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Molecular Characterization of Eurasian Watermilfoil, Northern Milfoil, and the Invasive Interspecific Hybrid in Michigan Lakes

ANN P. STURTEVANT[1], N. HATLEY[1], G. D. PULLMAN[2], R. SHEICK[1], D. SHOREZ[1], A. BORDINE[1], R. MAUSOLF[1], A. LEWIS[1], R. SUTTER[1] AND A. MORTIMER[1]

ABSTRACT

The presence of invasive aquatic plants can be detrimental to the ecology of lakes and reservoirs. The distribution of the introduced plant pest Eurasian watermilfoil (*M. spicatum*), the native Northern milfoil (*M. sibiricum*), and the invasive interspecies hybrid (*M. spicatum* × *M. sibiricum*) was surveyed in 14 Michigan lakes and one lake in northern Indiana. Although *M. spicatum*, *M. sibiricum*, and the hybrid are morphologically similar, they can clearly be distinguished from each other by sequencing the nuclear rRNA internal transcribed spacer region (rRNA ITS). We described 11 mutations that can be used to unambiguously identify *M. spicatum*, *M. sibiricum*, and the hybrid at the DNA level. We identified additional mutations that can be used to differentiate between variants within a species. Both invasive species, *M. spicatum* and the hybrid, are widespread in Michigan lakes. *M. sibiricum* was rarely found in the lakes studied. *M. spicatum* and the hybrid were found to coexist in the majority of the lakes studied, in direct contrast to previous studies.

Key words: aquatic plants, internal transcribed spacer, invasive plants, *Myriophyllum sibiricum, Myriophyllum spicatum* L., *M. spicatum* x *M. sibiricum*, rRNA ITS.

INTRODUCTION

The uncontrolled growth of invasive aquatic plants seriously impacts the recreational value and ecology of lakes and reservoirs. Invasive aquatic plants interfere with recreational boating and other water sports, can clog irrigation and other water conveyances, and can contribute to declining property values (Halstead et al. 2003, Deamund et al. 2004). Efforts to contain aquatic nuisance plants have cost property owners millions of dollars each year (Deamund et al. 2004, Getsinger et al. 2002).

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is an invasive plant pest that was introduced into the United States in the 1940s from Europe and Asia. Since its introduction *M. spicatum* has spread throughout the United States and Canada and has become one of the most widely distributed aquatic plant nuisance species. Eurasian watermilfoil is one of the most common plant pests in Michigan surface waters. *Myriophyllum spicatum* can form dense mats of floating vegetation, which may seriously interfere with recreation, impede the use of boat docks and marinas, and foul beaches.

Invasive aquatic plant species have more than an economic impact on water resources. They also can seriously reduce biological diversity, compromise the value of critical fish habitats, and destabilize aquatic ecosystems (Keast 1984, Madsen et al. 1991). *Myriophyllum spicatum* has a detrimental effect on native aquatic plant communities. It tolerates a wide range of habitats and can easily colonize new or disturbed areas. *Myriophyllum spicatum* grows very rapidly, forms dense canopies, which shades other plant species and deprives them of the light essential to their growth, and has been shown to decrease both the species richness and the species density of plants in areas it has invaded (Madsen et al. 1991, Boylen et al. 1999, Madsen 2005). Frequently *M. spicatum* forms thick stands where only one or a few native plant species also occur, and in some circumstances may negatively impact the macroinvertebrate and fish populations (Smith and Barko 1990).

Myriophyllum spicatum, the Eurasian watermilfoil, is closely related to another species of watermilfoil found in Michigan, the northern milfoil (*Myriophyllum sibiricum* Komarov), which is native to Michigan and other parts of North America. The two species are differentiated from each other according to the number of leaflet pairs per leaf, stem diameter, shape of the leaf apex, color, and the presence of winter buds (Crow and Helquist 2002). Lake management practitioners typically differentiate between the species by counting the leaflet pairs on each rachis based on the suggestion that the native *M. sibiricum* has only 5 to 12 pairs of leaflets per leaf while the Eurasian watermilfoil *M. spicatum* has 12 to 20 pairs (Gleason and Cronquist 1991). However, many Michigan plant specimens have an intermediate number of leaflet pairs, or a combination of characteristics, which makes species identification imprecise. Interspecific hybrids between the northern milfoil and the Eurasian watermilfoil have been reported in Wisconsin, Minnesota, Idaho, Michigan, and Washington (Moody and Les 2002, 2007). The presence of the interspecific hybrid makes species identification on the basis of leaflet number even less reliable because the leaf morphology of the hybrid is intermediate to that of the parents (Moody and Les 2007). Flowers of both species are produced in terminal spikes, above the surface of the water; however, the flowers are similar in appearance and are only present

[1]University of Michigan-Flint, Department of Biology, 264 Murchie Science Building, Flint, MI 48502. E-mail: asturt@umflint.edu.

[2]Aquest Corporation, 1110 South Drive, Flint, MI 48503. Received for publication October 31, 2008 and in revised form August 5, 2009.

for a brief period during the growing season. A difference between *M. sibiricum* and *M. spicatum* is the presence of turions. *Myriophyllum spicatum* does not produce turions; *M. sibiricum* does, but only during certain times of the year. The absence of turions is therefore not a useful feature for identification.

Some reports suggest that the hybrid watermilfoil *(M. spicatum* × *M. sibiricum*) is even more invasive than the Eurasian watermilfoil due to "hybrid vigor" (Moody and Les 2002). In fact, the ability of the two species to hybridize with each other may be responsible for the creation of a unique invasive, nuisance plant. Once a hybrid is formed by sexual reproduction between the two species, the gene combinations that make the plant invasive become established in the population. The presence of a variety of milfoil genotypes can have significant implications for lake managers.

Eurasian watermilfoil reproduces primarily by a form of vegetative propagation called fragmentation. Pieces of stem break off the plant and are carried to a new location, where they take root and rapidly grow into new plants genetically identical to the original (Voss 1985, Madsen 2005). Therefore, *M. spicatum*, *M. sibiricum* and the hybrid can easily be spread from one body of water to the next by boats and trailers. The watermilfoil hybrid can rapidly spread throughout a geographical region by vegetative propagation, while maintaining the specific gene combinations that makes it an aggressive nuisance plant.

There is considerable genetic variation among watermilfoil populations (Moody and Les 2002, 2007). Because of the difficulty of identifying watermilfoil species based on leaf morphology, accurate identification of watermilfoil species frequently requires characterization at the DNA level. DNA sequencing of the nuclear ribosomal RNA internal transcribed spacer (rRNA ITS) region is a standard tool used to analyze the phylogenetic relationships between organisms, especially at the family, genera, and species levels (Baldwin et al. 1995). Ribosomal ITS sequences are the most widely used sequence in studies of plant molecular phylogeny (Alvarez and Wendel 2003). Mutations tend to accumulate in the ITS region because it does not code for either rRNA or protein (Baldwin et al. 1995). Because the ribosomal ITS sequences are nuclear-encoded they are inherited biparentally (Alvarez and Wendel 2003), making the ribosomal ITS sequence useful in studies on hybrid speciation (Alvarez and Wendel 2003, Andreasen and Baldwin 2003, Koch et al. 2003). Ribosomal ITS sequences have successfully been used to distinguish between *M. spicatum*, *M. sibiricum*, and the hybrid at the molecular level (Moody and Les 2002) using the universal primers designed by White et al. (1990). Molecular characterization of the ribosomal ITS region has been used to identify genotypes of watermilfoil found in Minnesota, Wisconsin, Connecticut, and Florida but has only recently been used to study watermilfoil in Michigan (Moody and Les 2002, 2007).

The purpose of this project was to (1) use DNA sequencing of the ribosomal ITS region to identify genetic variants of *M. spicatum* and *M. sibiricum* present in Michigan lakes, and (2) document the presence of *M. spicatum*, *M. sibiricum*, and the interspecific hybrid (*M. spicatum* × *M. sibiricum*) in a number of Michigan lakes. We were especially interested in determining if the aggressive interspecies hybrid is present in Michigan and whether the two invasive plant species, *M. spicatum* and the hybrid, coexist in the same lake.

MATERIALS AND METHODS

Plant Sampling

Plants were collected from 14 Michigan lakes and one Indiana lake (Table 1), mostly located in the lower peninsula of Michigan, primarily in southeastern Michigan, excepting Fife and Pleiness lakes in northwestern Michigan; Townline Lake in central Michigan; and Dewart Lake in northern Indiana. These lakes were chosen based on previous reports of herbicide failure. Approximately 1 gallon of plant material was collected. The apical meristems and terminal leaflets were excised from each individual plant and stored at -80 C.

TABLE 1. DISTRIBUTION OF *MYRIOPHYLLUM SPICATUM*, *M. SIBIRICUM* AND THE INTERSPECIES HYBRID *M. SPICATUM* × *M. SIBIRICUM* IN MICHIGAN AND INDIANA LAKES

Lake	County, State (Latitude, Longitude)	Sample Number	Species		
			M. spicatum	Hybrid	*M. sibiricum*
Big	Oakland, MI (42.723°N, 83.520°W)	13	13	0	0
Bronson	Lapeer, MI (43.093°N, 83.385°W)	5	1	4	0
Dewart	Kosciusko, IN (41.370°N, 85.774°W)	7	0	6	1
Fife	Grand Traverse, MI (44.568°N, 85.345°W)	7	6	1	0
Kent	Oakland, MI (42.513°N, 83.676°W)	4	1	3	0
Lobdell	Genesee & Livingston, MI (42.791°N, 83.845°W)	48	2	46	0
Mirage	Washtenaw, MI (42.092°N, 83.395°W)	2	2	0	0
Pine	Genesee, MI (42.796°N, 83.768°W)	5	3	2	0
Pleiness	Mason, MI (43.858°N, 86.245°W)	11	11	0	0
Portage Lake East Colony Canal	Washtenaw, MI (42.428°N, 83.911°W)	6	6	0	0
Stony Creek	Macomb & Oakland, MI (42.717°N, 83.090°W)	16	13	3	0
Tipsico	Oakland, MI (42.717°N, 83.677°W)	12	2	8	2
Townline	Mecosta & Montcalm, MI (43.455°N, 85.204°W)	7	0	7	0
White	Oakland, MI (42.669°N, 83.564°W)	3	0	3	0
Whitmore	Livingston & Washtenaw, MI (42.089°N, 84.116°W)	16	8	8	0

A small number of plants from each lake were analyzed initially; the remaining plant samples collected from each lake were stored at -80 C. Lakes that provided plant samples with unusual genetic variants were more heavily sampled, as were lakes that appeared to contain plant samples of both *M. spicatum* and the interspecific hybrid (*M. sibiricum* × *M. spicatum*). A large number of plants were analyzed from Lobdell Lake because it was also part of another study.

DNA Extraction

DNA was prepared from the apical meristems and terminal leaves of plant samples. Floral tissue was also used for DNA extraction, when present. The CTAB extraction method (Doyle and Doyle 1987, Lodhi et al. 1994, Bekesiova et al. 1999, Tel-Zur et al. 1999) was used with the following modifications: plant tissue was ground in microfuge tubes using disposable plastic pestles (Kontes Scientific Glassware/Instruments) and a small amount of sterile sand. We added 200 µl CTAB Extraction Buffer (20 mM Na_2EDTA, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 2% CTAB [hexadecyltrimethylammonium bromide], 1% PVP [polyvinylpyrrolidone], 0.2% β-mercaptoethanol) at 60 C to the tube, and the tissue was further ground with a pestle and resuspended by vortexing. An additional 200 µl CTAB Extraction Buffer (60 C) was transferred to the tube, and the plant tissue was ground a third time. The final concentration of the extract was adjusted to 1% sodium sarcosyl, vortexed, and incubated at 60 C for 30 to 60 min. The sample was extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 13,000 × g for 10 min to separate the phases. The upper aqueous phase was transferred to a clean microfuge tube. An equal volume of 5 M NaCl was added to the sample followed by the addition of two-thirds volume of ice cold isopropanol. The tube was inverted to mix, and the sample was incubated on ice for 15 min. The sample was centrifuged at 3,000x g for 3 min, then the speed was increased to 5,000 × g for 5 min. Following this procedure a small, white pellet containing DNA was expected to form in the bottom of the tube. If no visible pellet was produced at this step, the sample was centrifuged at 13,000 × g for 15 min. The supernatant had two layers with a thick pellicle at the interface; the pellicle and supernatant were discarded. The pellet was washed with 75% ethanol then centrifuged at 13,000 × g for 5 min. The air-dried pellet was resuspended in 100 µl TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA). We added 10 µl RNAse A (10 mg/ml) and incubated the sample at 37 C for 30 min. Two volumes of dH_2O, 1/10 volume 3M Na acetate pH 5.3, and 2.5 volumes 100% ethanol was added. The sample was incubated on ice for 15 min, or at -20 C overnight to precipitate the DNA. The sample was centrifuged at 13,000 × g for 15 min. The pellet was air dried and resuspended in 100 µl dH_2O.

Characterization of Nuclear rDNA

The internal transcribed spacer region 1 (ITS1), 5.8S rRNA, and internal transcribed spacer region 2 (ITS2), located between the nuclear 18S and 26S ribosomal RNA (rRNA) genes, were analyzed to study nuclear inheritance (White et al. 1990, Baldwin et al. 1995; Figure 1). We used PCR to amplify the entire ITS1, 5.8S rRNA and entire ITS2 regions, using the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White et al. 1990). Reaction conditions were: 1X Promega GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM ITS4 primer, 0.2 µM ITS5 primer and 0.5 units Promega GoTaq Flexi DNA Polymerase, in a total volume of 25 µl. The PCR conditions were: denaturation at 95 C for 5 min, followed by 50 cycles of 95 C for 1 min, 50 C for 1 min, 72 C for 2 min, and a final extension at 72 C for 10 min. The PCR results were analyzed by gel electrophoresis on 0.8% agarose, 100 mM TBE gels. Samples that yielded the expected 750 bp band were cloned into the pCR4-TOPO plasmid vector (Invitrogen) using the manufacturers directions, and used to transform *Escherichia coli* Top10 cells. Plasmid DNA was isolated from bacteria using the QIAprep Spin Miniprep kit (Qiagen). Plasmid DNA was digested with EcoRI and analyzed by gel electrophoresis to confirm the presence of the insert. Plasmids that contained PCR fragments were sent to the DNA Sequencing Core facility of the University of Michigan in Ann Arbor for automated sequencing using Applied Biosystems DNA Sequencers (Model 3730). The T3 and T7 primers complementary to vector sequences were used as sequencing primers. Sequences were aligned using Lasergene 6 (DNAstar) and compared to sequences of *M. spicatum* and *M. sibiricum* in GenBank. To be considered a true genetic variant as opposed to a PCR error, the sequence must be present in more than one plant sample, from more than one lake. In this study, a sequence also needed to be present in a minimum of four clones to be included as a genetic variant. New genetic variants of the (rRNA) ITS1, 5.8S RNA and ITS2 region from *M. sibiricum* and *M. spicatum* were deposited in GenBank as accession numbers FJ426346-FJ426357. Because hybrid plants contain one chromosome from each of their parents, and each plasmid contains the DNA sequence derived from only one molecule of DNA from one parent, it was necessary to sequence multiple plasmids from each plant sample to determine whether it was a hybrid or homozygous species. Between four and eight plasmids from each plant sample were sequenced to determine whether the plant was *M. sibiricum* or *M. spicatum*. Theoretically, only two plasmids per plant need to be sequenced to determine if the plant sample is the hybrid. In practice, we normally sequenced between three and eight plasmids per plant sample before designating the plant as being a hybrid. This means that to molecularly characterize five plants, between 20 and 40 clones must be sequenced. The

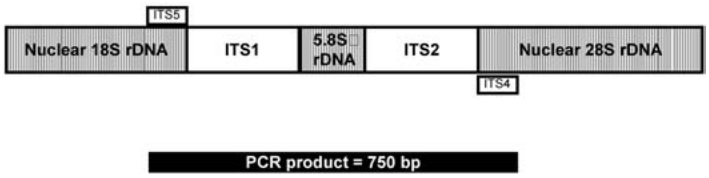

Figure 1. The internal transcribed spacer (ITS) region of nuclear rRNA genes. The region between the primers ITS5 and ITS4 was amplified by PCR to produce a PCR product of approximately 750 bp. The PCR product contains sequences corresponding to the entire ITS1, 5.8S rRNA and ITS2 regions. Note: regions not drawn to scale.

time and cost involved precludes the characterization of large numbers of plants per lake at this time.

Phylogenetic Analysis

Sequences of *M. sibiricum* and *M. spicatum* containing the entire ITS1, 5.8S rRNA and ITS2 regions were aligned using the Megalign module of Lasergene 6 and adjusted manually. Gaps were treated as a fifth base. Parsimony analysis was done using PAUP 4.0B8 (Sinauer) using heuristic searches with random addition (1000 replicates) and TBR (tree bisection reconnection) with unordered, equally weighted characters. A consensus tree was generated. Bootstrap analysis was performed using PAUP 4.0B8 with 500 replicates for analysis (Swofford 2005, Harrison and Langdale 2006).

RESULTS AND DISCUSSION

Sequence Analysis of Watermilfoil Nuclear rRNA ITS1 and ITS2 Region

Sequences were aligned using the Seqman II module of Lasergene 6 (DNASTAR, Inc.), and adjusted manually; ITS sequence data were deposited in Genbank (FJ426346-FJ426357). The watermilfoil sequences described in this paper were compared to previously described ITS sequences from *M. sibiricum* and *M. spicatum* (AF513838- AF513839; Moody and Les 2002) and (DQ786012-DQ786027; Moody and Les 2007). The entire ITS1, 5.8S rRNA, and ITS2 sequences were compared, when available. The *Haloragis erecta* sequence (AF513841) was used as an outgroup for phylogenetic analysis.

Among the *M. spicatum* and *M. sibiricum* sequences there were 756 aligned bases. The ITS1 region was 263-265 bp, the 5.8S rRNA was 160 bp, and the ITS2 region was 220 to 222 bp long. The remaining nucleotides correspond to part of the 18S rRNA and 28S rRNA regions to which the primers were made. Two types of mutations were commonly found, single nucleotide polymorphisms (SNP) and indels. An SNP is a mutation where one base is substituted for a different base; an indel is a position in the DNA sequence where one or more extra bases have been inserted or deleted, compared to the other sequence studied. Although *M. sibiricum* and *M. spicatum* are very closely related, we could detect 18 SNP and 4 indels (Table 2). Of these, 10 SNP and 1 indel are diagnostic (marked with an asterisk in Table 2) for a particular species; the mutation is always found associated with one species while a different sequence is always found in the other species. The other mutations are used to differentiate between variants within a species. These mutations are either not exclusively found in one species, or, while they are found exclusively in one species, only occur in a subset of the variants of the species.

Most of the variation occurred in ITS1 and ITS2. Because these regions do not code for protein or rRNA, they are hot spots for variation. There were eight SNP and three indels in ITS1 and nine SNP and one indel in ITS2. The 5.8S rRNA region contained few variations, none of which were informa-

TABLE 2. MUTATIONS FOUND IN THE RIBOSOMAL RNA ITS1 AND ITS2 REGIONS OF *MYRIOPHYLLUM SIBIRICUM* AND *M. SPICATUM* NUCLEAR DNA.

	Myriophyllum sibiricum variants						*Myriophyllum spicatum* variants					
Site	Sib MI1	Sib MI2	Sib MI3	Sib MI4	Sib MI5	Sib MI6	Spi MI1	Spi MI2	Spi MI3	Spi MI4	Spi MI6	Spi MI7
80*	T	T	T	T	T	T	C	C	C	C	C	C
102	G	G	G	A	G	G	G	G	G	G	C	C
105	C	C	C	C	C	C	A	C	C	C	C	C
131	A	A	A	A	A	A	G	A	G	G	G	G
140*	T	T	T	T	T	T	C	C	C	C	C	C
142	C	C	C	C	—	C	C	C	C	C	C	C
161	—	—	—	—	G	—	G	G	G	G	G	G
170	C	C	C	C	C	C	C	C	C	T	T	T
181*	—	—	—	—	—	—	T	T	T	T	T	T
209*	G	G	G	G	G	G	A	A	A	A	A	A
273*	G	G	G	G	G	G	A	A	A	A	A	A
413	C	C	C	C	C	C	C	C	C	C	C	T
515*	T	T	T	T	T	T	A	A	A	A	A	A
518*	T	T	T	T	T	T	C	C	C	C	C	C
552*	T	T	T	T	T	T	C	C	C	C	C	C
559*	A	A	A	A	A	A	C	C	C	C	C	C
582	G	G	G	G	G	C	G	G	G	G	G	G
633*	T	T	T	T	T	T	C	C	C	C	C	C
673*	T	T	T	T	T	T	C	C	C	C	C	C
679	C	T	T	C	C	C	C	C	C	C	C	C
684	C	T	C	C	C	C	C	C	C	C	C	C
689	—	—	—	—	T	—	—	—	—	—	—	—
690	—	—	—	—	T	—	—	—	—	—	—	—

The location of the mutation in the *M. spicatum* sequence is listed in the first column. Diagnostic sequences used to distinguish between *M. sibiricum* variants (Sib MI1-Sib MI6) and *M. spicatum* variants (Spi MI1-Spi MI7) are marked with an asterisk. A missing base (indel) is indicated by —.

tive for species identification; however, one SNP in this area was used to distinguish between variants within a species. We have identified six variants of the *M. sibiricum* sequence (*M. sibiricum* SibMI1-6) and six variants of the *M. spicatum* sequence (*M. spicatum* SpiMI1-4, SpiMI6-7) present in Michigan watermilfoil populations. Our rRNA ITS1 and ITS2 sequences are similar, but not identical, to previously published sequences (Moody and Les 2002, 2007). Our sequence *M. spicatum* SpiMI1 is most similar to *M. spicatum* FL36 (AF513839; Moody and Les 2002), and our sequence *M. sibiricum* SibMI5 is most similar to *M. sibiricum* CA51 (AF513838; Moody and Les 2002).

In addition to the *M. spicatum* and *M. sibiricum* variants (Table 2), chimeric sequences were present at a low frequency. A hybrid plant will contain some ITS sequences identical to one of the *M. spicatum* variants as well as additional ITS sequences identical to one of the *M. sibiricum* variants. A chimeric sequence is a combination of *M. spicatum* and *M. sibiricum* sequences in the same molecule of DNA. For example, for the first 250 nucleotides of the ITS region the chimeric sequence may be identical to one of the *M. sibiricum* sequences, then change to a sequence identical to one of the *M. spicatum* sequences. The reverse is also possible. The 5' end of the rRNA ITS region may consist of *M. spicatum* sequences while the 3' end may contain *M. sibiricum* sequences. Chimeric sequences have previously been described in the rRNA ITS regions in other plant hybrids (Moody and Les 2002, 2007, Alvarez and Wendel 2003, Andreasen and Baldwin 2003, Koch et al. 2003). These chimeric sequences are most likely due to recombination or concerted evolution between parental sequences in hybrid plants. They may also be the product of PCR-mediated recombination (Cronn et al. 2002).

The genetic variants described above are not evenly distributed among the lakes studied (Table 3). The focus of this study was to identify the presence or absence of *M. sibiricum*, *M. spicatum*, and the interspecies hybrid in the lakes sampled. It was not meant to be a detailed survey of *Myriophyllum* genetic variants and their frequency of distribution in each lake because only a small number of plant samples were analyzed from each lake; however, some generalizations can be made. Among the *M. sibiricum* variants, *M. sibiricum* SibMI1, *M. sibiricum* SibMI2, and *M. sibiricum* SibMI5 are the most widespread in the lakes studied. *Myriophyllum sibiricum* SibMI6 was the most frequently sampled form of *M. sibiricum* in Lobdell Lake but was only occasionally found elsewhere. *Myriophyllum spicatum* SpiMI1 and *M. spicatum* SpiMI3 were found in every lake studied, while *M. spicatum* SpiMI4 was found in 13 of the 15 lakes. In some cases a shortened DNA sequence was obtained from full length clones of the rRNA ITS region. These shortened sequences (*M. sibiricum* SibShort and *M. spicatum* SpiShort; Table 3) were missing up to 140 nucleotides at the 5' end of the sequence and so lacked one or more of the mutations used to distinguish between genetic variants within a species. Although these sequences were not full length, they contained at least 600 nucleotides used to unambiguously identify the sequence as *M. sibiricum* or *M. spicatum*. Shortened sequences of both *M. sibiricum* variants (4%) and *M. spicatum* variants (29%) were obtained.

Phylogenetic analysis of *M. spicatum* and *M. sibiricum* resulted in one tree of 122 steps. A strict consensus tree was generated using *Haloragis erecta* as the outgroup. Bootstrap analysis was done using 500 replicates. Numbers above the branches of the tree are bootstrap percentages for clades found in the 50% majority rule consensus tree (Figure 2). The consistency index (ci) was 0.9836 and the retention index (ri) was 0.9765. There were 767 total characters (756 nucleotides + 11 gaps). Of the 116 variable characters, 21 were parsimony-informative. The *M. sibiricum* variants are clearly separated into a different clade from the *M. spicatum* variants.

Distribution of *M. sibiricum*, *M. spicatum* and the Hybrid in Michigan Lakes

Watermilfoil plants were collected from 14 Michigan lakes and one lake in Indiana (Table 1). All of the Michigan samples were from the Lower Peninsula, primarily from southeastern Michigan. Both invasive species of watermilfoil, *M. spicatum* and the interspecies hybrid (*M. sibiricum* x *M. spicatum*), are widespread in Michigan lakes. The northern milfoil, *M. sibiricum*, was only found in samples from two of the lakes studied, Tipsico Lake, in Oakland County, Michigan, and Dewart Lake, Indiana. When present, *M. sibiricum* was a minor constituent of the watermilfoil samples collected from a lake. The majority of watermilfoil plants in samples collected from the lakes studied were either *M. spicatum* or the hybrid (Table 1). Some of the lakes appeared to exclusively contain *M. spicatum* in their watermilfoil population. Only *M. spicatum* was found in the watermilfoil samples from Big Lake, Mirage Lake, Portage East Colony Canal, and Pleiness Lake. Only the hybrid watermilfoil was found in watermilfoil samples collected from Townline and White lakes., Because our current method of molecularly characterizing the plants is laborious, however, we were only able to sequence DNA from a relatively small number of plants from these lakes (Table 1). Both *M. spicatum* and *M. sibiricum*, as well as the interspecies hybrid, might be found in these lakes if a larger number of plants were characterized.

In the majority of lakes studied (8 of 15), both the interspecies hybrid and *M. spicatum* were found in the watermilfoil samples collected. In some lakes (Bronson, Kent, Lobdell, and Tipsico) the hybrid was the most abundant form of watermilfoil, while *M. spicatum* and *M. sibiricum* were minor constituents of the sample. In other lakes (Fife and Stony Creek) M. spicatum was dominant, and the hybrid was found less frequently. The hybrid and *M. spicatum* seemed to be relatively equal in abundance in samples from Pine and Whitmore lakes. Due to the nature of DNA sequence analysis, it is relatively simple to identify a plant as being a hybrid. Theoretically, only two clones per plant need to be sequenced to determine a hybrid. If one sequence is an *M. spicatum* variant and the other is an *M. sibiricum* variant, the plant must be a hybrid. However, it is much more difficult to prove that a plant sample only contains DNA sequences from a single species. There is always the possibility that no matter how many individual clones are sequenced per plant sample, the next sequence might show that the sample was actually a hybrid. A minimum of four sequences was obtained before identifying a plant as being *M. spicatum* or *M. sibiricum*. Although unlikely, some plant samples designated as *M. spica-*

TABLE 3. DISTRIBUTION OF *MYRIOPHYLLUM SIBIRICUM* AND *M. SPICATUM* VARIANTS IN MICHIGAN AND INDIANA LAKES.

			Myriophyllum sibiricum variants							*Myriophyllum spicatum* variants						
Lake	Number of Plants	Number of Sequences	Sib MI1	Sib MI2	Sib MI3	Sib MI4	Sib MI5	Sib MI6	Sib Short	Spi MI1	Spi MI2	Spi MI3	Spi MI4	Spi MI6	Spi MI7	Spi Short
Big	13	63	0	0	0	0	0	0	0	20	1	15	6	3	2	16
Bronson	5	13	1	0	1	0	5	0	0	2	0	1	1	1	0	1
Dewart	7	33	5	0	0	5	7	1	2	4	1	4	2	0	0	2
Fife	7	36	1	0	0	0	0	0	0	15	1	7	8	0	2	2
Kent	4	19	4	0	0	0	4	0	0	6	0	1	0	2	0	2
Lobdell	48	216	9	10	0	0	6	63	4	27	0	22	4	9	7	55
Mirage	2	11	0	0	0	0	0	0	0	2	3	5	0	0	0	1
Pine	5	21	0	2	0	0	0	0	0	8	1	1	2	2	2	3
Pleiness	11	59	0	0	0	0	0	0	0	14	15	11	1	0	0	18
Portage Lake East Colony Canal	6	28	0	0	0	0	0	0	0	8	0	5	5	0	1	9
Stony Creek	16	77	1	2	0	0	0	0	0	21	0	17	7	0	2	27
Tipsico	12	67	18	2	0	0	5	0	2	10	1	9	11	4	0	5
Townline	7	37	14	0	7	0	0	0	0	3	0	1	4	2	0	6
White	3	11	1	3	0	0	0	0	0	1	0	4	2	0	0	0
Whitmore	16	54	4	2	0	1	0	8	0	14	0	6	3	3	1	12
Total	162	745	58	21	8	6	27	72	8	155	23	109	56	26	17	159

The distribution of the *M. sibiricum* (Sib MI1 - Sib MI6) and *M. spicatum* (Spi MI1 - Spi MI7) variants in the lakes studied. For each plant sample, between two and eight DNA sequences were analyzed. In some cases shortened sequences (designated *M. sibiricum* SibShort and *M. spicatum* SpiShort) were obtained. The distribution of chimeric sequences is not shown. A total of 162 plants and 745 sequences were analyzed.

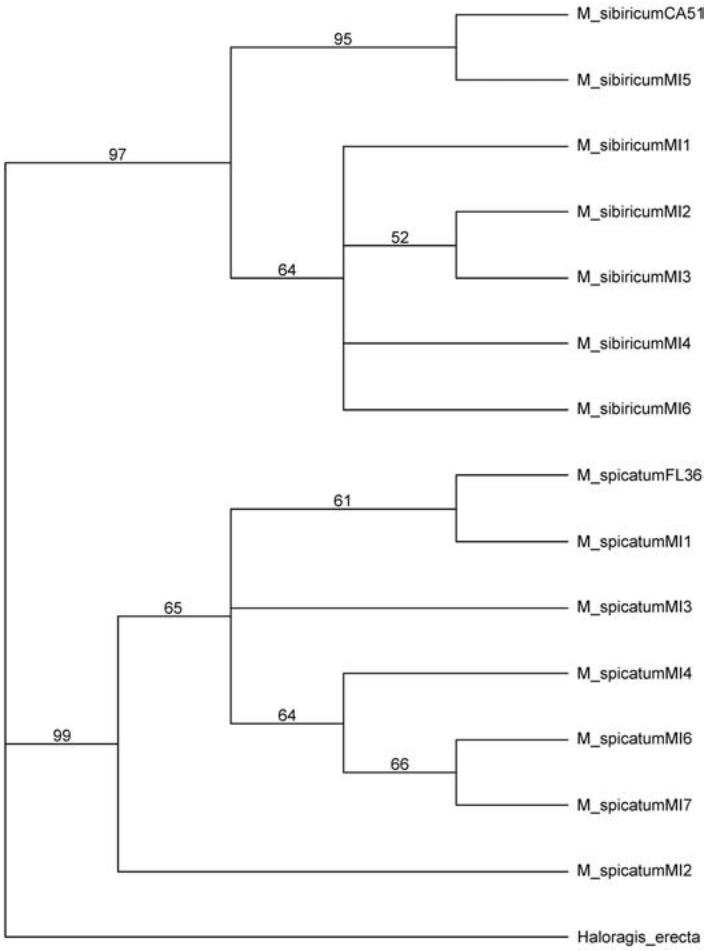

Figure 2. 50% majority rule consensus tree of *Myriophyllum sibiricum* and *M. spicatum* sequences found in Michigan. The single most parsimonious tree (122 steps) was computed from the entire ITS1, 5.8S and entire ITS2 sequences (CI: 0.9836, RI: 0.9765). Numbers above branches are bootstrap percentages.

tum or *M. sibiricum* could later prove to be hybrids if a greater number of plasmids per plant were sequenced. However, *M. spicatum* and the interspecies hybrid were found to coexist in eight different lakes (Table 1). It is extremely unlikely that all 36 *M. spicatum* samples from these lakes would turn out to be hybrids.

The co-occurrence of *M. spicatum* and the interspecific hybrid (*M. spicatum* × *M. sibiricum*) in eight Michigan lakes is in direct contrast to a previous report (Moody and Les 2007), where *M. spicatum* and the hybrid did not coexist in the Minnesota lakes studied. Moody and Les did note the occurrence of *M. spicatum* and the hybrid in some of the other lakes studied, but this was relatively rare, and it was observed in only 3 of the 28 lakes sampled in Idaho, Wisconsin, and Washington. Moody and Les (2007) suggested that the first invasive species to reach a lake, either *M. spicatum* or the interspecific hybrid, rapidly reproduces and becomes the dominant species of watermilfoil, thus preventing the other species from later becoming established in the lake. An alternate explanation is that while the first species to reach a lake will most likely be more abundant than the newly introduced species, both may thrive under the right circumstances. The presence of one invasive species does not necessarily prevent the proliferation of the other. Some environmental conditions may favor the hybrid, while others may favor *M. spicatum*. The dominant watermilfoil in a lake may alternate between *M. spicatum* and the hybrid over time, depending on growing conditions and reintroduction of watermilfoil from recreational watercraft. There may also be differences in the watermilfoil population in different parts of the same lake.

We have described 11 mutations (10 SNP and 1 indel) that can be used to unambiguously identify *M. spicatum*, *M. sibiricum*, and the interspecific hybrid at the DNA level. We have found an additional 10 SNP and 3 indels that can be used to differentiate between variants within a species. Both invasive species, *M. spicatum* and the interspecific hybrid, are widespread in the plant samples collected from the lakes studied. *Myriophyllum sibiricum* was rarely found in the samples collected. The presence of one invasive species in a lake did not preclude the presence of the other invasive species in the same lake. *Myriophyllum spicatum* and the hybrid were found to co-occur in a number of the lakes studied.

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