J. Phycol. **42,** 142–154 (2005)
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DOI: 10.1111/j.1529-8817.2006.00169.x

EVALUATING THE MORPHOSPECIES CONCEPT IN THE SELENASTRACEAE (CHLOROPHYCEAE, CHLOROPHYTA)[1]

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Isolates of the genera *Monoraphidium* Kom.-Legn., *Ankistrodesmus* Corda and *Raphidocelis* Hindák emend. Marvan et al. were cultured from two areas in Minnesota and North Dakota, USA. These isolates were identified to species level (when possible), using light microscopy and standard monographs and then characterized by 18S rDNA sequence analysis. Phylogenetic analyses indicated that in some cases, 18S rDNA sequences from these isolates were very similar, but not identical to the sequences of other isolates of the same morphospecies from different parts of the world. However, some isolates that were identified as the same species actually belong to different lineages within the Selenastraceae, whereas other isolates with identical or nearly identical 18S rDNA sequences possessed rather different morphologies. Overall, our data suggest that the application of a broad morphospecies concept to the Selenastraceae has resulted in an underestimation of the species diversity of this family and probably erroneous conclusions about the distribution of species.

Key index words: ***Ankistrodesmus*****;** ***Monoraphidium*****; morphospecies;** ***Raphidocelis*****; Selenastraceae**

Abbreviations: **ANWR, Arrowwood National Wildlife Refuge; ISP, Itasca State Park**

The Selenastraceae are ubiquitous freshwater green algae with uncertain taxonomy (Krienitz et al. 2001). The commonly observed genera in this family, *Ankistrodesmus*, *Selenastrum*, *Monoraphidium*, and *Kirchneriella*, are generally spindle-shaped, either straight or curved, and reproduce exclusively by autosporulation. Although genera are delimited by apparently distinguishable morphological features such as solitary or colonial habit, presence of mucilage pads, and overall cell shape (Table 1), these features have been shown to be ambiguous at the genus level by 18S rDNA sequence analysis (Krienitz et al. 2001). Members of this family are almost always included in phytoplankton floras where identification, frequently to species, has been performed by light microscopy. However, the results of Krienitz et al. (2001) bring into question the accuracy of these identifications.

Recent molecular studies of freshwater green algae have indicated considerable cryptic diversity among some genera and species, most notably *Choricystis* Fott (Hepperle and Krienitz 2001, Hepperle and Schlegel 2002, Fawley et al. 2005) and *Chlorella* Beijerinck (Huss et al. 1999, Krienitz et al. 2004). These studies have mostly employed 18S rDNA or *rbc*L sequence analysis. Both of these genes generally evolve too slowly to be useful for resolving species-level relationships, which would require examining highly variable regions such as the ribosomal ITS (An et al. 1999, van Hannen et al. 2000, Krienitz et al. 2004). Thus, the studies that have been conducted using 18S rDNA and *rbc*L have probably revealed only a portion of the actual diversity present.

The only molecular investigation of the diversity among the Selenastraceae is that of Krienitz et al. (2001). They examined 18S rDNA sequences from 11 named species representing the genera *Ankistrodesmus*, *Kirchneriella*, *Monoraphidium*, *Podohedriella*, and *Quadrigula*. Their results indicated monophyly for the Selenastraceae, but the two genera (*Ankistrodesmus* and *Monoraphidium*) for which they examined multiple species were both polyphyletic. No commonly employed features such as colony formation, cell shape, autospore arrangement, or ultrastructure of the pyrenoids were useful for delimiting the lineages of the Selenastraceae. However, Krienitz et al. (2001) stated that these characters are or may be useful for species-level identifications.

The goal of this study was to evaluate the use of morphology for species-level identification of members of the Selenastraceae. For this investigation, we isolated unialgal cultures from sites in Arrowwood National Wildlife Refuge (ANWR), North Dakota (USA) and Itasca State Park (ISR), Minnesota (USA). Light microscopy-based floras for these two areas have identified numerous species from this family from both locations (Meyer and Brook 1968, Phillips and Fawley 2002a,b). Some overlap between the two floras exists at the species level. By isolating these organisms into culture, we were able to critically compare species identifications by light microscopy with the results of molecular analyses using 18S rDNA sequences.

MATERIALS AND METHODS

Site descriptions. ANWR is located in the mixed-grass prairie region of east-central North Dakota. Within the refuge, four natural shallow lakes and wetlands on the James River

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TABLE 1. Characteristics of the genera included in this study.

Ankistrodesmus Corda—colonial in mucilage with fusiform cells in fasicles (parallel bundles) in which the cells are in contact. Straight or slightly curved, isopolar cells lacking visible pyrenoids.
Kirchneriella Schmidle—cells solitary, with mucilage around each cell. Cells isopolar, arctuate to lunate or spirally twisted, with a pyrenoid.
Monoraphidium Komárková-Legnerová—cells solitary, lacking mucilaginous envelope. Cells fusiform and isopolar, without visible pyrenoids (naked pyrenoids).
Ourococcus Grobety—cells solitary, without mucilage. Similar to *Monoraphidium* except possessing an obvious pyrenoid. Sometimes merged into *Keratococcus* Pascher.
Podohedriella Hindák—solitary fusiform cells lacking mucilage. Heteropolar, without visible pyrenoid.
Quadrigula Printz—colonial in mucilage with cells that lie parallel to one another (as in *Ankistrodesmus*) but are clearly separate, never touching. Cells generally with rounded ends; otherwise similar to *Ankistrodesmus*.
Raphidocelis Hindák emend. Marvan et al.—as for *Kirchneriella*, but without visible pyrenoids. Frequently with incrustations of the cell wall.

From Marvan et al. (1984), except *Podohedriella* from Hindák (1988) and *Ourococcus* from Shubert (2003).

have been stabilized by weirs. Four sites on three lakes, Arrowwood Lake, Mud Lake, and Jim Lake, were sampled for this study. Surface water samples (or just below the ice) were collected from the ANWR during three consecutive winters, 1994–1997 and monthly during 1995.

Samples were also collected from six sites in ISP, Minnesota, including Lake Itasca (mesotrophic), Mary Lake (mesotrophic), West Twin Lake (moderately dystrophic), North Deming Pond (dystrophic), an unnamed dystrophic pond referred to as Tower Pond and an unnamed moderately eutrophic pond referred to as Picnic Pond. Phytoplankton and tychoplankton samples were taken from these sites once during each season from fall, 2000 through summer, 2001. Itasca State Park is located at the transition of the prairie, hardwood forest, and northern coniferous forest and is notable as the headwaters of the Mississippi River. For more complete descriptions of the sites and their physical and chemical characteristics, see Phillips and Fawley (2002a, b) and Fawley et al. (2004).

Algal isolations. Algae were isolated using the technique of Phillips and Fawley (2000) and Fawley et al. (2004). Cultures were maintained on agar slants and grown in liquid media with continuous fluorescent illumination (approximately 50–75 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) at 15° C for microscopy and DNA extraction.

Light microscopy and identification. Light microscopy employed a Nikon E600 microscope (Nikon, Melville, NY, USA) equipped with a 60× plan apochromatic objective and differential interference optics. Digital images were captured with a Pixera digital camera (Pixera Corp., Los Gatos, CA, USA). Komárková-Legnerová (1969), Prescott (1973), Hindák (1977), Komárek and Fott (1983), Hindák (1984), Nygaard et al. (1986), Hindák (1988), Korshikov (1987) and John and Tsarenko (2002), were used for species identification.

Molecular characterization. Genomic DNA was extracted from the isolates, using the technique of Phillips and Fawley (2000) or Fawley and Fawley (2004). The 18S rDNA was amplified by PCR using the protocol of Fawley and Fawley (2004) with the primer sets NS1 (White et al. 1990) and 18L (Hamby et al. 1988), NS1-X and 18L-X (Fawley and Fawley 2004) or Med A and Med B (Medlin et al. 1988). The 18S rDNA was then sequenced (Fawley et al. 2004) using the primers NS-1, NS-3, NS-5, NS-7 (White et al. 1990), NS1-X and 18L-X (Fawley and Fawley 2004), 18G, 18H, 18J, 18L (Hamby et al. 1988), and a new primer, NS3a (5′ AA-GTCTGGTGCCAGCAGCC 3′). Sequences were analyzed with the Staden Package (Staden 1996) and aligned using GeneDoc version 2.6.000 (Nicholas et al. 1997) and MacClade 4.03 (Maddison and Maddison 2000). Additional published sequences from the Selenastraceae and representatives of *Scenedesmus* and *Desmodesmus* (outgroup taxa) were also included (Table 2), as well as the sequence from *Ourococcus multiporus*, a taxon shown to be allied with the Selenastraceae by Buchheim et al. (2001). All characters were unambiously aligned. The alignment has been submitted to the EMBL-Align database (Lombard et al. 2002) and is also available from the authors. Phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford 2002). Modeltest 3.5 (Posada and Crandall 1998) was used to determine the optimal model of evolution for these data. Maximum likelihood (ML) analysis with the GTR + G + I model employed rates for the A–G transitions of 2.9284 and the C–T transitions of 4.6155. The substition rates for the A–T, G–T, A–C, and G–C transversions were 1.0. The gamma distribution for this data set was 0.6665 and the proportion of invariable sites was 0.7549. Maximum parsimony (MP) analysis was conducted with the transition/transversion ratio set from the data using the reweight function in PAUP. The analysis included 1812 total characters, of which 220 were variable and 115 were parsimony informative. Bootstrap analyses were performed with 100 repetitions for ML and 1000 repetitions for MP. Pair-wise differences among sequences, both number of nucleotide substitutions and percent differences, were determined using PAUP. A neighbor-joining analysis using the total substitutions distance matrix was performed with PAUP. This neighbor-joining analysis was performed only to graphically illustrate the substitution differences among sequences and not as an inference of phylogeny. As such, bootstrap analysis was not performed.

RESULTS

Characterization by light microscopy. Seven isolates from ANWR and 11 isolates from ISP identified as Selenastraceae were characterized for this study. The Arrowwood isolate, AS 3-5 (isolated from Arrowwood Lake, 24 February 1995), was identified as *Monoraphidium minutum* (Fig. 1). The cells were cylindrical and either arcuate or sigmoid shaped. The cells were rounded at the ends and no pyrenoids were observable. Mucilage was not observed. The diameter of the cell helix ranged from 5.8 to 12.2 μm and cell width ranged from 2.4 to 5.6 μm. The fragment of the mother cell membrane was unseparated resulting from a partial longitudinal rupture.

The Arrowwood isolate MDL 1/12-3 (from Mud Lake, 12 January 1996) was identified as *Raphidocelis subcapitata* (formerly referred to *Selenastrum carpricornutum*, see Nygaard et al. 1986) (Fig. 1). The

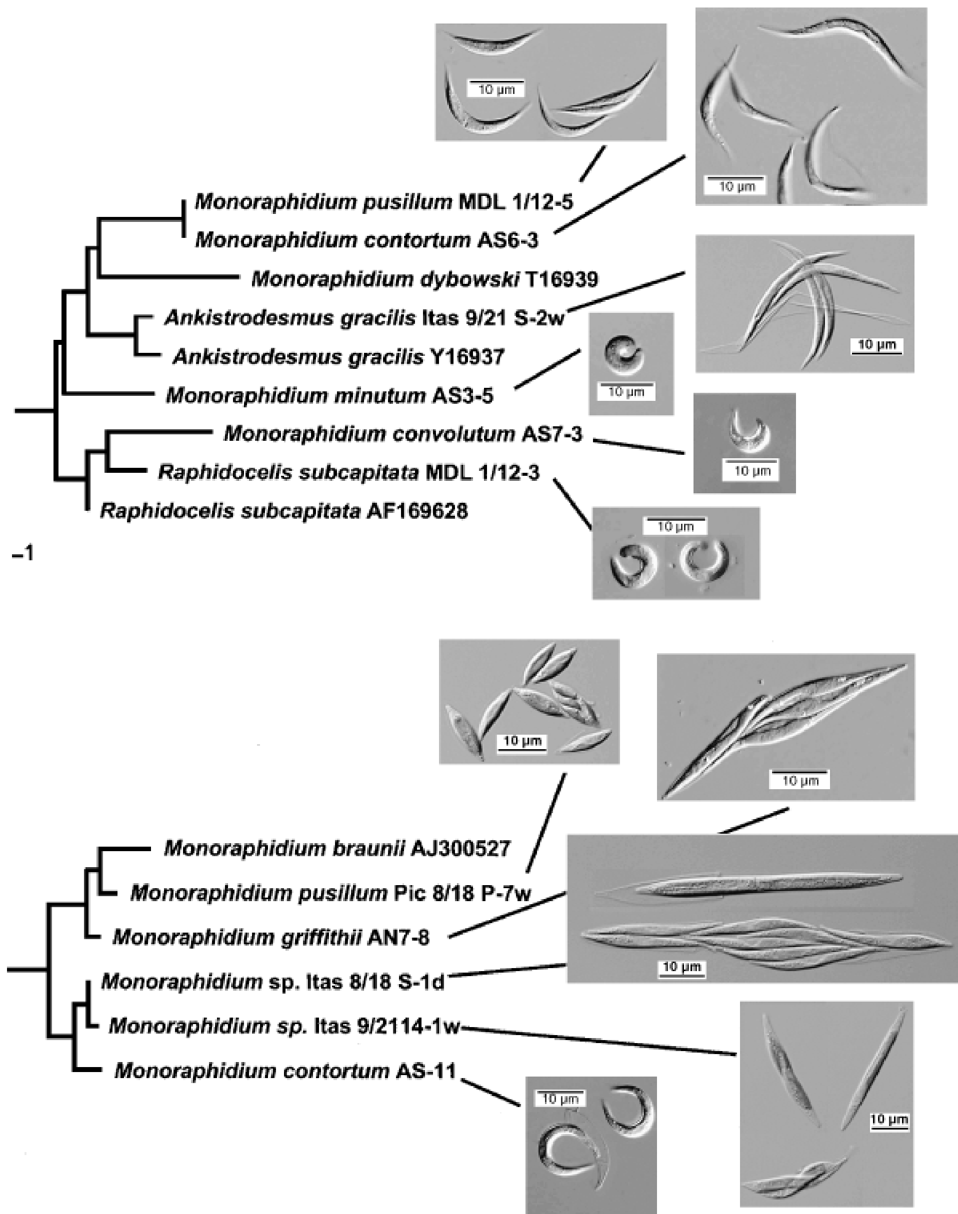

FIG. 1. Dendrogram depicting partial results of a neighbor-joining analysis of 18S rDNA sequences from Selenastraceae isolates, and accompanying light micrographs. Two sections of the dendrogram that included new isolates are shown. The analysis employed a distance matrix constructed using total number of substitutions between sequences and illustrates the total sequence differences. Branch lengths indicate absolute numbers of substitutions among sequences from isolates and published sequences.

arcuate- or sigmoid-shaped cells were surrounded by conspicuous mucilage. Cell dimensions were 5.6–11.7 µm helical diameter and 1.9–3.6 µm cell width. The ends of the cells frequently appeared subcapitate as a result of the helical twisting of the cell ends. Autospore production was similar to AS 3-5, with the fragment of the mother cell membrane unseparated resulting from a partial rupture. In this case, the rupture produced an irregular opening not located on the longitudinal axis.

One ANWR isolate, AN 7-8 (from Arrowwood Lake, July 18, 1995), was identified as *M. griffithii* (Fig. 1). The cells were straight to nearly straight and fusiform. The cells came to a gradual point and no pyrenoid was visible. The average cell length and width were 36.5 and 3.6 µm. Cell length ranged from 27.7 to 63.3 µm, and cell width ranged from 2.4 to 7.3 µm. The fragment of the mother cell membrane was separated into two halves by an equatorial rupture. This isolate could also be compared to *M. intermedium* Hindák (Hindák 1984), which it resembles in the shape of the apices, but the cells of our isolate are larger than for *M. intermedium*.

Arrowwood isolate MDL 1/12-5 (from Mud Lake, 12 January 1996) was identified as *M. pusillum* (Fig. 1), but appeared in many ways intermediate between *M. con-*

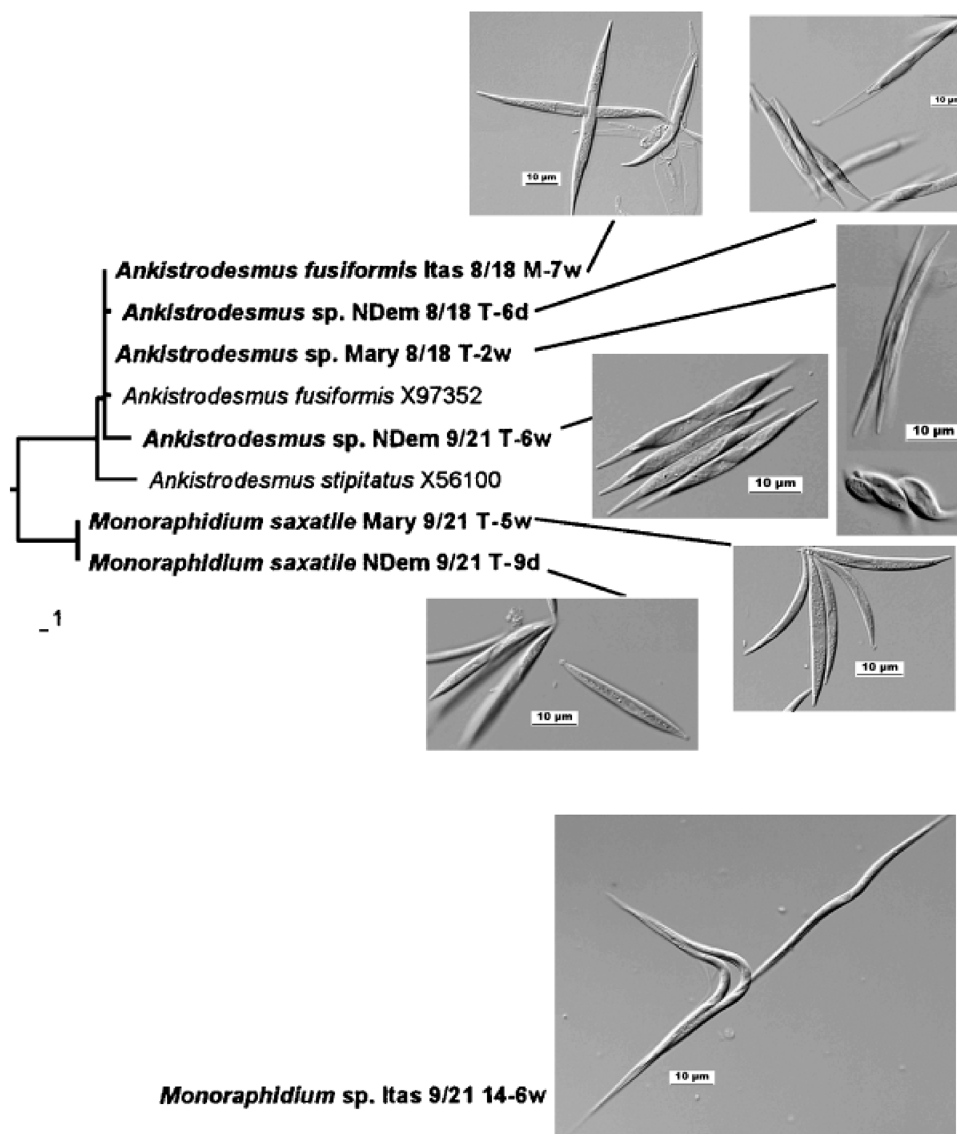

Fig. 2. Dendrogram depicting partial results of a neighbor-joining analysis of 18S rDNA sequences from Selenastraceae isolates, and accompanying light micrographs. One section of the dendrogram that included new isolates is shown. The analysis employed a distance matrix constructed using total number of substitutions between sequences and illustrates the total sequence differences. Branch lengths indicate absolute numbers of substitutions among sequences from isolates and published sequences. *Monoraphidium* sp. Itas 9/21 14-6w, which was resolved as a separate basal lineage, is also shown.

tortum and *M. pusillum*. Cells were typically curved or slightly arcuate, as in *M. contortum*, but only slightly so. This shape is within the range described for *M. pusillum*. Cells ranged from 13.4 to 32.3 μm long × 1.8 to 4.9 μm wide.

One isolate from ANWR, AS 7-3 (from Arrowwood Lake, 18 July 1995), was identified as *M. convolutum* (Fig. 1). The cells were arcuate or widely fusiform and the ends gradually came to a point. The ends of the cells did not lie within one plane and pyrenoids were visible. The average cell length and width were 7.3 and 2.8 μm. Cell length ranged from 5.3 to 11.6 μm, and cell width ranged from 1.7 to 4.6 μm. The fragment of the mother cell membrane was unseparated resulting from a partial longitudinal rupture. Autospores tended to have sharper ends than that of adult cells.

Two ANWR isolates, AS-11 (from Arrowwood Lake, December 1994) and AS 6–3 (from Arrowwood Lake, 29 June 1995), were identified as *M. contortum* although, the isolates were morphologically distinct (Figs. 1 and 2). Cells of AS-11 were slightly arcuate to arcuate, tapered gradually to fine points, with dimensions 10.2–27.4 μm long × 2.5–5.8 μm wide. The ends of the cells were in separate planes. When viewed in two dimensions, the ends of the cells appeared to nearly touch, which is not typical for *M. contortum*. Cells of AS 6-3 varied greatly from straight to markedly curved. The cells were narrowly or broadly fusiform

and pointed. Many cells were sigmoid as is typical for *M. contortum*. No pyrenoid was visible within the chloroplast. Cell length ranged from 11.5 to 35.0 μm, and cell width ranged from 1.3 to 6.5 μm.

Itasca isolate Pic 8/18 P-7w (from Picnic Pond phytoplankton, 18 August 2001) was identified as *M. pusillum* (Fig. 1), but differed somewhat from the Arrowwood *M. pusillum* isolate and the description of Komárková-Legnerová (1969). The Itasca isolate was shorter than the Arrowwood isolate, with overall dimensions 11.1–19.4 μm × 2.7–4.5 μm for adult cells. These dimensions are actually more in keeping with the original description of *M. pusillum*. In addition, autospores of the Itasca isolate were much broader than illustrated by Komárková-Legnerová. However, Komárková-Legnerová indicates that this species is highly variable. The isolate can also be compared to *M. braunii*, but the cells of our isolate are much shorter than those of that species, and the somewhat finger-like processes at the ends of the cell also distinguish our isolate from *M. braunii*. Isolate Pic 8/18 P-7w has a shape similar to that of *M. neglectum* Heynig and Krienitz (1982), but our isolate is much smaller and the mucilage pads characteristic of *M. neglectum* were never observed. Thus, we place this isolate in the highly variable species, *M. pusillum*.

Isolate Itas 9/21 S-2w (from Lake Itasca phytoplankton, 21 September 2000) was identified as *Ankistrodesmus gracilis* (Fig. 1). This isolate matched the description of *A. gracilis* in Komárková-Legnerová (1969) exactly. Cells were 30 to 35 μm × 1.9 to 3.7 μm, strongly arcuate, and typically occurred in bundles of four cells.

Isolate Itas 8/18 S-1d (from Lake Itasca phytoplankton, 18 August 2001, Fig. 1) could not be placed with confidence in any species of Komárková-Legnerová (1969) or Korshikov (1987). Cells were 41–68 μm × 2.8–3.7 μm, cylindrical or slightly narrowed at the center, with ends of older cells tapering to finger-like ends. Young cells were more spindle-shaped and often somewhat sigmoid. This shape most strongly resembles *M. braunii*, except in that species, the cells are broader than that for our isolate. *Monoraphidium braunii* also has a prominent pyrenoid (Komárková-Legnerová 1969), a feature not seen in our isolate.

Isolate Itas 9/21 14-1w (from Lake Itasca phytoplankton, September 21, 2000), *Monoraphidium* sp. (Fig. 1), somewhat resembles *M. braunii* and *M. obtusum* (Korsh.) Kom.-Legn., with straight cells with ends of the cells that are clearly rounded. Overall dimensions (34 to 52 μm × 2.6 to 3.9 μm) are within the range of *M. braunii*. However, the autospores are spirally twisted and straighten with age. Autosporangia become inflated and produce four or eight autospores. In addition, mucilage is secreted from one end of the cell, a feature not mentioned for either *M. braunii* or *M. obtusum* (Komárková-Legnerová 1969).

Itasca isolate NDem 9/21 T-6w (from North Deming Pond tychoplankton, 21 September 2000), *Ankistrodesmus* sp. (Fig. 2), somewhat resembled *Ankistrodesmus densus* Korsh. in colony form, but differed from the description of Komárková-Legnerová (1969) in that cells were not cylindrical, but rather were constricted at the midpoint and somewhat shorter than the description. Autospores often formed spiral bundles that persisted as a colony of adult cells. In other cases, the cells stayed together in parallel bundles of three or four cells. Overall cell size ranges from 27 to 55 μm × 1.7 to 3.5 μm.

Isolate Itas 8/18 M-7w (from Lake Itasca phytoplankton, 18 August 2001) strongly resembled *Ankistrodesmus fusiformis* (Fig. 2). Cells were 42–56 μm × 2.5–3.7 μm and generally straight or nearly so. Cells often remained together in groups of 2 to 4, either in parallel bundles or irregularly crossed. Our isolate differs from typical *A. fusiformis* in that many of the colonies were present as parallel bundles, whereas in typical *A. fusiformis*, the cells of colonies are crossed (Komárková-Legnerová 1969, Korshikov 1987). In the formation of parallel bundles, our isolate resembles *A. falcatus* (Corda) Ralfs, which has been considered conspecific with *A. fusiformis* by Hindák (1988) due to variability in colony form.

Isolate NDem 8/18 T-6d (from North Deming Pond tychoplankton, 18 August 2001) also resembled *A. fusiformis* in some respects (Fig. 2). Cells were 35–40 μm × 2.6–4.1 μm, nearly linear to somewhat spirally twisted. A distinguishing feature of this isolate was the production of mucilage from one pole of many of the cells, which often gave the appearance of a capitate end similar to that of the genus *Podohedra* Düringer. In addition, no cruciate colonies typical for *A. fusiformis* were observed. In fact, organized colonies of any type were infrequent, although some parallel bundles of cells were observed. Many "colonies" were simple loosely connected cells, often in linear series or somewhat so. These characteristics do not match any of the described species of *Ankistrodesmus*.

Mary 8/18 T-2w was isolated from a tychoplankton sample from Mary Lake in ISP, 18 August 2001. Most cells were in parallel bundles of generally four cells, similar to *A. falcatus* (Corda) Ralfs (Fig. 2). Occasionally, cells were spirally twisted and in groups of four cells twisted together. A few group of cells formed irregular cruciate patterns, similar to *A. fusiformis*. This isolate was re-plated and individual colonies were checked to see if multiple taxa were mixed together in the original isolate, but all re-isolates possessed the three colony morphologies. Individual cells were quite thin relative to their length, with dimensions 40–56 μm × 1.7–3.1 μm, and nearly straight or slightly arcuate to somewhat helical. This isolate fit the description of *A. falcatus*, although the presence of the spirally twisted colonies, never mentioned by Komárková-Legnerová for this species, makes this identification questionable. The isolate could also be identified as *A. stipitatus* by the criteria of Komárková-Legnerová (1969), but the cells are not of the same dimensions and that taxon also lacks the diversity of colony form.

TABLE 2. GenBank accession numbers of 18S rDNA sequences used in this study.

Taxon	GenBank accession number
Ankistrodesmus bibraianus (Reinsch) Kors.	Y16938
***Ankistrodesmus fusiformis* Corda Itas 8/18 M-7w**	**AY846370**
Ankistrodesmus fusiformis	X97352*
***Ankistrodesmus gracilis* (Reinsch) Korsh. Itas 9/21 S-2w**	**AY846371**
Ankistrodesmus gracilis	Y16937*
***Ankistrodesmus* sp. Mary 8/18 T-2w**	**AY846372**
***Ankistrodesmus* sp. NDem 8/18 T-6d**	**AY846373**
***Ankistrodesmus* sp. NDem 9/21 T-6w**	**AY846374***
Ankistrodesmus stipitatus (Chod.) Kom.-Legn.	X56100
Kirchneriella aperta Teiling	AJ271859*
Monoraphidium braunii (Näg. in Kütz.) Kom.-Legn.	AJ300527
***Monoraphidium contortum* (Thur.) Kom.-Legn. AS-11**	**AY846375***
***Monoraphidium pusillum* MDL 1/12-5**	**AY846376**
***Monoraphidium convolutum* (Corda) Kom.-Legn. AS7-3**	**AY846377**
Monoraphidium dybowskii; (Wolosz.) Hindák et Kom.-Legn.	Y16939
***Monoraphidium griffithii* (Berk.) Kom.-Legn. AN7-8**	**AY846378**
***Monoraphidium minutum* (Näg.) Kom.-Legn. AS3-5**	**AY846380**
Monoraphidium neglectum Heynig et Krienitz	AJ300526
***Monoraphidium contortum* (Printz) Kom.-Legn. AS6-3**	**AY846382**
***Monoraphidium pusillum* Pic 8/18 P-7w**	**AY846383**
***Monoraphidium saxatile* Kom.-Legn. Mary 9/21 T-5w**	**AY846384***
***Monoraphidium saxatile* NDem 9/21 T**-9d	**AY846385**
***Monoraphidium* sp. Itas 8/18 S-1d**	**AY846386**
***Monoraphidium* sp. Itas 9/2114-1w**	**AY846387**
***Monoraphidium* sp. Itas 9/21 14-6w**	**AY846379**
Monoraphidium terrestre (West et G.S. West) Kom.-Legn.	Y17817*
Ourococcus multisporus Bischoff et Bold	AF277648
Podohedriella falcata (Düringer) Hindák	X91263
Quadrigula closterioides (Bohlin) Printz	Y17924*
***Raphidocelis subcapitata* (Korsh) Nygaard et al. MDL 1/12-3**	**AY846381***
Raphidocelis subcapitata	AF169628*
Desmodesmus communis (Hegew.) Hegew.	X73994
Scenedesmus obliquus (Turp.) Kütz.	X56103

Bold font indicates sequences generated in this study. Asterisks indicate sequences with 1 or more putative introns.

Isolates Mary 9/21 T-5w (from Mary Lake tychoplankton, 21 September 2001) and NDem 9/21 T-9d (from North Deming Pond tychoplankton, 21 September 2001) matched almost perfectly the description of *M. saxatile* Komárková-Legnerová (Fig. 2). Cells dimensions were 26–37 μm × 2.4–3.8 μm. Older cells were linear and younger cells tended to be arcuate, but never sigmoid. In some cells, a small pyrenoid was visible. Cells were typically attached to each other at one end by a conspicuous pad of mucilage; mucilage rarely was apparent at both ends of a cell. All of these features are consistent with *M. saxatile*, as is the tychoplanktonic habitat.

Cells of isolate Itas 9/21 14-6w (from Lake Itasca phytoplankton, 21 September 2000) were very long and thin, with dimensions 70–88 μm × 2.4–5 μm (Fig. 2). The cells become somewhat inflated before autospore production. Cells were typically straight with the ends drawn out to fine points; however, autospores just released from the mother cell sometimes were somewhat bent or twisted because the two parts of the mother cell wall did not completely separate. In many cases, cells remained attached in a semi-linear sequence. In shape and dimensions, this isolate resembles *M. komarkovae* Nygaard (*M. setiforme* (Nygaard) Komárková-Legnerová in Komárková-Legnerová 1969). However, in *M. komarkovae*, the mother cell wall cleanly separates into two parts, which is not the case for our isolate. Another species, *A. acicularis* (A. Braun) Korsh., which Komárková-Legnerová reduced to synonomy with *M. griffithii,* is similar in shape and size to our isolate. However, once again the separation of the mother cell wall in that species is into two parts, which is not the case for our isolate. Therefore, this isolate cannot be referred to any named species.

Analyses of 18S rDNA sequence data. Eighteen 18S rDNA sequences were generated from the isolates examined. Table 2 indicates the GenBank accession numbers of these new sequences. The pair-wise differences in nucleotide sequences (excluding introns) are shown in Table 3. Mary 9/21 T-5w and NDem 9/21 T-9d, both identified as *M. saxatile*, possessed identical 18S rDNA coding sequence, but Mary 9/21 T-5w possessed putative Group 1 introns that were lacking in NDem 9/21 T-9d. Itas 8/18 M-7w, *A. fusiformis*, and Mary 8/18 T-2w, *A.* sp., also produced identical sequences. The maximum variation among18S rDNA coding regions observed among the Selenastraceae was 3% between the sequence from Arrowwood isolate AS 7-3, identified as *M. convolutum*, and the published sequence for *Quadrigula closterioides*.

Phylogenetic analyses of the new Selenastraceae sequences plus the sequences included in the analyses of Krienitz et al. (2001) and a set of sequences from the

TABLE 3. Pair-wise difference matrix for 18S rDNA from Selenastraceae.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Ankistrodesmus bibraianus* ABY16938		0.0129	0.0134	0.0146	0.0151	0.013	0.0134	0.0129	0.0134	0.0128	0.0186	0.0134	0.0184	0.0214	0.024	0.0141
2. *Ankistrodesmus fusiformis* Itas 8/18 M-7w	23		0.0006	0.0152	0.0157	0	0.0006	0.0022	0.0039	0.0185	0.0197	0.0168	0.0201	0.0243	0.0247	0.0164
3. *Ankistrodesmus fusiformis* X97352	24	1		0.0146	0.0151	0.0006	0.0011	0.0028	0.0045	0.019	0.0192	0.0162	0.0195	0.0236	0.024	0.0158
4. *Ankistrodesmus gracilis* Itas 9/21 S-2w	26	27	26		0.0028	0.0152	0.0157	0.0152	0.0146	0.0208	0.0255	0.0191	0.0109	0.0156	0.0157	0.0209
5. *Ankistrodesmus gracilis* Y16937	27	28	27	5		0.0158	0.0162	0.0157	0.0162	0.0212	0.0261	0.0196	0.0103	0.0138	0.0156	0.0215
6. *Ankistrodesmus* sp. Mary 8/18 T-2w	23	0	1	27	28		0.0006	0.0023	0.0039	0.0186	0.0197	0.0169	0.0202	0.0243	0.0242	0.0165
7. *Ankistrodesmus* sp. NDem 8/18 T-6d	24	1	2	28	29	1		0.0028	0.0045	0.019	0.0203	0.0173	0.0207	0.0248	0.0246	0.0169
8. *Ankistrodesmus* sp. NDem 9/21 T-6w	23	4	5	27	28	4	5		0.0061	0.0179	0.0192	0.0162	0.0201	0.0242	0.0241	0.0158
9. *Ankistrodesmus stipitatus* X56100	24	7	8	26	29	7	8	11		0.0201	0.0203	0.0173	0.0218	0.026	0.0263	0.0169
10. *Kirchneriella aperta* AJ271859	23	33	34	37	38	33	34	32	36		0.018	0.0162	0.0201	0.0167	0.024	0.0152
11. *Monoraphidium braunii* AJ300527	32	34	33	44	45	34	35	33	35	31		0.011	0.0234	0.0251	0.0273	0.0058
12. *Monoraphidium contortum* AS-11	24	30	29	34	35	30	31	29	31	29	19		0.0218	0.0225	0.0257	0.0073
13. *Monoraphidium pusillum* MDL 1/12-5	32	35	34	19	18	35	36	35	38	35	40	38		0.0196	0.0149	0.0213
14. *Monoraphidium convolutum* AS7-3	37	42	41	27	24	42	43	42	45	29	43	39	34		0.0167	0.022
15. *Monoraphidium dybowski* T16939	43	44	43	28	28	43	44	43	47	43	47	46	26	29		0.0271
16. *Monoraphidium griffithii* AN7-8	25	29	28	37	38	29	30	28	30	27	10	13	37	38	48	
17. *Monoraphidium* sp. Itas 9/21 14-6w	24	35	34	36	37	35	36	34	37	24	22	20	33	38	43	15
18. *Monoraphidium minutum* AS3-5	25	26	27	19	20	26	27	28	27	32	41	31	26	29	33	34
19. *Monoraphidium minutum* MDL 1/12-3	29	33	32	19	20	33	34	33	35	35	33	31	25	17	24	30
20. *Monoraphidium neglectum* AJ300526	29	32	31	30	32	31	32	30	33	35	31	24	34	37	38	24
21. *Monoraphidium contortum* AS6-3	32	35	34	19	18	35	36	35	38	36	40	38	0	34	27	37
22. *Monoraphidium pusillum* Pic 8/18 P-7w	28	32	31	40	41	32	33	31	33	28	7	14	36	41	46	3
23. *Monoraphidium saxatile* Mary 9/21 T-5w	22	22	23	31	30	22	23	21	27	29	36	28	32	46	45	29
24. *Monoraphidium saxatile* NDem 9/21 T-9d	22	22	23	31	30	22	23	21	27	29	36	28	32	46	45	29
25. *Monoraphidium* sp. Itas 8/18 S-1d	23	27	26	35	36	27	28	26	28	28	14	5	39	38	45	8
26. *Monoraphidium* sp. Itas 9/2114-1w	24	28	27	36	37	28	29	27	29	29	15	4	40	39	46	9
27. *Monoraphidium terrestre* Y17817	29	33	32	34	36	32	33	31	36	30	31	22	33	42	34	26
28. *Ourococcus multisporus* AF277648	22	32	33	36	37	32	33	31	33	30	26	20	42	39	47	17
29. *Podohedriella falcata* X91263	26	26	25	25	26	26	27	25	27	34	30	20	32	36	38	23
30. *Quadrigula closterioides* Y17924	52	42	43	43	45	41	42	42	43	50	51	47	45	52	43	51
31. *Selenastrum capricornutum* AF169628	22	26	27	20	24	25	27	27	28	29	23	22	25	18	26	20

TABLE 3. (Continued).

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1. *Ankistrodesmus bibraianus* ABY16938	0.0134	0.0141	0.0166	0.0164	0.018	0.0157	0.0123	0.0123	0.0129	0.0134	0.0162	0.013	0.0145	0.0291	0.0134
2. *Ankistrodesmus fusiformis* Itas 8/18 M-7w	0.0197	0.0148	0.0188	0.0181	0.0197	0.018	0.0124	0.0124	0.0152	0.0157	0.0185	0.019	0.0146	0.0236	0.0159
3. *Ankistrodesmus fusiformis* X97352	0.019	0.0153	0.0183	0.0176	0.0192	0.0173	0.0129	0.0129	0.0145	0.0151	0.0179	0.0195	0.014	0.024	0.0165
4. *Ankistrodesmus gracilis* Itas 9/21 S-2w	0.0202	0.0108	0.0108	0.017	0.0107	0.0224	0.0174	0.0174	0.0196	0.0202	0.0191	0.0213	0.014	0.0241	0.0123
5. *Ankistrodesmus gracilis* Y16937	0.0207	0.0113	0.0114	0.0181	0.0101	0.0229	0.0168	0.0168	0.0201	0.0207	0.0201	0.0219	0.0145	0.0251	0.0146
6. *Ankistrodesmus* sp. Mary 8/18 T-2w	0.0197	0.0148	0.0188	0.0176	0.0198	0.018	0.0124	0.0124	0.0152	0.0158	0.018	0.019	0.0146	0.0231	0.0154
7. *Ankistrodesmus* sp. NDem 8/18 T-6d	0.0201	0.0153	0.0194	0.0181	0.0203	0.0184	0.0129	0.0129	0.0157	0.0162	0.0184	0.0195	0.0151	0.0235	0.0165
8. *Ankistrodesmus* sp. NDem 9/21 T-6w	0.019	0.0158	0.0188	0.017	0.0197	0.0173	0.0118	0.0118	0.0145	0.0151	0.0173	0.0184	0.014	0.0235	0.0165
9. *Ankistrodesmus stipitatus* X56100	0.0207	0.0153	0.02	0.0187	0.0214	0.0184	0.0151	0.0151	0.0157	0.0162	0.0201	0.0195	0.0151	0.024	0.0171
10. *Kirchneriella aperta* AJ271859	0.0134	0.0181	0.02	0.0198	0.0203	0.0156	0.0162	0.0162	0.0156	0.0162	0.0168	0.0178	0.019	0.0279	0.0177
11. *Monoraphidium braunii* AJ300527	0.0128	0.0239	0.0192	0.018	0.0232	0.0041	0.0209	0.0209	0.0081	0.0087	0.018	0.0155	0.0174	0.0296	0.0146
12. *Monoraphidium contortum* AS-11	0.0112	0.0175	0.0177	0.0136	0.0214	0.0078	0.0157	0.0157	0.0028	0.0022	0.0123	0.0118	0.0112	0.0263	0.0134
13. *Monoraphidium pusillum* MDL 1/12-5	0.0189	0.015	0.0145	0.0197	0	0.0207	0.0184	0.0184	0.0224	0.023	0.0189	0.0249	0.0184	0.0258	0.0157
14. *Monoraphidium convolutum* AS7-3	0.0219	0.0168	0.0099	0.0216	0.0196	0.0236	0.0265	0.0265	0.0219	0.0225	0.0242	0.0231	0.0208	0.03	0.0114
15. *Monoraphidium dybowski* T16939	0.024	0.0186	0.0137	0.0215	0.0152	0.0257	0.0252	0.0252	0.0252	0.0257	0.019	0.0279	0.0212	0.024	0.0159
16. *Monoraphidium griffithii* AN7-8	0.0085	0.0193	0.0172	0.0137	0.0209	0.0017	0.0164	0.0164	0.0045	0.0051	0.0147	0.0101	0.013	0.0288	0.0123
17. *Monoraphidium* sp. Itas 9/21 14-6w		0.0209	0.0177	0.017	0.0186	0.0089	0.0162	0.0162	0.0106	0.0112	0.0106	0.013	0.0156	0.0285	0.0134
18. *Monoraphidium minutum* AS3-5	37		0.0137	0.0178	0.0147	0.0209	0.0169	0.017	0.0181	0.0186	0.0215	0.0196	0.0169	0.0254	0.0154
19. *Monoraphidium minutum* MDL 1/12-3	31	24		0.0166	0.0143	0.0177	0.0222	0.0222	0.016	0.0165	0.02	0.0184	0.016	0.0257	0.0025
20. *Monoraphidium neglectum* AJ300526	30	31	29		0.0199	0.0159	0.0153	0.0153	0.013	0.0136	0.0153	0.0113	0.0034	0.0215	0.0143
21. *Monoraphidium contortum* AS6-3	33	26	25	35		0.0203	0.018	0.018	0.022	0.0225	0.0192	0.0249	0.018	0.0259	0.0154
22. *Monoraphidium pusillum* Pic 8/18 P-7w	16	37	31	28	36		0.0179	0.0179	0.005	0.0056	0.0156	0.0118	0.0145	0.0274	0.0134
23. *Monoraphidium saxatile* Mary 9/21 T-5w	29	30	39	27	32	32		0	0.0151	0.0157	0.0145	0.0166	0.0134	0.0252	0.0171
24. *Monoraphidium saxatile* NDem 9/21 T-9d	29	30	39	27	32	32	0		0.0151	0.0157	0.0146	0.0166	0.0134	0.0252	0.0165
25. *Monoraphidium* sp. Itas 8/18 S-1d	19	32	28	23	39	9	27	27		0.0006	0.0128	0.0112	0.0117	0.0268	0.0122
26. *Monoraphidium* sp. Itas 9/2114-1w	20	33	29	24	40	10	28	28	1		0.0123	0.0118	0.0123	0.0274	0.0122
27. *Monoraphidium terrestre* Y17817	19	38	35	27	34	28	26	26	23	22		0.0172	0.0151	0.0252	0.0159
28. *Ourococcus multisporus* AF277648	22	33	31	19	42	20	28	28	19	20	29		0.0107	0.0285	0.013
29. *Podohedriella falcata* X91263	28	30	28	6	32	26	24	24	21	22	27	18		0.0201	0.014
30. *Quadrigula closterioides* Y17924	51	45	45	38	46	49	45	45	48	49	45	48	36		0.025
31. *Selenastrum capricornutum* AF169628	22	25	4	23	25	22	28	27	20	20	26	20	23	41	

Numbers below the diagonal are the absolute number of substitutions and numbers above the diagonal are substitutions per site.

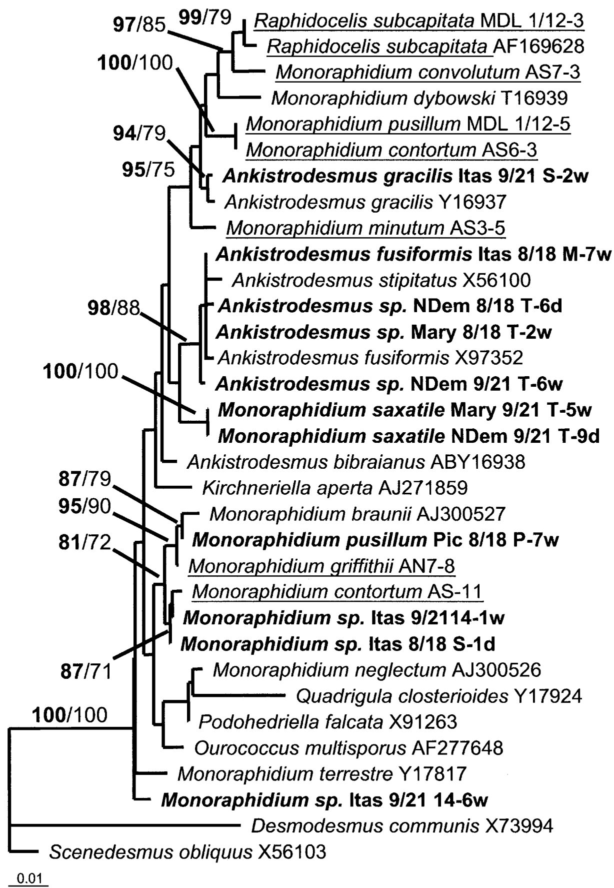

FIG. 3. Phylogram resulting from a maximum likelihood analysis of 18S rDNA sequences from the Selenastraceae, and including *Desmodesmus communis* and *Scendesmus obliquus* as outgroups. Bootstrap values for maximum likelihood are shown in bold; values for maximum parsimony are shown in plain text. Only values greater than 70% are given.

Chlorophyceae (results not shown) resulted in strong support for monophyly of the Selenastraceae, as already determined by Krienitz et al. (2001). Subsequent analyses utilized the apparent closest relatives of the Selenastraceae, *Scenedesmus* and *Desmodesmus*, as outgroup taxa. Portions of the results of a neighbor-joining analysis of these sequences are depicted in Figs. 1 and 2. The analysis depicts the actual substitution differences among the sequences from our isolates and the most similar published sequences. This analysis was intended to show the diversity among our isolates and does not represent a phylogenetic analysis. Thus, no bootstrap values are shown.

The results of the ML analysis are depicted in Fig. 3, with bootstrap values for both the ML analysis and the MP analysis shown. The results show excellent bootstrap support (100%) for the monophyly of the Selenastraceae, but there is little support for internal nodes. Bootstrap support greater than 70% was only achieved for a few terminal lineages. The low bootstrap support is probably due to the relatively small number of variable characters employed in the analyses. Among the Selenastraceae sequences, there were only 87 parsimony informative characters. Our results, like those of Krienitz et al. (2001), show no support for traditional generic concepts within the Selenastraceae.

More important for this study than the relationships among different lineages of the Selenastraceae is the comparison of molecular and morphological data. Some of our isolates, such as *A. fusiformis* Itas 8/18 M-7w, *A. gracilis* Itas 9/21 S-2w and *Raphidocelis subcapitata* MDL 1/12-3, produced 18S rDNA sequences very similar to the published sequences for the same taxa (Table 3, Fig. 1). In some cases, we analyzed multiple isolates that were referable to the same species, including *M. pusillum*, *M. contortum*, *M. minutum* and *M. saxatile*. Our 2 isolates of *M. saxatile*, Mary 9/21 T-5w and NDem 9/21 T-9d, share identical coding sequences, although they differ in intron content. The sequences from the 2 isolates identified as *M. pusillum* (MDL 1/12-5 and Pic 8/18 P-7w), however, differed by 36 substitutions (Table 3, Fig. 1) and were resolved in different lineages (Fig. 3). The sequence from *M. pusillum* MDL 1/12-5 actually was identical to that from isolate AS6-3, identified as *M. contortum*, (Table 3, Fig. 1). The sequence of Pic 9/19 P-7w, identified as *M.* sp., differed from *M. braunii* (AJ300527) by only 7 substitutions (Table 3, Fig. 1) and this isolate did possess some features similar to *M. braunii*. Sequences from the 2 isolates identified as *M. contortum* (AS6-3 and AS-11) differed by 38 substitutions (Table 3, Fig. 1) and were also resolved in different lineages (Fig. 3).

The additional unidentified isolates Mary 8/18 T-2w, NDem 8/18 T-6d, and NDem 9/21 T-6w were all resolved as sister taxa to *A. fusiformis* (X97352) and *A. stipitatus* (X56100) (Fig. 3). In fact, Mary 8/18 T-2w produced a 18S rDNA sequence identical to the sequence of the Itasca *A. fusiformis* isolate, Itas 8/18 M-7w (Table 3, Fig. 2).

Sequences from the isolates Itas 9/21 14-1w and Itas 8/18 S-1d differed from each other by only a single substitution and from the sequence of AS-11, identified as *M. contortum*, by 4 and 5 substitutions, respectively (Table 3, Fig. 1). All of these isolates, plus *M. griffithii* AN7-8, were resolved in a lineage that included *M. braunii* (Fig. 3). The final isolate examined in this study, Itas 9/21 14-6w (Fig. 2), which could not be identified to species, was resolved without significant bootstrap support as a basal taxon of the Selenastraceae (Fig. 3).

DISCUSSION

Morphological analysis by light microscopy of our 18 isolates from the Selenastraceae indicated that 12 of the isolates could be tentatively identified as species included in current monographs. Two isolates were identified as *Monoraphidium saxatile*, 2 as *M. contortum*, 2 as *M. pusillum* and one isolate each as *Ankistrodesmus fusiformis*, *A. gracilis*, *M. convolutum*, *M. minutum*, *M. griffithii*, and *Raphidocelis subcapitata*. Of these identifi-

cations, only the isolates referred to *M. saxatile*, *A. fusiformis*, *A. gracilis* and *R. subcapitata* fit the published descriptions without exception. In other cases, there were differences between our isolates and published descriptions, but these differences may have been undetectable without culturing. The multiple isolates identified as *M. contortum* and *M. pusillum* differed from each other, but still fit within the broad definitions of the species.

The named isolates from ANWR, with the exception of *R. subcapitata*, had been found previously from ANWR, with identifications based upon light microscopy from preserved natural samples (Phillips and Fawley 2002a,b). During periods of ice cover, *M. minutum* was reported as a frequently dominant species, *M. pusillum*, *M. contortum*, and *M. convolutum* were very frequent, and *M. griffithii* was only occasional found. Although *R. subcapitata* was not found in the previous study, it is very likely that specimens of that species would have been referred to *M. minutum*, which has very similar cell morphology but lacks mucilage. The mucilage would have been very difficult to see with the counting method employed. Additional species found from ANWR by light microscopy but not observed in this study included 4 species of *Ankistrodesmus*, only 1 of which, *A. bibraianus* (Reinsch) Korshikov, was commonly found.

Previous work on the Itasca State Park algal flora (Meyer and Brook 1968) indicated several species of Selenastraceae that were not detected in this study, including *A. falcatus* (Corda) Ralfs, *A. falcatus* var. *tumidus* (West et West) G. S. West, *A. spirilis* (Turner) Lemm., *M. griffithii* (as *A. falcatus* var. *acicularis* (Braun) G. S. West and 3 species of *Kirchneriella*. One of our isolates, Itas 9/21 14-6w, identified simply as *Monoraphidium* sp., was very similar to the description of *A. acicularis*, (see results) which was a later combination of *A. falcatus* var. *acicularis* (Korshikov 1987). Itas 9/21 14-6w may represent the taxon that Meyer and Brook (1968) referred to *A. falcatus* var. *acicularis*. Of the isolates that we identified from ISP, both *A. gracile* (as *Selenastrum westii* Smith) and *M. saxatile* (as *A. falcatus* var. *stipitatus* (Chod.) Lemm.) had previously been detected in the park. We were unable to assign additional isolates from ISP to any named species, with any confidence. We are also confident that additional taxa from the Selenastraceae are present in ISP. We have commonly observed *Quadrigula* sp. and additional *Ankistrodesmus* spp. among tychoplankton samples, but have not attempted species identification from field material.

Although *M. spiralis* and *M. griffithii* have both been reported from ISP and ANWR, we did not culture isolates from these 2 species from both sites. Otherwise, there is no reported overlap of the Selenastraceae from these locations. We did not also find any additional taxa that were presented at both sites in this study. The coccoid green algal communities of these locations have also been found to be largely distinct by analyses of 18S rDNA (Fawley et al. 2004).

Analysis of 18S rDNA sequences from our isolates and previously published sequences from the Selenastraceae indicate the monophyly of the family, but provide little internal resolution. Greater resolution would probably be achieved using more highly variable sequences such as *rbc*L or ribosomal ITS. However, analyses of 18S rDNA sequences allow an assessment of our morphology-based species identifications. The comparison of the relationships among our isolates based upon sequence analysis and the results of our morphological analysis indicate only partial concordance, with some striking contradictions. Our isolate identified as *A. fusiformis* differed from the published sequence for an *A. fusiformis*, SAG 2005 isolated from Germany, by only a single nucleotide. However, we also had additional isolates that differed somewhat from *A. fusiformis* in morphology, but that produced 18S rDNA sequences either identical or nearly identical to our *A. fusiformis* isolate. Another of our isolates, Itas 9/21 S-2w, was an excellent fit for the description of *A. gracilis*, and the 18S rDNA sequence of this isolate differed from the published sequence for an *A. gracilis* isolate from England, SAG 278-2, by five nucleotide substitutions. The isolate MDL 1/12-3, identified as *Raphidocelis subcapitata*, differed by only four substitutions from the published sequence for *R. subcapitata*, that was produced from an isolate, UTEX 1648, from a Norwegian lake. Our 2 isolates of *M. saxatile*, a species that has not been previously examined by molecular methods, produced identical 18S rDNA coding sequences, but differed in intron content. Although sequences from some of our isolates were very similar to published sequences from European isolates, in no case, the sequences were identical.

In other cases, the correspondence between morphological and molecular results was not as clear. We had 2 isolates, MDL 1/12-5 and Pic 8/18 P-7w, that were identified as *M. pusillum*, although with some reservations for both isolates. The 18S rDNA sequences of these 2 isolates differed by 36 substitutions and they were resolved in separate lineages of the Selenastraceae. On the other hand, *M. pusillum* isolate MDL 1/12-5 and isolate AS6-3, identified as *M. contortum*, produced identical 18S rDNA sequences. An additional isolate identified as *M. contortum*, AS-11, was also resolved in a separate lineage from AS6-3 by molecular analyses. Finally, the sequences generated from isolates Itas 8/18 S-1d, Itas 9/21 14-1w and AS-11 differed from each other by a maximum of five substitutions, but the morphology of AS-11 was dramatically different from the other two isolates. Isolate AS-11 was strongly curved and identified as *A. contortum* (although, not a perfect match), whereas the other 2 isolates were almost perfectly straight.

In several cases, the characters used to identify species were inconsistent with the molecular results. Not only did isolates referred to as the same species fall into different lineages, but also isolates with very similar 18S rDNA sequences occasionally possessed morphologies that would, without question, result in different

species identification. However, the isolates that we identified as the same species using current keys that possessed somewhat different morphologies were always placed in separate lineages by molecular analyses. For example, AS-11 and AS6-3 could both be considered *M. contortum* based on cell size, shape, and the gradually pointed cell ends. However, the cells certainly were not exactly the same and represent opposite extremes of the variability attributed to this species by Komárková-Legnerová (1969).

There is, or course, the possibility that analyses based only on the single gene, 18S rDNA, may be misleading. However, the conservative nature of the 18S rDNA makes this rather unlikely. It is more likely that there is even more variability among our isolates than that is revealed by these sequences. Additional studies utilizing a multigene approach should be conducted to enhance these results.

Our results indicate that the characters used to identify species within the Selenastraceae are not, in fact, defining species-level lineages. The characters considered by Komárková-Legnerová (1969) to be "good interspecific features" include: "(a) Shape of adult cells, (b) intensity of curvature . . ., (c) shape of the colonies with respect to the fascicles, (d) presence of a mucous layer and its consistence, (e) presence of a pyrenoid, (f) range of dimensions, and (g) termination of the cells." With this array of characters, one can, in fact, differentiate among our several isolates, including those that we considered to be conspecific based on morphology. However, these features are clearly not consistent within any specific lineage and do not provide clear information on the evolutionary relationships among isolates.

Our results can be interpreted in several ways. In some cases, one could argue that all of the organisms in a well-supported lineage represent a particular species and that the previous species definition was, in fact, not broad enough. For example, there is little morphological variation in cell shape among the well-supported lineage depicted in Fig. 2 (excluding the 2 *M. saxatile* isolates) that includes the previously published sequences from *A. fusiformis* and *A. stipitatus* as well as 4 of our isolates from ISP. These sequences vary by a maximum of 11 substitutions. The differences between the published descriptions of *A. fusiformis* and *A. stipitatus* are primarily length (*A. stipitatus* is longer) and colony form (*A. stipitatus* forms fasicles and *A fusiformis* cruciate or irregular aggregates). These features were also variable among our isolates from this lineage, in some cases, preventing us from placing a species name on isolates. Hindák (1988) also recognized the variability of the colony form, arguing that *A. fusiformis* and *A. falcatus* (Corda) Ralfs should be considered conspecific.

The well-supported lineage including *M. braunii* as well as our isolates, identified as *M. pusillum* (Pic 8/18 P-7w) and *M. griffithii* (AN7-8), vary by a maximum of 10 substitutions, or approximately the same variation as the *A. fusiformis*/*A. stipitatus* lineage. However, members of this lineage vary considerably in cell size and the shapes of the ends of the cells. It is unlikely that anyone describing taxa from morphological data would combine these isolates in a single taxon.

Even less divergent (based on sequence data) than the *M. braunii* lineage is the lineage comprising our isolates identified as *M. contortum* (AS-11), *M.* sp. (Itas 9/21 14-1w) and *M.* sp. (Itas 8/18 S-1d). The sequences for these isolates vary by a maximum of 5 substitutions, but the cells of AS-11 are strongly curved whereas those of the other 2 isolates are straight or very nearly so. Curvature is one of the primary characters used to delineate species of *Monoraphidium*. Thus, these isolates would never be considered the same species by morphological analysis.

Finally, two isolates, MDL 1/12-5 and AS6-3, possessed identical 18S rDNA coding sequences, but the morphologies varied significantly. The isolate MDL 1/12-5 was identified (with reservations) as *M. pusillum*. The cells were nearly straight to moderately lunate, and occasionally the ends were very slightly sigmoid. AS6-3 was identified with confidence as *M. contortum*, and the cells were highly recurved and sigmoid. These examples make it difficult to accept the idea that species need to be more broadly defined while retaining the same diacritic characters.

Another possible explanation for our observations is that species are too broadly defined by the morphological characters that are presently used. Thus, the variation among isolates that we observe at the molecular level is not concordant with the morphological species definitions. If this explanation is accurate, we would expect those isolates that matched the most narrowly defined species to be the best matches at the molecular level. The most narrowly defined taxa examined in this study were probably *A. fusiformis*, *A. gracilis*, and *A. saxatile*, which were defined by both cell and colony morphologies. Our isolates that matched the descriptions of *A. fusiformis* and *A. gracilis* did, in fact, produce 18S rDNA sequences that were quite similar to the published sequences for these species. However, the isolates identified as *A. fusiformis* were not monophyletic in our analyses, although 18S rDNA is not variable enough to be confident in this assessment.

Thus, we are left with the explanation that the diacritical characters used to delimit species in the Selenastraceae, at least for the polyphyletic genera *Ankistrodesmus* and *Monoraphidium*, are not reliable. Our results show examples of isolates with identical 18S rDNA sequences but morphological variation, and isolates with very different sequences but similar morphologies. The best explanation for the patterns of our results is that species are currently defined too broadly under the morphological concept. This result is not surprising. Some monographs on the Selenastraceae have mentioned high levels of variability among isolates referred to a single taxon, with the suggestion that this variability may actually represent multiple taxa (i.e. Komárková-Legnerová 1969, Nygaard et al. 1986).

Our results also indicate that it is very unlikely that a researcher would be able to accurately identify all Selenastraceae from natural samples. For our analysis, we essentially forced isolates into existing species definitions (based mostly on European isolates) where that was possible. However, we were unable to match several isolates with any named species. When examining a natural sample, it is often impossible to tell whether the range of variation observed represents one or multiple taxa, and frequently autospore production cannot be observed. However, the general tendency is to place a name on an organism when it falls within the broad taxon description. By considering that our results, in addition to those of Krienitz et al. (2001), suggest that both existing genera and species in the Selenastraceae are not well defined by existing criteria, then it is essentially impossible to accurately place a species name on a member of the Selenastraceae from a natural sample, using only characters visible with light microscopy.

Our results and those of Krienitz et al. (2001) point to the need for a massive effort to understand the diversity of the Selenastraceae and determine reliable characters for identification. This effort should employ multiple genes that are more variable than 18S rDNA in concert with morphological analyses. These organisms are so frequently encountered in water samples that it is, indeed, amazing that we have so little understanding of what constitutes a species and how to identify taxa at any level. The number of isolates that we could not assign to any described species as well as our observations of the variability of these organisms in natural samples lead us to speculate that there may be hundreds of taxa within the family world-wide. Our conclusions have far-reaching significance. Our results indicate that many of the diacritical characters used to identify species are not reliable. Our results also strongly suggest that the species-level diversity of the Selenastraceae has been greatly underestimated. As a result, species identified using existing monographs have probably been misidentified in many cases. Because our concept of species has been flawed, we really have very little understanding of the true distribution and ecology of any species of Selenastraceae.

We thank Matt Hoffman and Joni Johnson for technical assistance and Phillip McClean and Rian Lee for the use of the automated sequencer. We thank two anonymous reviewers who provided excellent comments that resulted in substantial improvements to the manuscript. This material is based upon work supported by the National Science Foundation under Grant Nos. DBI-0070387, DEB-0128952 and MCB-0084188. Additional support was provided by the North Dakota Water Resources Research Institute.

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