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Note

Comparison of molecular markers to distinguish genotypes of Eurasian watermilfoil, northern watermilfoil, and their hybrids

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INTRODUCTION

Managed aquatic plant taxa can exhibit genetic variation that is relevant to their growth, impacts, and control efficacy. For example, genotypes of hydrilla (*Hydrilla verticillata* L.f. Royle) differ in their degree of sensitivity to fluridone (Michel et al. 2004), and genetic screening of hydrilla populations can therefore be used to predict whether fluridone treatment will be efficacious on the specific genotypes present in a water body (Benoit and Les 2013). For most species, where, when, and how genetic variation will be important for management decisions and outcomes is still unknown. However, molecular markers can be used to quantify and monitor genetic variation across space and time, which holds potential to identify specific genotypes of interest (e.g., putatively herbicide-resistant genotypes) or changes in genetic variation that could signal important changes in population-level responses to management.

Invasive Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) is a highly managed aquatic plant in the United States. EWM hybridizes with native northern watermilfoil (*Myriophyllum sibiricum* Komarov; NWM), and EWM and hybrid genotypes can differ in their growth and herbicide response (Berger et al. 2012, 2015, Thum et al. 2012, LaRue et al. 2013, Netherland and Willey 2017, Taylor et al. 2017), prompting interest in determining which specific genotypes will respond best to specific control tactics. However, because genotypes are unable to be distinguished by morphology, distinguishing genotypes requires molecular markers.

Previous studies of genetic variation in EWM, NWM, and hybrid watermilfoil have used amplified fragment length polymorphisms (AFLPs) and microsatellite markers. AFLPs have been used to identify hybridization and demonstrate genetic diversity in EWM, NWM, and hybrids (Zuellig and Thum 2012, LaRue et al. 2013). AFLPs are relatively cheap, but they have limited precision to distinguish closely related genotypes from sequencing or scoring errors. This means that individuals that are the same genotype (i.e., ramets of the same genet) can be mistakenly considered as different genotypes because of these errors. Further, slight differences among laboratories in methods or scoring make it difficult to compare AFLP data collected from different laboratories. Microsatellite markers have also been used to study genetic variation in EWM, NWM, and hybrids (Wu et al. 2013, 2015a,b, Taylor et al. 2017, Guastello and Thum 2018). Microsatellite scoring is generally more precise and repeatable than AFLPs, but interlaboratory collaboration is still challenging because of slight differences in bench methods, scoring methods, and fragment analysis parameters. A potentially more serious limitation of microsatellites is the limited number of loci available to distinguish genotypes. This means that there is the potential for two unrelated individuals to share the same multilocus microsatellite genotype (MMG) by chance through sexual reproduction, as opposed to by common ancestry through asexual reproduction.

Next-generation sequencing methods for genotyping by sequencing (GBS), such as double digest restriction-associated DNA sequencing (ddRAD-Seq, Peterson et al. 2012), offer promise to improve molecular genotyping over AFLPs and microsatellites. These methods can produce hundreds to thousands of markers that could be better at distinguishing genotypes than the limited number of microsatellite markers. At the same time, since ddRAD-Seq is sequence based, it has the potential to be more precise and repeatable than AFLPs. To date, we are unaware of any studies of watermilfoil that have utilized ddRAD-Seq.

In this study, we compare genotype assignments using microsatellites, AFLPs, and GBS-single nucleotide polymorphisms (SNPs). Specifically, because there is the potential for two unrelated individuals to share the same MMG by chance instead of via common ancestry through asexual propagation, we tested whether individuals with the same MMG were estimated to be the same or different genotypes for AFLP and GBS-SNP markers.

MATERIALS AND METHODS

Sampling strategy and DNA extraction

Samples used for the genetic analysis in this study were collected for separate projects examining spatial and

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Table 1. Microsatellite multilocus genotype (MMG), lake of sample origin, county of origin, and number of individuals sampled for all watermilfoil used in this study. Samples are grouped by MMG, and lakes sharing those genotypes are listed in order.

Genotype (MMG)	Lake	County of Origin	Number of Individuals
HWM1	Coon Lake, MN	Anoka	9
HWM1	Elmo Lake, MN	Anoka	9
HWM2	Ham Lake, MN	Anoka	9
HWM3	Bald Eagle Lake, MN	Ramsey	2
HWM3	Lake Josephine, MN	Ramsey	2
HWM3	Otter Lake, MN	Anoka	2
HWM3	Fish Lake, MN	Dakota	2
HWM3	Bone Lake, MN	Washington	2
HWM3	South Lindstrom Lake, MN	Chisago	2
HWM4	Lake Minnetonka, North Arm Bay, MN	Hennepin	11
HWM4	Lake Minnetonka, Gray's Bay, MN	Hennepin	2
HWM4	Lake Minnetonka, St. Alban's Bay, MN	Hennepin	2
HWM5	Sage Lake, MI	Ogemaw	5
HWM5	Budd Lake, MI	Clare	4
HWM6	Townline Lake, MI	Montcalm	3
HWM6	Lake Templene, MI	Sherman	3
HWM6	Muskellunge Lake, MI	Montcalm	3
EWM1	Rebecca Lake, MN	Hennepin	11
EWM1	Bald Eagle Lake, MN	Ramsey	2
EWM1	Mitchell Lake, MN	Hennepin	2
EWM1	Coon Lake, MN	Anoka	2
EWM1	Big Marine Lake, MN	Ramsey	2
EWM1	Riley Lake, MN	Carver	2
EWM2	Fish Lake, MI	Sherman	14
EWM3	Jordan Lake, MI	Ionia	14
EWM4	Lansing Lake, MI	Ingham	14
NWM1	Bald Eagle Lake, MN	Ramsey	9
NWM2	Lake Minne-Belle, MN	Meeker	9
NWM3	Mitchell Lake, MN	Sherburne	9
NWM4	Spectacle Lake, MN	Isanti	9

temporal patterns of genetic diversity in watermilfoil in Michigan and Minnesota (R. A. Thum, unpub. data). Briefly, plant samples for those projects were collected by randomly sampling one plant from a rake toss at each location on a point-intercept grid of the littoral zone of the lake (e.g., see Parks et al. 2016). This sampling typically resulted in 50–100 plants collected from each lake. Then, approximately 20 plants were randomly subsampled for genetic analysis using microsatellite markers (see description of methods below). All of these samples were extracted using the Qiagen DNeasy Plant Mini Kit[1] following the standard plant protocol.

In this study, we specifically tested the hypothesis of whether individuals with the same MMGs represented ramets of the same genets (i.e., clones, or individuals that share common ancestry through asexual reproduction) versus having the same MMG by chance through sexual reproduction. Therefore, we chose individuals for this study by identifying individuals that shared the same MMG within and/or among lakes in the Michigan and Minnesota data sets described above. To test whether genotypes were the same because of chance versus ramets of the same genet, we collected AFLP and SNP data for 8 to 14 individuals sharing each unique MMG in our study (see Table 1). For each MMG, we also gathered molecular marker data on two to three duplicate DNA extractions to quantify scoring and sequencing error.

Microsatellite data collection and analysis

Eight microsatellite loci from Wu et al. (2013) were collected for the 192 samples in this project (Myrsp 1, Myrsp 5, Myrsp 9, Myrsp 12, Myrsp 13, Myrsp 14, Myrsp 15, and Myrsp 16). Each microsatellite locus was amplified using the protocols detailed in Wu et al. (2013). Fluorescently labeled microsatellite polymerase chain reaction products were sent to University of Illinois–Urbana-Champaign's Core Sequencing Facility for fragment analysis on an ABI 3730xl sequencer. Microsatellites were scored using GeneMapper (version 5.0). We identified unique MMGs using the R-package "PolySat" (Clark and Jasieniuk 2011). EWM, NWM, and hybrids are hexaploid, and therefore their exact genotypes cannot be determined because of potential differences in allele dosage in heterozygotes. Therefore, we treated microsatellite data as dominant (presence or absence of all possible alleles at each locus) (see also Wu et al. 2015a,b). We delineated distinct MMGs in PolySat using Lynch distances and a threshold of 0.

AFLP data collection and analysis

AFLP data were collected on 192 individuals (Table 1) using methods described in Zuellig and Thum (2012). We used three primer pairs during the selective amplification step of AFLPs, EcoRI-AGG/MseI-CAT, EcoRI-AGG/MseI-CAG, and EcoRI-AGG/MseI-CAC. AFLP data were run on an ABI3730xl DNA sequencer at the University of Illinois–

Urbana-Champaign Core Sequencing Facility. Fragment data were scored in GeneMapper version 5.0. We assessed the repeatability of AFLP loci using the methods of Ley and Hardy (2013) comparing duplicates using the SpAgeDi software (Hardy and Vekemans 2002). Loci with low repeatability ($F_{ST} \leq 0.80$) were removed from the data set, ultimately leaving us with 108 AFLP molecular markers.

We compared the estimated number of distinct AFLP genotypes with the number of distinct MMG genotypes and GBS genotypes (see below). We used AFLPop (Duchense and Bernatchez 2002) to distinguish genotypes while accounting for scoring error. We calculated the genetic distance between all individuals, and individuals that differed by less than the estimated scoring error rate (six differences) were considered to be the same genotype. To visualize relatedness among genotypes, and confirm that individuals belonging to the same MMG were most closely related to each other as opposed to other MMGs, we ran a principal coordinates analysis (PCoA) using GenAlEx version 6.503 (Peakall and Smouse 2006).

ddRAD data collection and analysis

We also used a next-generation GBS approach, ddRAD sequencing (Peterson et al. 2012), to genotype our 192 individuals. This method is particularly useful for species that do not have a reference genome, such as watermilfoil. Sequencing reads produced through ddRAD-Seq can be clustered together to create contigs leading to a consensus sequence of each locus in the data set; this *de novo* assembly is able to be done in the absence of a true reference genome. DNA from each individual was quantified using a Qubit v3 fluorometer, and 250 ng of total DNA was used for the DNA library preparation and sequencing, which was conducted at the University of Texas–Austin Genomic Sequencing and Analysis Facility. The libraries were prepared using EcoRI and SphI restriction enzymes, and 350-base pair (bp) fragments were selected. Data were sequenced using the Illumina HiSeq4000 for this project using a 2 by 125 paired-end run type.

We processed the ddRAD-Seq reads using a bioinformatics pipeline to produce a panel of SNPs for each of our 192 individuals. ddRAD data were analyzed using the GBS-SNP-calling reference optional pipeline (CROP) (Melo et al. 2016), with the addition of SWEEP (Clevenger and Ozias-Akins 2015) and TASSEL v5.0 (Bradbury et al. 2007) software on Montana State University's Hyalite Computing Cluster. First, raw sequencing reads were trimmed of Illumina adapters using Trimmomatic v0.33 (Bolger et al. 2014), part of the GBS-SNP-CROP. After trimming, reads were filtered for quality and demultiplexed on the basis of a unique barcode sequence for each individual. Next, reads were clustered and assembled into a mock reference using VSearch version 2.9.1 (Rognes et al. 2016) through GBS-SNP-CROP. In the clustering and mock reference assembly step, we used the following parameters: sequence identity of 0.90, read lengths of 300 bp, and a *P*-value threshold of 0.01 from PEAR version 0.9.8 (paired-end read merging [Zhang et al. 2014]). After a mock reference was created, we aligned the reads from each individual to the mock reference using Burrows–Wheeler aligner (BWA) version 0.7.12 (Li and Durbin 2010). SNPs were called with SAMtools version 1.7 (Li 2011), and reads were sorted and indexed with BWA. After SNPs were called they were then filtered using SWEEP (Clevenger and Ozias-Akins 2015), a tool designed to filter diploidized SNPs from polyploid SNPs.

The read processing and filtering performed above resulted in a final data set of 542 diploid SNPs. We used the R-Package "poppr" (Kamvar et al. 2014) to calculate genetic distances among all individuals. We used the genetic distance among duplicate DNA extractions to estimate a genotyping error rate, and used this as the threshold (four differences) to distinguish genets and ramets. We constructed a PCoA using TASSEL version 5.0 (Bradbury et al. 2007) to visualize relatedness among genotypes and confirm that individuals belonging to the same MMG were most closely related to each other as opposed to other MMGs. Finally, we compared estimated genets and ramets after correcting for sequencing error using SNPs with those estimated using MMGs and AFLPs.

RESULTS AND DISCUSSION

Microsatellites and AFLPs agreed on their estimates of genets and ramets. For AFLPs, individuals with the same MMG were most similar to one another (Figures 1A, 1C, and 1E). Moreover, the range of differences found between duplicates of the same individuals ($n = 32$) ranged from zero to six differences. All individuals with the same MMG, regardless of whether they were from the same or different lakes, differed by fewer than this, suggesting that individuals with the same MMG are ramets of the same genet. In contrast, individuals with different MMGs differed by greater than six AFLP markers. Similarly, the estimated genets and ramets for GBS agreed with those estimated by MMGs. Individuals with the same MMG were most similar to each other with GBS markers (Figures 1B, 1D, and 1F). Our GBS error rate among individuals with duplicate extractions was just four SNPs. As with AFLP molecular markers, individuals with the same MMG were never more than four SNPs different, whereas individuals with different MMGs were always greater than four SNPs different.

This study therefore provides evidence that microsatellite-, AFLP-, and SNP-based molecular markers are all effective at distinguishing ramets and genets in watermilfoil. Distinguishing genotypes of watermilfoil is important because different genotypes can respond differently to herbicides (Berger et al. 2012, 2015, Thum et al. 2012, Netherland and Willey 2017, Taylor et al. 2017). This recognition has sparked interest in characterizing the growth and herbicide response properties of different genotypes. For example, two lakes in our data set (Bald Eagle and Coon, (Table 1) contained distinct Eurasian and hybrid watermilfoil genotypes that would not likely be distinguished without molecular genotyping data (Moody and Les 2003, Parks et al. 2016). Since it is possible that different genotypes occurring in the same lake could exhibit different herbicide responses, herbicide studies of specific lakes should incorporate genotyping to ensure that different genotypes are tested separately.

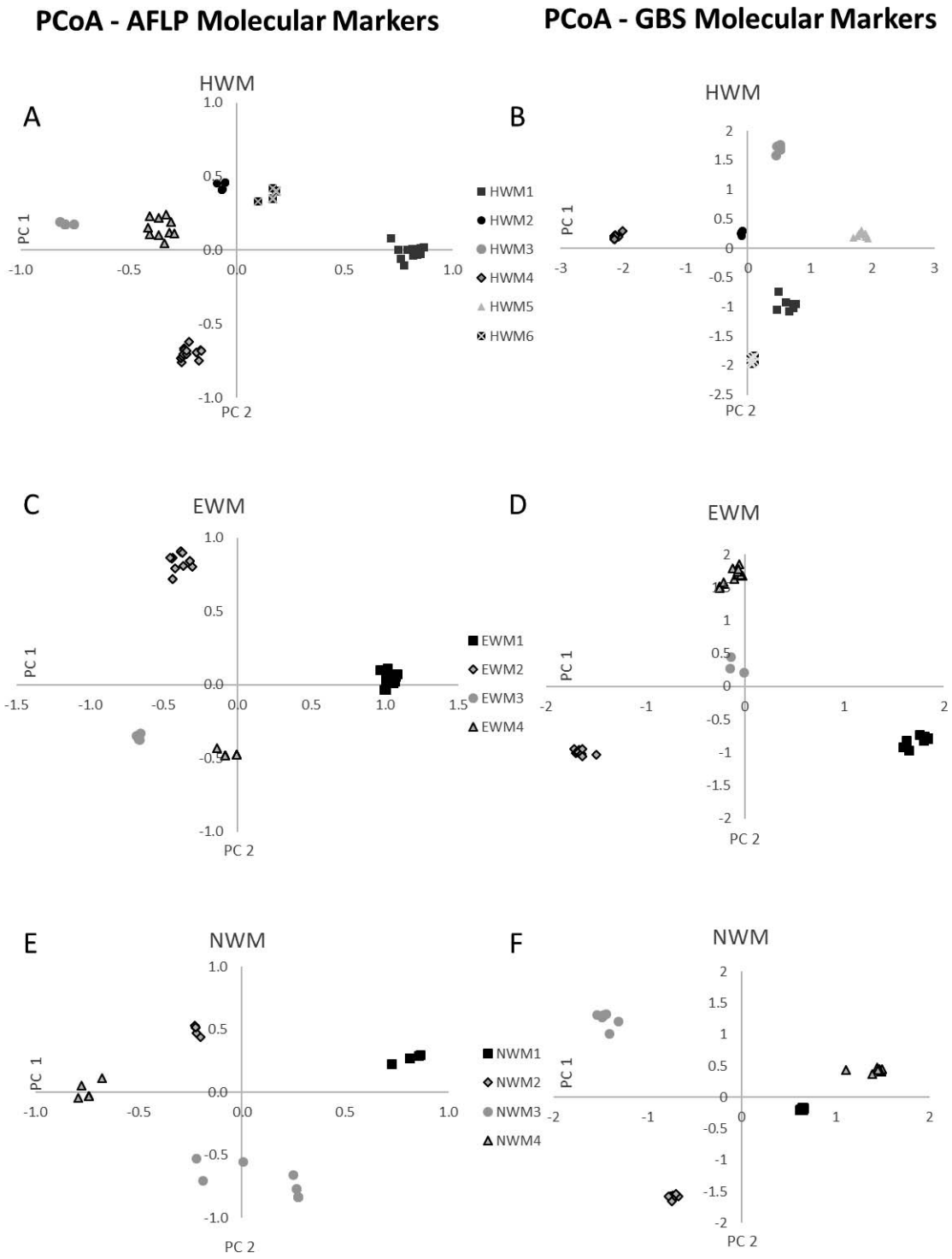

Figure 1. Principal coordinates analysis (PCoA) of amplified fragment length polymorphism (A, C, and E) and genotyping-by-sequencing (B, D, and F) molecular markers labeled by microsatellite multilocus genotype. PC 1 and PC 2 are the first two axes of the PCoA. In each panel, the first two PC axes combined explained the following percentages of the data: (A) 53.59%, (B) 47.80%, (C) 64.29%, (D) 70.74%, (E) 64.95%, and (F) 55.67%. Each symbol used corresponds to a microsatellite multilocus genotype (see Table 1), and each point on the graph represents an individual.

As we have shown here, the same genotype can also occur in multiple lakes (see Table 1). For example, we included a genotype in this study that was isolated from Townline Lake, MI and is known to exhibit fluridone resistance (see Berger et al. 2012, 2015, Thum et al. 2012) and diquat resistance (Netherland and Willey 2017). In an ongoing survey of genetic variation in Michigan, we identified this same MMG in several lakes (Thum, unpublished data), which raises concern that these lakes may also exhibit resistance to these herbicides. We therefore included individuals from

two other lakes found to have this MMG to test with additional markers (AFLPs and SNPs) whether these same lakes harbored the same genotype, as opposed to having the same MMG by chance. Indeed, AFLPs and SNPs identified individuals in different lakes with this same MMG as ramets of the same genet, indicating that plants in lakes with the same MMG share common ancestry through asexual (clonal) reproduction.

We recognize that individuals that share common ancestry through asexual reproduction may differ by somatic mutations that determine their response to treatment (e.g., Michel et al. 2004). Herbicide experiments are therefore the only way to be certain that individuals with the same molecular genotype, such as the Townline genotype, in fact exhibit the same level of resistance. Nevertheless, in the absence of herbicide information, it seems prudent for managers to assume that individuals that share ancestry via asexual reproduction will exhibit similar characteristics. Thus, our study illustrates the potential for molecular genotyping to identify genotypes that have been characterized previously, potentially eliminating the need for herbicide studies on each lake where a genotype is found, or at least providing important, interim information to managers unless and until a herbicide study proves otherwise.

Although this study provides evidence that microsatellite, AFLP, and SNPs can all distinguish ramets and genets, we believe that SNPs provide several advantages. In our study, the genotyping error rate for GBS was 0.7% (four of 542 SNPs differed among duplicate samples), whereas AFLP error rates were 5.6% (six of 108 markers). Although microsatellite error rates were negligible for our study because we chose individuals known to have the same MMG, we have estimated microsatellite error rates from autoscoring to be $\sim$ 15% (R. A. Thum, unpub. data). This difference in error rate can be attributed to SNPs being generated via DNA sequencing, whereas AFLPs and microsatellites are based on assays of DNA sequence via fragment analysis. AFLPs and microsatellites can therefore be more heavily affected by laboratory methods, instrumentation, and human judgement in comparison with DNA sequences. SNP data will therefore be more easily shared and integrated across laboratories.

A current challenge for using GBS methods in watermilfoil genotyping is the prohibitive cost and turnaround time. The development of a cheaper and faster SNP assay should therefore be a priority for genetic surveys and monitoring of watermilfoil. For example, SNP assays such as microhaplotyping (Kidd et al. 2013) offer promise to significantly reduce the cost per sample but retain the resolution and accuracy of SNP-based analyses (Kidd et al. 2013, Campbell et al. 2015).

SOURCES OF MATERIALS

[1]DNeasy Plant Mini Kit, Qiagen Corp., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

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