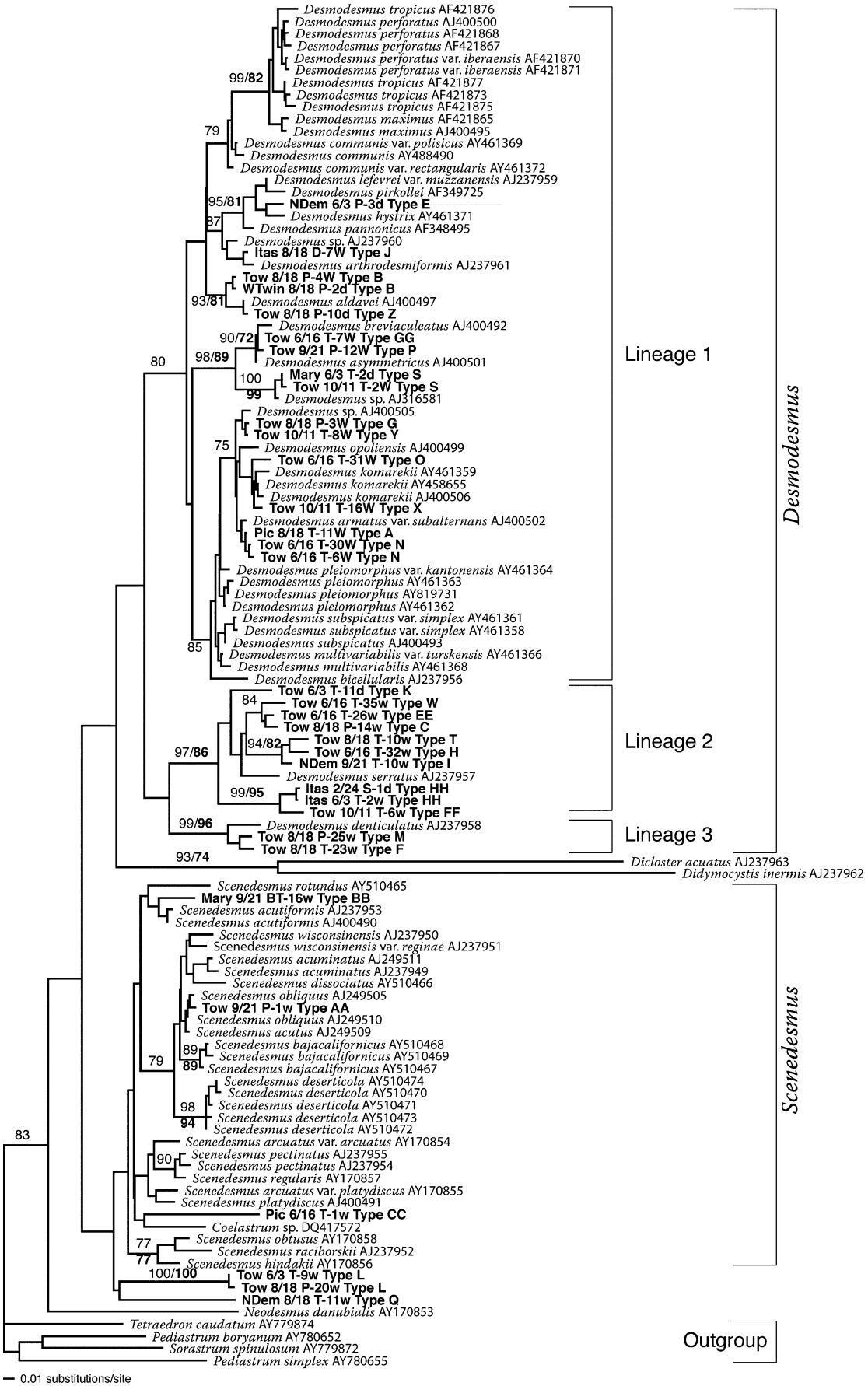
Figs 23–32. Light micrographs of *Desmodesmus* from Itasca State Park.
Fig. 23. Type W isolate Tow 10/11 T-1W.
Fig. 24. Type EE isolate Tow 6/16 T-26W.
Fig. 25. Type C isolate Tow 8/18 P-14W.
Fig. 26. Type HH isolate Itas 8/18 S-6d.
Fig. 27. Type FF isolate Tow 10/11 T-3W.
Fig. 28. Type T isolate Tow 8/18 T-10W.
Fig. 29. Type I isolate NDem 9/21 T-10W.
Fig. 30. Type II isolate Tow 6/16 T-32W.
Fig. 31. Type F isolate Tow 8/18 T-23W.
Fig. 32. Type M isolate Tow 8/18 P-25W.
Figs 33–36. Light micrographs of types from Itasca State Park allied with *Scenedesmus*.
Fig. 33. Type AA isolate Tow 9/21 P-1W.
Fig. 34. Type BB isolate Mary 9/21 BT-16W.
Fig. 35. Type L isolate Tow 8/18 P-20W.
Fig. 36. Type Q isolate NDem 8/18 T-11W.


Desmodesmus tropicus AF421876
Desmodesmus perforatus AJ400500
Desmodesmus perforatus AF421868
Desmodesmus perforatus AF421867
Desmodesmus perforatus var. iberaensis AF421870
Desmodesmus perforatus var. iberaensis AF421871
Desmodesmus tropicus AF421877
Desmodesmus tropicus AF421873
Desmodesmus tropicus AF421875
Desmodesmus maximus AF421865
Desmodesmus maximus AJ400495
Desmodesmus communis var. polisicus AY461369
Desmodesmus communis AY488490
Desmodesmus communis var. rectangularis AY461372
Desmodesmus lefevrei var. muzzanensis AJ237959
Desmodesmus pirkollei AF349725
NDem 6/3 P-3d Type E
Desmodesmus hystrix AY461371
Desmodesmus pannonicus AF348495
Desmodesmus sp. AJ237960
Itas 8/18 D-7W Type J
Desmodesmus arthrodesmiformis AJ237961
Tow 8/18 P-4W Type B
WTwin 8/18 P-2d Type B
Desmodesmus aldavei AJ400497
Tow 8/18 P-10d Type Z
Desmodesmus breviaculeatus AJ400492
Tow 6/16 T-7W Type GG
Tow 9/21 P-12W Type P
Desmodesmus asymmetricus AJ400501
Mary 6/3 T-2d Type S
Tow 10/11 T-2W Type S
Desmodesmus sp. AJ316581
Desmodesmus sp. AJ400505
Tow 8/18 P-3W Type G
Tow 10/11 T-8W Type Y
Desmodesmus opoliensis AJ400499
Tow 6/16 T-31W Type O
Desmodesmus komarekii AY461359
Desmodesmus komarekii AY458655
Desmodesmus komarekii AJ400506
Tow 10/11 T-16W Type X
Desmodesmus armatus var. subalternans AJ400502
Pic 8/18 T-11W Type A
Tow 6/16 T-30W Type N
Tow 6/16 T-6W Type N
Desmodesmus pleiomorphus var. kantonensis AY461364
Desmodesmus pleiomorphus AY461363
Desmodesmus pleiomorphus AY819731
Desmodesmus pleiomorphus AY461362
Desmodesmus subspicatus var. simplex AY461361
Desmodesmus subspicatus var. simplex AY461358
Desmodesmus subspicatus AJ400493
Desmodesmus multivariabilis var. turskensis AY461366
Desmodesmus multivariabilis AY461368
Desmodesmus bicellularis AJ237956
Tow 6/3 T-11d Type K
Tow 6/16 T-35w Type W
Tow 6/16 T-26w Type EE
Tow 8/18 P-14w Type C
Tow 8/18 T-10w Type T
Tow 6/16 T-32w Type H
NDem 9/21 T-10w Type I
Desmodesmus serratus AJ237957
Itas 2/24 S-1d Type HH
Itas 6/3 T-2w Type HH
Tow 10/11 T-6w Type FF
Desmodesmus denticulatus AJ237958
Tow 8/18 P-25w Type M
Tow 8/18 T-23w Type F
Dicloster acuatus AJ237963
Didymocystis inermis AJ237962
Scenedesmus rotundus AY510465
Mary 9/21 BT-16w Type BB
Scenedesmus acutiformis AJ237953
Scenedesmus acutiformis AJ400490
Scenedesmus wisconsinensis AJ237950
Scenedesmus wisconsinensis var. reginae AJ237951
Scenedesmus acuminatus AJ249511
Scenedesmus acuminatus AJ237949
Scenedesmus dissociatus AY510466
Scenedesmus obliquus AJ249505
Tow 9/21 P-1w Type AA
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Scenedesmus acutus AJ249509
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Scenedesmus deserticola AY510472
Scenedesmus arcuatus var. arcuatus AY170854
Scenedesmus pectinatus AJ237955
Scenedesmus pectinatus AJ237954
Scenedesmus regularis AY170857
Scenedesmus arcuatus var. platydiscus AY170855
Scenedesmus platydiscus AJ400491
Pic 6/16 T-1w Type CC
Coelastrum sp. DQ417572
Scenedesmus obtusus AY170858
Scenedesmus raciborskii AJ237952
Scenedesmus hindakii AY170856
Tow 6/3 T-9w Type L
Tow 8/18 P-20w Type L
NDem 8/18 T-11w Type Q
Neodesmus danubialis AY170853
Tetraedron caudatum AY779874
Pediastrum boryanum AY780652
Sorastrum spinulosum AY779872
Pediastrum simplex AY780655
0.01 substitutions/site
Lineage 1
Lineage 2
Lineage 3
Desmodesmus
Scenedesmus
Outgroup

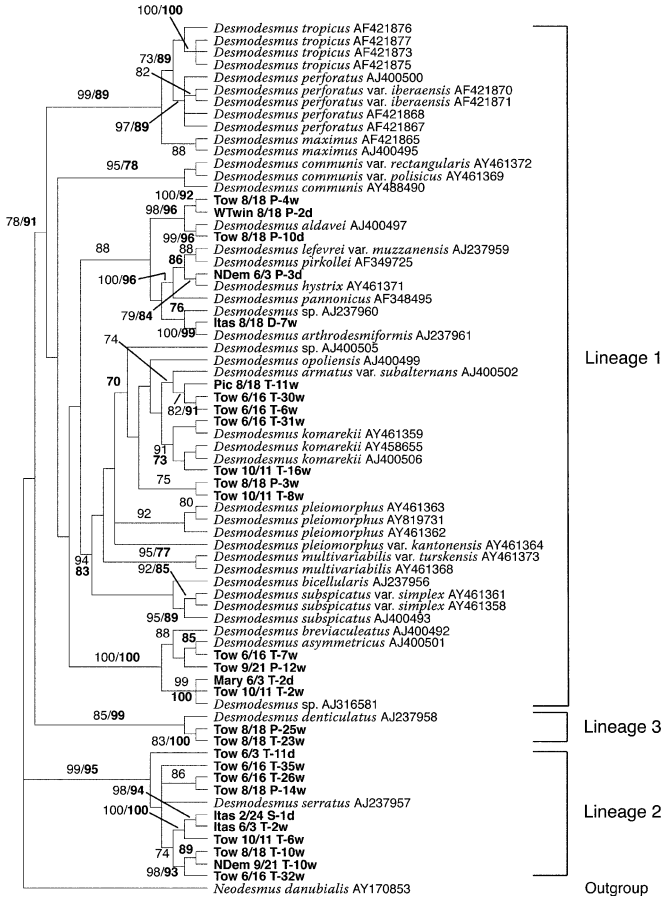

Fig. 39. Majority-rule consensus tree (110 most parsimonious trees) derived from maximum parsimony (MP) analysis of 71 internal transcribed spacer (ITS-2) rDNA sequences from *Desmodesmus* taxa aligned using the software MARNA (Siebert & Backofen 2005). The tree is rooted with the sequence from *Neodesmus danubialis.* This MP analysis employed the complete alignment with gaps treated as a fifth character state. Bootstrap values (1000 replicates) for the MP analysis greater than 70 are shown in bold. Bootstrap values for a neighbor-joining analysis of the same data set are shown in standard font.

No wall ornamentation was visible on cells of any of the ISP type F and M isolates using LM, but small granules could just be seen on old mothercell walls of type F. Both types F and M lacked any hint of the short teeth characteristic of *D. denticulatus* (Dillard 1989). These isolates would both be referred to *S. bijuga* (Turpin) Lagerheim based on morphology (Prescott 1967), but the ITS-2 sequence for authentic S. *bijuga* is not available.

Scenedesmus

Few isolates allied with any known *Scenedesmus* species (Fig. 2). The ITS-2 sequence of type AA differed by 2 to 3 bases from the ITS-2 sequences of *S. acutus* Meyen and *S. obliquus.* Unicells and 2-celled coenobia were found for type AA (Fig. 33). Cells of 2-celled coenobia were linearly arranged. Cells were narrowly to broadly lemon-shaped, with thickenings at the cell poles, and cell length ranged from 7.1 to 12.5 μm in width and from 11.7 to 16.2 μm in length. These cells are larger than typical for *S. obliquus.* (Hegewald 1989)

Sequence type BB isolates allied with *S. acutiformis* Schröder; however, ITS-2 sequences differed by 19 bases (Fig. 2). Cells of type BB were large coccoids, ranging from 11.6 to 19.3 μm in width, mostly unicellular, but sometimes found in groups of two (Fig. 34). The morphological description of *S. acutiformis* is quite different from the observed morphology of type BB. *S. acutiformis* is described as fusiform cells with sharply pointed apices (Dillard 1989). Intermediate cells of *S. acutiformis* have a single longitudinal ridge, although terminal cells have two to four longitudinal ridges (Dillard 1989).

Of those isolates identified as *Scenedesmus* that did not ally with any published sequence, most (types CC and L) were unicellular (Figs 35, 37). Cells were oval to spherical, and exhibited a large size range. Type L ranged from 3.6 to 4 μm in width and from 4.6 to 5.7 μm in length (Fig. 35). Type CC ranged from 6.4 to 9.6 μm in width and from 12.9 to 15.7 μm in length (Fig. 37). One of the ISP *Scenedesmus* isolates, type Q, produced coenobia of more than 2 cells (Fig. 36). The cells had an alternate arrangement and ranged from 5.2 to 8.8 μm in width and from 9.2 to 13.4 μm in length. This morphology is similar to *S. bijuga* var. *alternans* (Prescott 1967; Hegewald & Silva 1988).

Phylogenetic analysis

Monophyly of the Scenedesmaceae was supported only with a bootstrap value of 80% in the global NJ analysis (Fig. 38). However, there were 57 nucleotide differences between the most similar sequence from the Scenedesmaceae and the Hydrodictyaceae (outgroup). *Neodesmus danubialis* Hindák is basal to the rest of the Scenedesmaceae (Fig. 38). The ITS-2 sequence for *Neodesmus danubialis* differed by 52 nucleotides from the most similar *Scenedesmus* sequence. *Dicloster acuatus* Jao, Wei & Hu and *Didymocystis inermis* (Fott) Fott formed a lineage basal to *Desmodesmus,* but without support. ITS-2 sequences for these species differed from the most similar *Scenedesmus* or *Desmodesmus* sequence by at least 74 nucleotides.

Monophyly was not supported for either *Desmodesmus* or *Scenedesmus* in the global ITS-2 analysis (Fig. 38). The most similar sequences from the genera *Scenedesmus* and *Desmodesmus* differed by 38 nucleotides. Within *Desmodesmus,* three lineages were supported in the NJ analysis, and many small terminal lineages were supported in both NJ and MP analyses (Fig. 38). Within S*cenedesmus,* many terminal lineages were supported in NJ and MP analyses (Fig. 38), but relationships among these lineages were not resolved. Nucleotide differences within these terminal lineages ranged from one to seven nucleotides between the sequences of *S. deserticola* Lewis & Flechtner strains to 18 nucleotides between *S. hindakii* (Hegewald & Hanagata) and *S. raciborskii* Wołoszyńska sequences.

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Fig. 38. Phylogram derived from neighbor-joining (NJ) analyses of 113 internal transcribed spacer (ITS-2) rDNA sequences from members of the Scenedesmaceae. The NJ tree was rooted with members of the Hydrodictyaceae. Bootstrap values (1000 replicates) from NJ (standard font) and maximum parsimony analyses (bolded) are shown. Only bootstrap values over 70 are shown.

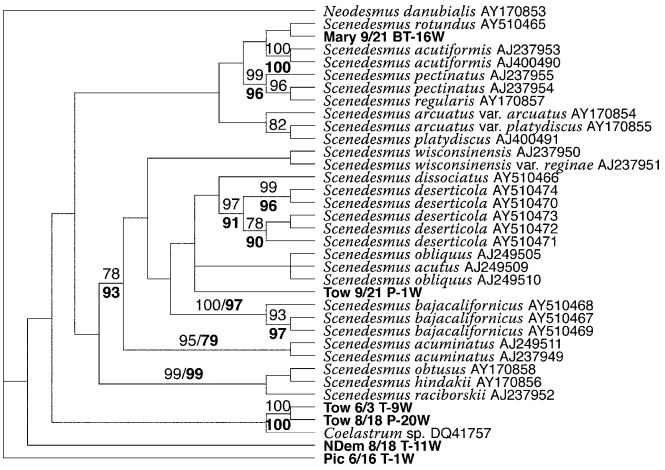

Fig. 40. Majority-rule consensus tree (3 most parsimonious trees) derived from maximum parsimony (MP) analysis of 35 internal transcribed spacer (ITS-2) rDNA sequences from *Scenedesmus* and related taxa aligned using the software MARNA (Siebert & Backofen 2005). The tree is rooted with the sequence from *Neodesmus danubialis.* This MP analysis employed the complete alignment with gaps treated as a fifth character state. Bootstrap values (1000 replicates) for the MP analysis greater than 70 are shown in bold. Bootstrap values for a neighbor-joining analysis of the same data set are shown in standard font.

Phylogenetic analyses indicated three distinct lineages within *Desmodesmus.* Lineage one included the majority of sequences and had only 80% bootstrap support for the NJ analysis in the global phylogeny (Fig. 38). However, support for this lineage increased to 91% in the MP analysis when sequences were aligned using MARNA and gaps were treated as a fifth state (Fig. 39). Two basal lineages within *Desmodesmus* were strongly supported. One lineage, lineage 2, included 19 ISP isolates representing eight ITS types and *D. serratus* (Fig. 38, 39). The second lineage (lineage 3) included four ISP isolates representing two sequence types, along with *D. denticulatus* (Fig. 38, 39). The ISP isolates included in lineage 3, types F and M, have morphologies consistent with *S. bijuga,* a species that lacks wall ornamentation (by LM) and has not been transferred to *Desmodesmus.* Bootstrap support for monophyly of *Desmodesmus* was lacking for both analyses (Fig. 38, 39). Overall, the analysis using the MARNA-derived alignment produced a much more defined phylogeny than did the global analysis, with higher bootstrap support for several lineages in the MP analysis than were seen in the initial global analysis.

The global analysis suggested that *Scenedesmus* is not monophyletic, with *Coelastrum* sp. within the *Scenedesmus* lineage. However, a subsequent analysis employing the alignment derived from MARNA indicated that *Coelastrum* sp. is basal to the greater *Scenedesmus* lineage (Fig. 40). Another difference between the two analyses is that two ISP isolates, NDem 8/18 T-11w and Pic 6/16 T-1w, were resolved as basal to *Scenedesmus* in the MARNA-derived analysis. The MARNA analysis also showed greater support for individual lineages within *Scenedesmus* than did the global analysis, but monophyly of *Scenedesmus* still was not supported by bootstrap analyses (Fig. 40).

DISCUSSION

We found 34 ITS-2 sequence types in the lakes and ponds of Itasca State Park. Sequences from 27 of the types differed by at least eight nucleotide substitutions from each other, whereas three sets (corresponding to 7 types) of types only differed by seven substitutions or less. Lewis & Flechtner (2004) showed that their isolates of *Scenedesmus,* which were morphologically identical but differed by more than eight substitutions, should be considered distinct species. If we use this criterion, our diversity analysis suggests that these 34 types could correspond to 31 distinct taxa. ITS-2 sequences for five of the ITS types identically matched ITS-2 sequences previously published in GenBank, and 12 ITS types differed in ITS-2 sequence by only two to seven substitutions from published sequences. By the criteria of Lewis & Flechtner (2004), these 12 ISP types could be referred to these described species. However, there were multiple ISP sequence types that possessed sequences that differed by less than eight nucleotides from the same species. Based on these molecular analyses, the following nine taxa are present among our isolates: *D. armatus* var. *subalternans, D. multivariabiis* var. *turskensis, D. arthrodesmiformis, D. asymmetricus, D. hystrix, D. pirkollei, D. bicellularis, D. cuneatus,* and *S. obliquus.* Other ISP isolates also may match other described taxa for which ITS-2 sequences are not yet available

Using light microscopy, we critically compared our isolates to the published descriptions for species that were closely related based on ITS-2 sequence analysis. Our results showed that those isolates with ITS-2 sequences that exactly matched sequences from described species were also morphologically identical (or very nearly so) to those species. Taxa that matched with our isolates were *Desmodesmus hystrix, D. pirkollei,* and *D. bicellularis* and *D. multivariabilis* var. *turkensis.* Finally, one of our isolates produced an ITS-2 sequence that was an exact match to the sequence of an unnamed and undescribed *Desmodesmus* sp., NIOO-MV5.

Those ISP isolates with ITS-2 sequences that differed from GenBank sequences by two to seven nucleotides did not always match the morphology of their sister taxa. Some of our types, such as the one that was very similar to the GenBank sequence for *D. asymmetricus* (listed in GenBank as *S. jovais,* UTEX 2444), matched quite well with the morphology of the type culture. In most cases, isolates with sequences that differed by two to seven nucleotides from GenBank sequences possessed very similar cell and coenobial shapes to the named taxon, and in some cases matched the published description quite well. Morphologies perhaps can be compared more rigorously among ISP isolates than to published descriptions because ISP isolates were grown under the same conditions, and very subtle differences in morphology could be detected by comparing closely related types. Those isolates that differed by two to nine nucleotides in ITS-2 sequences differed very little, if at all, in morphology. The majority of the differences were in cell size and shape, two characters that other studies have suggested are not useful in species descriptions (An *et al.* 1999). The ISP isolates were found to enlarge during autosporic reproduction, so a range of cell size was found regularly in cultures. On the other hand, one pair of isolates differed by only four nucleotides, but had very different morphologies. Cells of type H were ovoid with long spines at the

apices of terminal cells and on some intermediate cells. Cells of type G, on the other hand, were oval and elongate with ridges encircling the cells and lining the edge of intermediate cells.

Sequence differences greater than nine nucleotides usually meant more differences in morphology. Isolates that differed by nine to 15 nucleotides generally had the same cell shape and arrangement, but had differences in wall ornamentation. For example, type I and type II differed by 11 nucleotides. They had the same cell shape and arrangement, but type I had short teeth at the cell apices, whereas type II was covered in large warts. Similarly, type A and type O differed by 15 nucleotides. The cell shape and arrangement were the same, but these isolates differed in the number of spines per cell and spine placement on the cell.

Morphological differences, such as differences in cell shape and wall ornamentation, became very noticeable as the ITS-2 sequence differences exceeded 15 nucleotides. For example, types P and S differed by 17 nucleotides. Cells of type P were larger and more elongate than those of type S and type P cells had distinctive wall appendages not present in type S. An exception is seen among some of the isolates that are related to *D. serratus.* There were large nucleotide differences, ranging from 24 to 26 nucleotides, but little morphological variation among the isolates of types K, W, C, and EE. Cell shape and arrangement were the same for all isolates, and cell sizes were similar. Types EE and C differed by 24 bases, but they had similar wall ornamentation and only differed in size.

Differences in morphology between isolates of different lineages, thus between isolates with a large number of base differences, were easily detected. These morphological differences suggest that LM may be useful for distinguishing between lineages. Differences in the types of wall ornamentation as well as cell size and shape were seen between different lineages. Isolates of lineage one had cells with rounded apices and some sort of arrangement of spines or spine-like projections. Isolates of lineage 2 had cells with pointed apices, and short spiky projections or little wall ornamentation that was visible with LM. Isolates of lineage three had large, broad oval cells with no wall ornamentation that was visible with LM. However, because the full range of phenotypic plasticity is not known for these genera, it is uncertain if these morphological differences are stable.

Thus, our results indicate that isolates with very similar ($<$ 7 substitutions) ITS-2 sequences usually, but not always, have very similar morphologies. We chose to consider an isolate conspecific with a named taxon when they differed by seven or fewer ITS-2 substitutions and possessed identical morphologies. When ITS-2 sequences differed by more than six substitutions, or the morphologies differed, then our isolate was not placed within that taxon. However, we recognize that this distinction is still subjective and ultimately expect to use different techniques to describe new species. However, the purpose of this study was not to describe species but rather to assess diversity.

How well does the diversity detected in this study compare to the diversity detected in previous studies of this region? In a floristic study of the lakes and ponds of Itasca State Park using LM, Meyer and Brook (1968) found six taxa of *Scenedesmus,* including two taxa now referred to as *Desmodesmus* [*S. quadricauda* and *S. serratus* (Corda) Bohlin]. Four taxa still considered to be in the genus *Scenedesmus* also were found by Meyer and Brook (1968): *S. arcuatus* Lemmermann, *S. arcuatus* var. *platydisca* G.M. Smith, *S. bijuga* var. *alternans* (Reinsch) Hansgirg and *S. dimorphus* (Turpin) Kützing. *Scenedesmus dimorphus* has been referred to as *S. obliquus* by Hegewald (1989). Using molecular techniques as well as LM, we detected much more diversity than was detected by Meyer and Brook (1968). One type found in this study, type AA, also could be referred to as *S. obliquus* under Hegewald's definition of the species, but our isolate differed markedly in morphology from the description of *S. dimorphus,* which is characterized by lunate outer cells (Prescott 1962). One of our unidentified types, type F, could be referred to as *S. bijuga* (Turpin) Lagerheim, but not *S. bijuga* var. *alternans,* whereas another type, type Q, fit the morphology of *S. bijuga* var. *alternans* fairly well. Prescott (1962) listed 41 taxa (species and varieties) of *Scenedesmus* (including *Desmodesmus*) from the Western Great Lakes Area of Wisconsin and Michigan, a very large study area with habitats that could be considered comparable to those of ISP. We found nearly as many apparent taxa (31) from ISP. It is not likely that almost as much diversity exists in Itasca State Park as in the entire Great Lakes region. A study of the *Scenedesmus*-like algae of the Ukraine in which many of the taxa were studied by both LM and SEM, and some isolates by ITS-2 sequence analysis, revealed 68 taxa (Tsarenko *et al.* 2005). The results of Tsarenko *et al.* (2005) included some taxa from *Pseudodidymocystis* Hegewald & Deason, *Enallax* Pasch. and *Didymocystis* Korsh. in addition to *Scenedesmus* and *Desmodesmus.* These results from the Ukraine also show that very careful work is needed to describe all the diversity of the coccoid algae from a region.

These comparisons suggest that studies based on LM alone often underestimate diversity. In addition, taxa very commonly reported from many floristic studies, such as *S. acuminatus* (Lagerheim) Chodat, *D. opoliensis* and *S. quadricauda* [renamed *Desmodesmus maximus* (W. & G.S. West) Hegewald] were not detected among our isolates. Of commonly reported taxa with published ITS-2 sequences, only one, *S. obliquus,* was found in this study, and the sequence of our isolate was not identical to the published sequence.

On the other hand, five of our isolates produced ITS-2 sequences that were identical to the sequences from isolates from very different locations. For example, our sequence type U produced an ITS-2 sequence identical to the published sequence from the type culture of *D. pirkollei* that was isolated from a site in Bali. Moreover, our isolate was morphologically consistent with *D. pirkollei.* Additional ISP isolates had ITS-2 sequences very similar to those of isolates from distant locations. These results suggest that at least some taxa are widely distributed, a conclusion consistent with the cosmopolitan hypothesis of the distribution of eukaryotic microorganisms as elaborated by Finlay (2002). Similar results have been reported recently for some taxa in the Hydrodictyaceae that have been examined using sequence analysis (Buchheim *et al.* 2001; McManus & Lewis 2005). However, similar studies of other green algae, such as *Choricystis* (Skuja) Fott (Fawley *et al.* 2005 and unpublished data) and colonial green flagellates (Coleman *et al.* 1994; Coleman 2001) have revealed the possibility of distinct biogeographies for these organisms. There are at least two explanations for the different patterns observed for different green algal linages. Firstly, some fresh-

water microalgae may be cosmopolitan, whereas others are not. Dispersal may be much more rapid for some types of microalgae than for others. Secondly, most freshwater microalgae may be cosmopolitan, but the diversity is much greater than previously thought, with rather narrow environmental requirements for each specific taxon. Investigating these possibilities requires much more extensive sampling from diverse areas similar to Itasca State Park in multiple locations around the world.

The lakes and ponds of Itasca State Park represent very diverse habitats. Thirteen of the 34 different sequence types that we characterized from ISP were represented by only a single isolate. Of the 21 types with multiple isolates, 14 were cultured from only a single water body. Most of our sampling effort focused on Tower Pond and, not surprisingly, 12 of the 21 types with multiple isolates were isolated only from that site. No type was isolated from every site, although one type, type J, was isolated from every site except Picnic Pond. Type J is an as yet unidentified species allied with *D. arthrodesmiformis.* Types A and N possessed very similar ITS-2 sequences, but these two types were isolated from different sites, with type A not found in Tower Pond. These results suggest that some of the isolates with very similar sequences may have limited distributions among the sites. However, because of the uneven sampling effort, our distribution data must be considered very tentative.

Phylogenetic analysis of the ITS-2 data for our isolates combined with additional published sequences provides some support for the monophyly of the Scenedesmaceae, but does not provide robust support for monophyly of the genera *Scenedesmus* and *Desmodesmus.* Instead, our results suggest that *Desmodesmus* may be a composite of closely related lineages. Observations by LM indicate that some of these lineages may be supported by morphological characters. The results of the phylogenetic analyses also suggest that additional species, such as *S. bijuga,* that still are considered allied with *Scenedesmus,* actually are included in lineages of *Desmodesmus.* However, the ITS-2 data set represents only a limited number of nucleotides, and because of the rapid evolutionary rate, the ITS-2 region generally is considered to have limited utility for phylogenetic inference above the genus level (e.g. McManus & Lewis 2005). Our results point out the need to investigate the phylogeny of this family using sequences from multiple loci.

In conclusion, our results clearly show that using culture techniques and ITS-2 sequence data reveals a much higher level of diversity among *Scenedesmus* and *Desmodesmus* than had been revealed using LM from field samples. In addition, our results reveal that isolates that possess identical ITS-2 sequences are also morphologically identical or very nearly so. The critical morphological comparisons necessary for species identification are extremely difficult from field material, especially if the community is composed of multiple species. Therefore, culturing and sequence analysis actually may be the easiest method for species identification for these genera.

Although more isolates from all sites need to be evaluated, there is an indication that ITS-2 sequence types that differ by only a few nucleotide substitutions sometimes have restricted distributions in ISP. However, at this time, it is impossible to tell whether the different distributions are based on the physical and biological characteristics of the different water bodies or instead represent different microhabitats within each lake or pond. In either case, our results suggest that these organisms are probably rather finely adapted to their environment. Sampling additional locations from each site might result in both additional diversity and a better understanding of the distribution of each taxon. It is also clear from our results that some species of *Desmodesmus* and *Scenedesmus,* at least as defined by ITS-2 sequences and morphology, have wide geographic distributions.

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