

An analysis of genetic variability of *Myriophyllum spicatum* in Madison County Lakes

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INTRODUCTION

An investigative project was conducted using randomly amplified polymorphic DNA (RAPD) analysis to investigate the genetic diversity of *Myriophyllum spicatum* from Leland Pond and Lake Moraine in Madison County, New York. This species of milfoil was first introduced to North America in the late 19th century around the Chesapeake Bay area (Aiken, 1979), although some authors believe that it came as late as the 1940's (Couch and Nelson, 1985). *Myriophyllum spicatum* is an aquatic macrophyte native to Europe, Asia and Northern Africa (Couch and Nelson, 1985), and reproduces primarily through vegetative fragmentation, which occurs throughout most of the year. These fragments typically develop adventitious roots at the nodes prior to separation from the parent plant (Aiken, 1979). Adventitious roots are also formed on fragmented plants, which have been broken off of the parent plant by physical disturbances, either natural or man-made (Aiken, 1979). Natural physical disturbances include the mining by *Euhrychiopsis lecontei*, the milfoil weevil, and weather conditions causing extreme water movement. Human disturbances include harvesting and recreational activities such as boating, water skiing and jet skiing.

Sexual reproduction also occurs in *Myriophyllum* sp. by means of flowering and seed production (Patten, 1956). Aiken (1979) states that cross-pollination is the favored mode of reproduction. However, few milfoil flowers have been noted in central New York State, whereas adventitious roots are common in all lakes thus far studied by researchers at the SUNY Oneonta Biological Field Station in Cooperstown, NY (Lord, 2000).

Genetic diversity within or between closely related species can be investigated at the molecular level by comparison of their DNA. The physical basis of heredity and the role of mutation are known to generate differences between individuals of the same species whether they exist in close proximity or in geographically isolated areas. Random amplification of polymorphic DNA (RAPD) is a procedure that has been developed in which nanogram amounts of DNA can be used, without prior knowledge of the DNA sequence, to determine genetic differences. These differences may provide a key toward controlling the spread and dominance of milfoil.

What we believe to be *M. verticillatum* (positive confirmation pending location of flowers and fruits) has been naturally occurring in Leland Pond for more than one season. This plant is a native North American species of milfoil that is unobtrusive and unobjectionable to lakeside residents and lake users. Conversely, *M. spicatum* is an aggressive exotic species of milfoil that typically out-competes all other submerged

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aquatic plants within its community. *Myriophyllum spicatum* has not been known to exist in Leland Pond until 1999, when a State sponsored public boat launch site was placed there. Since there is a possibility that hybridization could occur between these two species of milfoil, this could potentially interfere with the control efforts of lake-users. This is a possibility only if there is sexual reproduction of milfoil occurring in the lake. This preliminary study seeks to determine if there is sexual reproduction occurring, which would produce an array of genotypes, or if there is asexual reproduction occurring, which will produce genetically identical or nearly identical organisms. Any asexually reproducing organism will be genetically identical to the parent plant unless a somatic mutation is revealed.

Furnier (1995) observed genetic variation in Eurasian watermilfoil within and among North American lakes using randomly amplified polymorphic DNA markers. Variation was found within lakes for two different species of milfoil, *M. spicatum* and *M. sibiricum*. Asexual (clonal) reproduction was found to play an important role in determining the genetic structure of the populations. This clonal structure was much more prevalent in *M. spicatum* than in *M. sibiricum* (Furnier, 1995). Attempts are being made to replicate the methodologies used to amplify DNA so as to determine genetic variability within one species of milfoil (*M. spicatum*). The results may provide information to controlling the rapid spread of EWM in North America.

Lake Moraine has historically had problems with the infestation of Eurasian watermilfoil. This lake has been part of an ongoing study by the Biological Field Station since 1997 (Bennet et al., 1998; Harman *et al.* 1999; 2000; 2001). The most recent application of the herbicide Sonar[®] was initiated on 14 May 2001 and successfully completed on 30 June, after which *M. spicatum* was nearly eliminated and most other macrophyte species were impacted to varying degrees. On 30 June, a small population of EWM stems with little growth was found. There is a possibility that these remaining stems could be resistant to the chemical, due to some mutation or slightly modified genomic structure.

Genetic diversity within the exotic plant species Eurasian watermilfoil (*Myriophyllum spicatum*) is of interest due to the implications of control methods on this aggressive exotic aquatic macrophyte. Genetic variability promotes adaptability, which can select for forms that are resistant to conventional control measures. One competitive variation of milfoil believed to exist includes thin-stemmed Eurasian watermilfoil, whose advantage over its predecessor seems to be the diameter of the stem (Lord, 2000). Because *Ehurychiopsis lecontei* is physically too large to burrow into the thin-stemmed milfoil, this biological control agent is denied pupation sites. Another competitive variation could be that which has become resistant to fluridone (1-methyl-3-phenyl-5(3-(trifluoromethyl)phenyl)-4(1H)-pyridone[C₁₉ H₁₄F₃NO]) after treatment (Burns, 2001 Personal Communication). If these species enter into an ecosystem, the methods of control now being used will no longer be effective.

METHODS

Sampling

Five samples of actively growing *M. spicatum* plants were collected by hand from a boat in the shallow water of Leland Pond, and five samples of persisting *M. spicatum* were collected by divers in the deep water of Lake Moraine. Plants were brought to the surface and placed in zip-lock bags and put on ice for transport. In the laboratory the samples were rooted in autoclaved sediment, collected from a location with dense Eurasian water-milfoil beds. These were placed in 4-liter plastic cylinders for growth under fluorescent light for two weeks (until new growth was evident).

Laboratory methodologies refer to Puregene[®] DNA isolation kit protocol:

Cell Lysis:

1. 40-mg samples of apical meristem were ground using a mortar and pestle and added to a 1.5-ml tube.
2. 600 μ l of cell lysis solution (Tris[hydroxymethyl] aminomethane and ethylenediaminetetraacetic acid and sodium dodecyl sulfate) was added to each sample and vortexed for 1-3 seconds to wet the tissue.
3. Samples were incubated at 65°C for 60 min.

RNAse Treatment:

1. 3 μ l RNAse A Solution was added to the cell lysate (to digest the RNA).
2. Samples were inverted 25 times and incubated at 37°C for 15 min.

Protein Precipitate:

1. Samples were cooled to room temperature.
2. 200 μ l protein precipitate solution (ammonium acetate) was added to the RNAse A-treated cell lysate.
3. Products were vortexed at high speed to mix uniformly
4. Products were centrifuged at 14,000 rpm for 3 minutes. Precipitated proteins formed a tight brownish green pellet.

DNA Precipitation:

1. Supernatant containing DNA was poured into a 1.5 ml tube that contained 600 μ l of 100% isopropanol.
2. Samples were mixed gently by inverting.
3. Products were centrifuged at 14,000 rpm for 1 minute; DNA was then visible as a small pellet that ranged in color from off white to light green.
4. Supernatant was poured off and 600 μ l of 70 % ethanol was added to the DNA pellet.
5. Products were centrifuged at 14,000 rpm for 1 minute and ethanol carefully poured off.
6. Samples were allowed to air dry for 15 minutes.

DNA Hydration;

1. 100 μ l DNA Hydration Solution (Tris [hydroxymethyl] amino- methane and ethylenediaminetetraacetic acid) was added to each DNA pellet.
2. Samples were placed in a 65°C water bath in shaker for 1 hour. Alternatively, DNA was allowed to rehydrate overnight at room temperature on shaker.

3. DNA was stored at -4°C .

After the DNA was isolated, electrophoresis was run to determine the integrity of the genomic DNA. Electrophoresis was run on a 0.5% agarose gel, which contains .25 grams agarose in 50 ml TAE 1x (Tris Acetate EDTA). One μl Ethidium Bromide was added to the gel solution before solidification to visualize the DNA. Ten μl of DNA and 3 μl of load solution was added to sample wells and 13 μl lambda and lambda/ hinde III were added to sample wells as size markers. The electrophoresis was run at 100 volts for 1 hour.

Once the integrity of the DNA was established, polymerase chain reactions (PCRs) were performed in a thermocycler using set F primers from Operon technologies. The PCR reactions were run following the methodologies of Furnier *et al.* (1995). 1.5 μl of 2.5 mM dNTP, 1.5 μl of 10x reaction buffer, 0.6 μl of 5 μM oligonucleotide primer, 0.4 μl of 0.75 unit Taq polymerase, 2.5 μl of 10 ng/ml Plant DNA and 8.5 μl of deionized distilled water were combined. The thermocycler was run for 1 minute 30 seconds (1:30) at 92.5°C , followed by 45 cycles of 1:00 at 92.5°C , 2:00 at 36°C , and 2:00 72°C for a total incubation time of 225 minutes. All temperatures were run at maximum speed except for the transition from 36°C to 72°C , which runs at 4 seconds/ 1°C .

Products of the PCR were separated by electrophoresis and the sizes of the amplified fragments were determined by comparison with DNA standards, which were electrophoresed on the same gel. The gels were stained with Ethidium bromide and photographed.

RESULTS

The banding patterns of the electrophoresed products were analyzed by viewing the photographs (i.e. Figure 1) obtained from the gel. The results from the set of random primers used in this study to amplify fragments from the milfoil genome have been inconclusive. Within the myriad of 10mer (10 base pair) oligonucleotide primers available, primer set F was said to have previously worked to recognize *Myriophyllum sp.* nucleotide sequences (Ofelt, 2001). Portions of samples that have been electrophoresed did not reveal any banding patterns on the final gel; therefore, it is assumed that the short oligonucleotide primers did not recognize any nucleotide sequences in the RAPD's which were taken from *Myriophyllum spicatum*. There are many factors that must be accounted for in amplifying DNA in a polymerase chain reaction. Since changes in the amount of magnesium involved in the reaction may affect the amplification, an additional 5mM Mg^{2+} (0.5ml) was added to the original reaction and tested. Since the cleanliness of the DNA may affect the PCR, original milfoil DNA was cleaned a second time with QAI PCR purification kit. The amount of primer concentration used in the reaction process may have to be altered to properly amplify the sequence; therefore, 4 times the original concentration was used in a second reaction, using 2.4 additional ml of primer solution. There could have been contamination in the nucleotide mix, so a new nucleotide mix was used in a second reaction with the original milfoil DNA. To test the austerity of the agarose gel and primers, previously isolated bovine DNA was amplified and electrophoresed. Not one of the second reactions, when electrophoresed, came up with

results. Due to the limited time available to conduct this project, only 5 samples were collected from each location. This amount was expected to be sufficient to get this project underway.

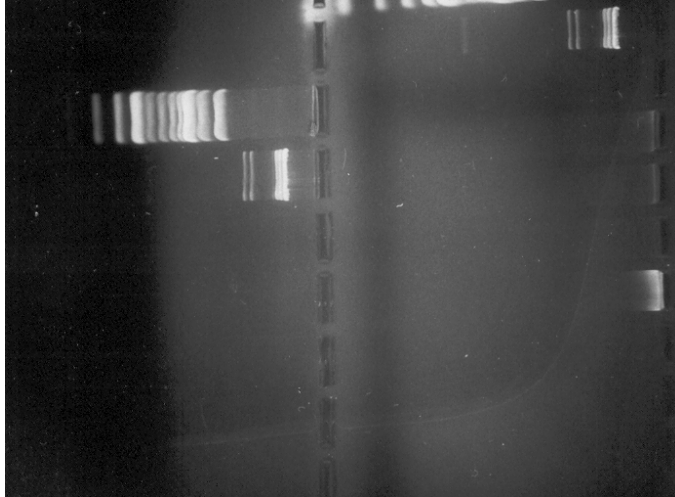


Figure 1. Photograph of 1.2% agarose electrophoresis gel showing Lambda and Lambda-Hinde III size markers.

DISCUSSION

Genetic variability of Eurasian watermilfoil occurring in and among lakes is an important issue related to the management of that plant. State sponsored public boat launch sites throughout the upstate NY region may increase the spread of exotic plant and animal species. If there are differences in the genotype of the same species of milfoil in one area, then that set of genes could easily be transmitted to other lakes in the region. The introduction of genetically distinct fragments of milfoil into a lake could, through outbreeding, increase the variability of the population there. If genes that promote resistance to conventional management techniques are incorporated, than those techniques may, over time, become ineffective. If such variability is found to exist, then precautions to avoid further introductions of milfoil should be made even if that species is already present or dominant

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