GENETIC ANALYSIS OF INVASIVE EURASIAN WATERMILFOIL IN IDAHO WATERS

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Background:

Eurasian watermilfoil is a high priority invasive aquatic plant species in Idaho, and identifying the best means to control its distribution, abundance, and spread are high priorities for many states across the northern tier of the United States.

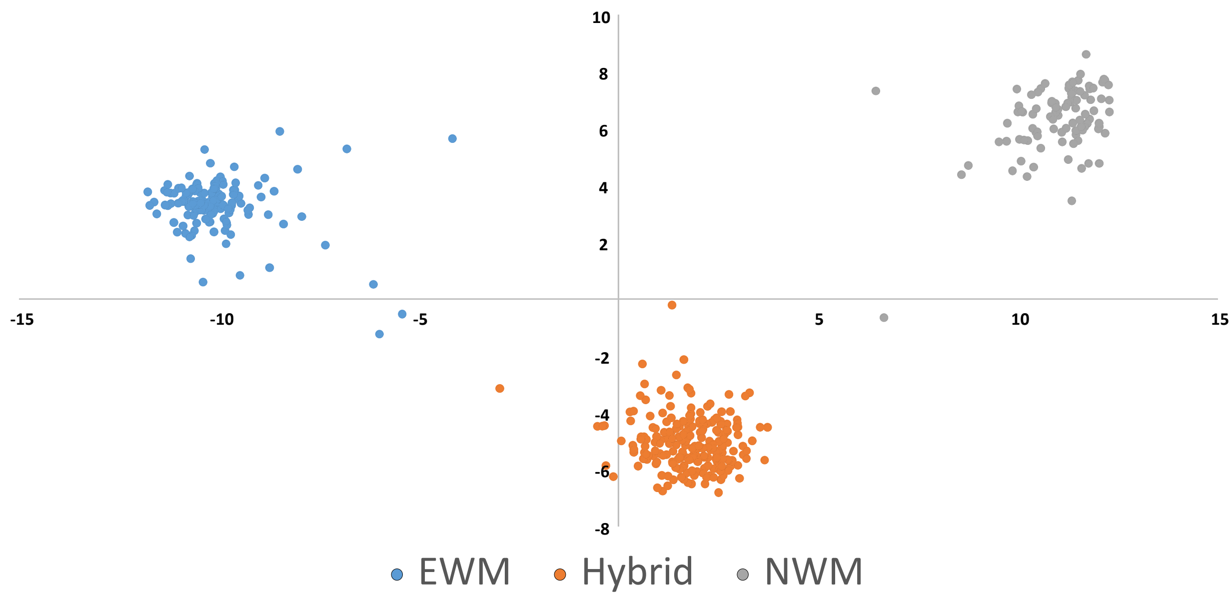
There are currently a number of well-established control methods for Eurasian watermilfoil, which typically rely heavily on a small number of herbicides. However, short and long term efficacy of specific control techniques can vary from population to population. While many site-specific physical and chemical conditions can contribute to this variation, it is becoming increasingly clear and accepted that not all "Eurasian watermilfoils" are the same. Some grow more aggressively than others, and some are more difficult to manage and control than others (e.g., Berger et al. 2012; Thum et al. 2012). For example, Eurasian watermilfoil in the broadest sense is composed of pure and hybrid forms, because Eurasian watermilfoil frequently hybridizes with native northern watermilfoil. It is clear that hybrid watermilfoils can be more invasive than pure Eurasian watermilfoil (LaRue et al 2013a; Parks et al. 2016). However, there is considerable genetic diversity among hybrid watermilfoils (Zuellig and Thum 2012), and distinct hybrid genotypes can exhibit distinct growth characteristics and herbicide responses (Glomski and Netherland; Thum et al 2012; Berger et al 2012; Taylor et al. submitted). While there is evidence that hybrid watermilfoils generally exhibit more "vigorous" vegetative growth compared to Eurasian watermilfoil, and numerous observations from experienced managers that hybrids frequently represent unique management challenges, there are varied opinions among aquatic plant managers about whether increased invasiveness in hybrids is the rule or the exception. In addition, at least two genetically distinct types of Eurasian watermilfoil occur in North America (Zuellig and Thum 2012), and nothing is currently known about whether these distinct types vary in their growth and response to specific control techniques.

Given the potential for genetic and ecological diversity within and among populations identified as "invasive Eurasian watermilfoil", an important first step in the development of a comprehensive management plan for a geographic area is to carefully describe the genetic diversity that is present within and among invaded water bodies. Such description can be useful when identifying potential factors influencing any variation in growth or management response among populations, or within the same population over time. In addition, genetic studies can help identify specific lineages for further, detailed, experimental study to compare and contrast patterns of growth and herbicide response. These studies can ultimately inform which herbicides to use or not use for specific populations.

Recently, Idaho has identified troublesome populations of invasive Eurasian watermilfoil. For example, nuisance watermilfoil in Hayden Lake has been difficult to control over the past several years with auxinic herbicides, whereas initial treatments with auxinic herbicides seemed very effective. Anecdotally, this shift in auxinic herbicide efficacy is associated with a shift in the composition of the plants from pure Eurasian to hybrid watermilfoil. In addition, the Coeur d'Alene Lake system has been managed in recent years for Eurasian watermilfoil, and hybrid watermilfoil is suspected to occur in this system, too. However, it is unclear whether the Eurasian and/or hybrid watermilfoil in the Coeur d'Alene Lake system is the same or different from that in Hayden Lake. Similarly, it is unknown how the watermilfoils in other nearby systems (e.g., Pend Oreille and upstream waterbodies on the Clark Fork River system, Cocolalla Lake, and Priest Lake) compare with the watermilfoils in Hayden Lake and Coeur d'Alene Lake. Finally, it is unclear whether the watermilfoils present in Idaho and nearby regions are distinct from other areas where Eurasian and hybrid watermilfoil are common and actively managed (e.g., the Great Lakes basin states: MI, WI, MN).

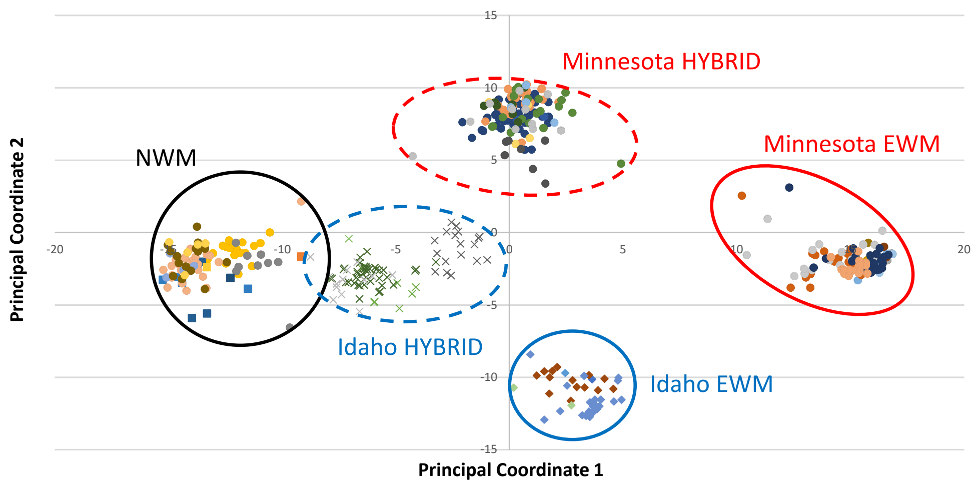
The primary objective of this project was to genetically characterize populations of watermilfoils in several key waterbodies in Idaho (Hayden Lake, Coeur d'Alene Lake, Pend Oreille, Cocolalla Lake, and Priest Lake) and an upstream waterbody on the Clark Fork River system (Noxon Reservoir). Specifically, we surveyed populations to determine whether pure Eurasian, native northern, and hybrid watermilfoils were present, and whether they were genetically similar or different among these waterbodies. To put the genetic diversity of these waterbodies into a broader perspective, we included watermilfoils sampled from other parts of the country, especially the Great Lakes basin where these taxa are also common. We used a combination of molecular markers – Amplified fragment length polymorphisms (AFLPs) and microsatellites. We collected AFLP information to 1) assign individuals to taxa (Eurasian – EWM; northern – NWM; hybrid – HWM), and 2) compare overall genetic relatedness to reference taxa from other parts of North America. We used microsatellite markers to unambiguously assign individuals to specific, discrete genotypes, and we consider individuals with the exact same microsatellite genotype to be the same genetic individual (i.e., a "clone"). We used this information to determine how much clonal diversity was present within and among lakes, and whether lakes shared genotypes.

Assigning individuals as Eurasian, northern, or hybrid watermilfoil – The first step in our genetic analysis was to assign each individual to a species (Eurasian vs northern) or hybrid (Eurasian x northern). The figure below shows the results of a Discriminant Analysis of Principal Components (DAPC) (Jombart 2008, Jombart and Ahmed 2011) using 216 amplified fragment length polymorphism markers (AFLPs; see Zuellig and Thum 2012 and LaRue et al. 2013) with the number of groups set to three. The DAPC analysis clearly distinguishes Eurasian (EWM; blue circles) from northern watermilfoil (NWM; gray circles) and hybrid watermilfoil (Hybrid; orange circles). We used this analysis to classify each plant as Eurasian (EWM), northern (NWM), or hybrid in the accompanying table below, which summarizes the taxonomic composition of each water body as estimated by samples for which we processed AFLPs



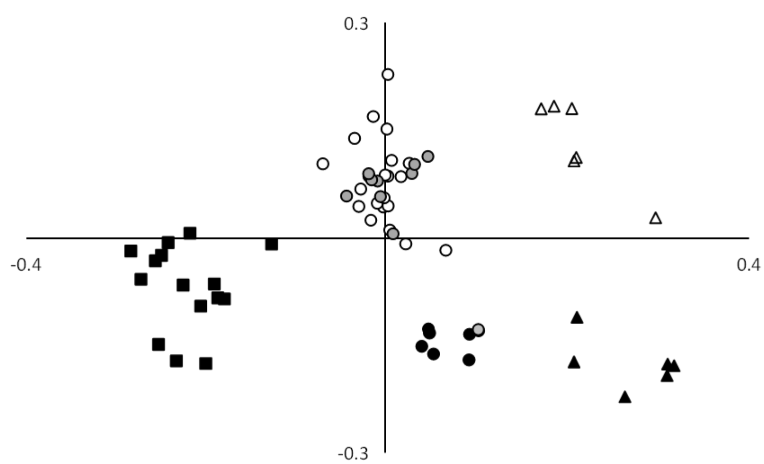
	EWM	NWM	HWM
Hayden			42
Coeur d'Alene	14		18
Pend Oreille	23	8	2
Noxon		1	13
Cocolalla	1	3	7
Priest	2	1	
Cabinet	1		

Genetic divergence between Idaho watermilfoil and other populations across the United States – While a comprehensive geographic analysis of Eurasian, northern, and hybrid watermilfoil is beyond the scope of this contract, we did perform some comparisons of Idaho populations of watermilfoil to populations from other parts of the country (with an emphasis on the Great Lakes basin, where watermilfoil commonly occurs and is commonly managed). Our first analysis used AFLPs. In addition, we conducted a second analysis using genotype information from eight microsatellite markers developed by Wu et al. (2013) (Myrsp1, Myrsp5, Myrsp9, Myrsp12, Myrsp13, Myrsp14, Myrsp15, and Myrsp16).



The accompanying figure shows the results of a Principal Coordinates Analysis (Peakall and Smouse, 2006 and 2012) of Minnesota and Idaho populations using AFLP data. These data clearly illustrate that EWM sampled from Idaho are distinct from those sampled from Minnesota. And, concomitantly, the hybrids sampled from Idaho are distinct from those sampled from Minnesota.

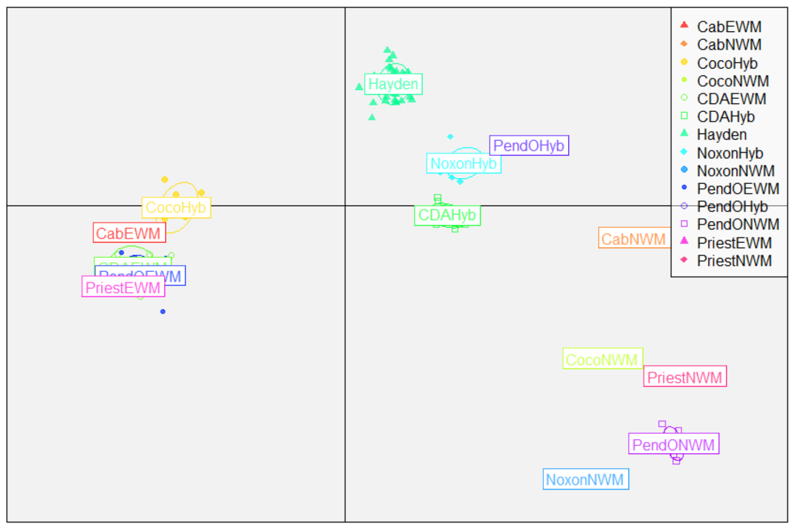
The accompanying figure shows a principal coordinates analysis of microsatellite genotypes for hybrid watermilfoils analyzed using the PolySat R package (Clark and Jasieniuk 2011). Comparison with microsatellite data from other Eurasian (triangles), northern (squares), and hybrid (circles) watermilfoils collected from several populations across the northern tier of the United States indicated two clearly distinct groups (biotypes) of Eurasian watermilfoil(open and closed triangles in accompanying figure), consistent with the two distinct groups of Eurasian watermilfoil identified in Zuellig and Thum (2012). Eurasian watermilfoil collected from Coeur d'Alene, Pend Oreille, Cocolalla, and Priest grouped out with the second group of Eurasian watermilfoil. Concordantly, we identified two distinct groups of hybrid watermilfoils, which we interpret as hybridization between northern watermilfoil and the two genetically distinct biotypes of Eurasian watermilfoil, as each hybrid group showed a clear affinity to one of two genetically distinct clusters of EWM. Hybrid genotypes from

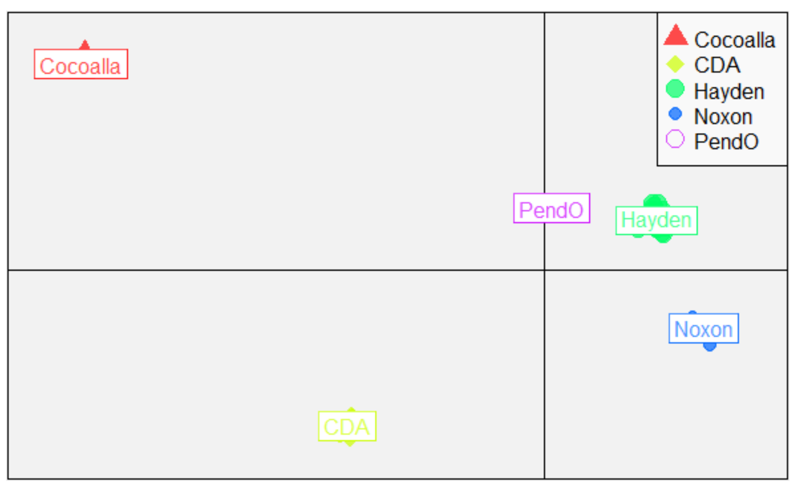


Coeur d'Alene grouped out with the first group of hybrids, whereas hybrid genotypes collected from Hayden, Cocolalla, Pend Oreille, and Noxon Reservoir grouped out with the second group. Northern watermilfoil genotypes are shown in closed squares. (Gray circles are specific genotypes for which we collected vegetative growth data in Taylor et al., submitted.)

While a thorough geographic analysis would be required to rule out the possibility that some or all of the Idaho hybrids have been introduced from elsewhere, these data suggest that Idaho hybrids result from independent hybridization event(s) that occurred in or near Idaho. As we are constantly adding samples to our database from different geographic regions, we will provide updates as to how Idaho hybrids relate to hybrids from other geographic regions upon request.

Genetic relationships among hybrids from different Idaho lakes – Idaho hybrids occurring in different water bodies were genetically distinct from one another, as illustrated by the accompanying figures, which represent DAPCs based on 216 AFLP markers (see earlier section on "Assigning individuals as Eurasian, northern, or hybrid watermilfoil"). The top panel of the accompanying figure shows all plants sampled in Idaho – broken out by both water body and taxon (EWM, NWM, Hyb). The bottom panel shows only hybrids (i.e., is "zoomed in" on the hybrid populations), to illustrate the relationships among populations based on AFLPs. In both cases, it is clear that the genetic composition of populations differ from one another, because similar populations would overlap on the figures.





The differences in genetic composition among hybrids collected from different lakes are also clear from the microsatellite data. We found different hybrid genotypes in each lake, and no two hybrid plants from different lakes had the same microsatellite genotype. (Note, however, that two Eurasian microsatellite genotypes were shared between Noxon Reservoir and CDA Lake.)

Below, we provide a detailed summary of the genetic diversity (based on microsatellites) for each lake.

Hayden Lake is dominated by hybrid watermilfoils, and we identified a single microsatellite genotype in the 48 samples for which we collected microsatellite data. This suggests that Hayden Lake is dominated by a single clone that has propagated vegetatively throughout the lake. We did not find this same microsatellite genotype in any of the other lakes.

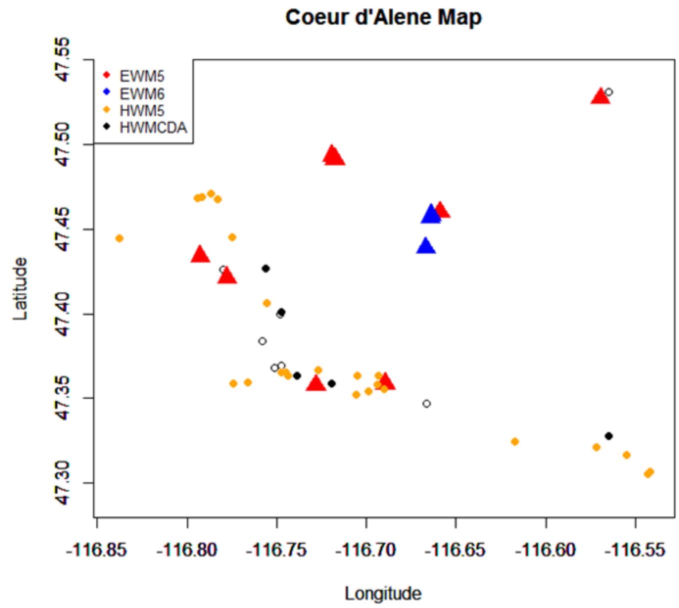
Hayden Lake may have undergone a large shift from dominance by pure Eurasian watermilfoil to dominance by hybrid watermilfoil over the past several years. Initial treatment with the auxinic herbicides 2,4-D and triclopyr were highly effective (Wersal et al. 2010), but treatment with auxinic and contact herebicides have had limited success in recent years. Although genetic methods were not used to characterize watermilfoil in Hayden Lake over time, anecdotally it appears that the initially successful herbicide treatments occurred when the population was dominated by pure Eurasian watermilfoils, and that the shift in efficacy corresponds to a shift in taxonomic composition from dominance by pure Eurasian to dominance by hybrid watermilfoils. Given that the Hayden Lake genotype exhibits unusually fast vegetative growth, and reduced response to auxinic herbicides, it seems likely that the specific hybrid genotype in Hayden Lake is dominant due to natural selection for vegetative growth rate and/or tolerance to auxinic herbicide.

One of the main interests in the genetic relatedness among Idaho hybrids was to determine whether the same genotype present in Hayden Lake occurs in other Idaho lakes since knowledge of herbicide response by genotypes in one waterbody would presumably translate to knowledge of those same genotypes occurring in a separate waterbody. Based on our genetic analysis, it does not appear that the same genotype found in Hayden Lake is present in other lakes, and efforts should be made to both prevent the spread of the Hayden Lake genotype to other waterbodies and routinely screen other waterbodies for the presence of “Hayden Lake genotype”. **However, it is important to note that the lack of the specific Hayden Lake hybrid genotype in other water bodies does not mean that other hybrid genotypes will not possess the same or similar characteristics of fast vegetative growth and auxinic herbicide tolerance, as different clones may still share the relevant genes for these traits.**

Control of hybrid watermilfoil in Hayden Lake remains a significant management challenge. Although the lake is currently dominated by a single genotype, we recommend continued genetic monitoring of Hayden Lake, as it is possible the genetic composition could rapidly change over time. For example, we do not know whether Hayden Lake has a diverse seed bank that could serve as a source for recruitment of new genotypes that may have different growth and herbicide response characteristics. We recommend field and laboratory trials with different control techniques that use the genotype as the experimental unit of interest. For example, herbicide screens should be conducted on specific genotypes (e.g., any newly-identified genotypes resulting from continued genetic monitoring).

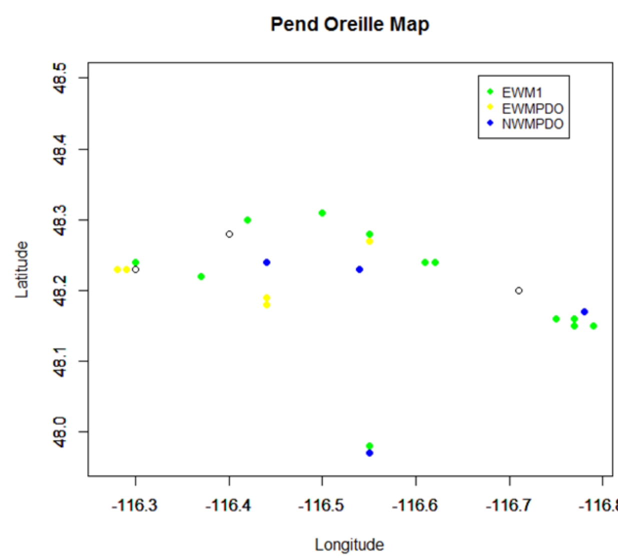
Coeur d’Alene Lake contains a mixture of pure and hybrid Eurasian watermilfoil. We found two distinct microsatellite genotypes in our 11 Eurasian watermilfoil samples (73% and 23% relative frequency, respectively; EWM5 and EWM6 in the accompanying figure). These two Eurasian watermilfoil microsatellite genotypes were also found in Noxon Reservoir. We found five unique microsatellite genotypes in our 29 hybrid watermilfoil samples. However, the hybrid watermilfoil samples were dominated by one microsatellite genotype (83% of individuals were one genotype). The other four hybrid microsatellite genotypes were found only once or twice (shown as black circles in the accompanying figure).

Interestingly, the distribution of pure versus hybrid Eurasian watermilfoil is structured across this large lake system. Specifically, samples of watermilfoils were dominated by hybrid watermilfoils in areas that have been actively managed over the past several years (Round Lake, Chatcolet Lake, and South CDA Lake). The specific underlying cause for the association between hybrid watermilfoil occurrence and management is not clear, but could be related to more vigorous growth and/or lower control efficacy. Evaluations of treatment efficacy in Coeur d'Alene Lake should aim to quantitatively determine whether efficacy is the same or different on pure versus hybrid Eurasian watermilfoil, and the system should be closely monitored to determine whether rates of growth and spread differ between pure and hybrid watermilfoil. In addition, laboratory herbicide studies should be conducted to compare pure versus hybrid watermilfoils from Coeur d'Alene Lake, and inform the development and implementation of specific control techniques.



Since CDA Lake is dominated by one microsatellite genotype, most of the treated areas contained only this hybrid genotype. However, we did find four other microsatellite genotypes. It is unclear whether these distinct hybrid genotypes differ in their growth and/or herbicide response characteristics. Continued genetic monitoring, combined with laboratory growth and herbicide studies, could be useful for determining whether specific genotypes have a competitive advantage related to growth or control techniques.

Pend Oreille appears to be dominated by pure Eurasian watermilfoil, with hybrid watermilfoil comparatively rare. We found four microsatellite genotypes in our 22 Eurasian watermilfoil samples, but one genotype was dominant (77% relative frequency; EWM1 in accompanying figure). The remaining EWM genotypes were only found once each (i.e., each yellow circle in the accompanying figure is a different genotype). None of these Eurasian watermilfoil genotypes were shared with any other lakes. We only processed microsatellite data for one hybrid individual, and its genotype was also not shared with any other lakes. Finally, each of the six northern watermilfoils



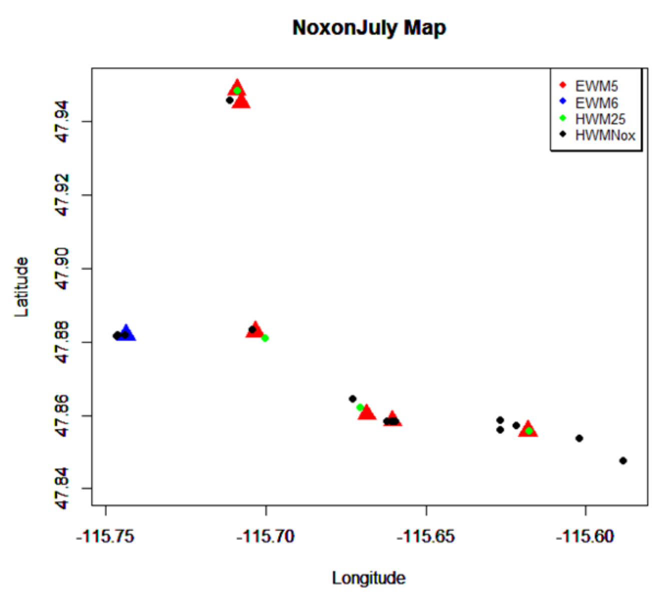
that we collected microsatellite data for were distinct genotypes that were not shared with any other water bodies (each blue circle in the accompanying figure represents a unique northern watermilfoil genotype).

Since hybrid watermilfoil is present, it isn't clear why it is not more common, like it is in Hayden and Coeur d'Alene Lakes. It is possible that the hybrid genotypes present in Pend Oreille do not grow as vigorously and/or the plants are more susceptible to specific control techniques that have been implemented in Pend Oreille. Alternatively, it is possible that there has not been much hybridization, or that hybrid watermilfoils have not had as much time to spread around the system as in the other lakes where they are common. These alternative hypotheses could be tested with laboratory studies. Nevertheless, the distribution and abundance of pure versus hybrid watermilfoil in Pend Oreille should be carefully monitored.

We did not have an extensive sample from **Cocolalla Lake**, but our sample was dominated by hybrid watermilfoils. We found two unique hybrid watermilfoil genotypes in the nine hybrid samples we collected microsatellite data for, and neither of these were shared with any other lake. We were unable to get microsatellite data from the single Eurasian watermilfoil sample from Cocolalla Lake. I am unaware of the management history of Cocolalla Lake, and it therefore isn't clear whether there is any relationship between watermilfoil composition and management history. As with the other lakes, careful genetic monitoring of watermilfoils in Cocolalla Lake, with an emphasis on quantitative evaluation of any control efficacy, is warranted.

Our two samples from **Priest Lake** identified only pure Eurasian watermilfoil, and we did not collect microsatellite data for these samples. Since both Eurasian and northern watermilfoil are found in Priest Lake, there is the potential for hybridization. As such, the watermilfoil population in this lake should be carefully monitored.

Finally, we found both pure and hybrid watermilfoil in **Noxon Reservoir**. We found five microsatellite genotypes among nine Eurasian watermilfoil samples. Two of these Eurasian genotypes were shared with CDA Lake (EWM5 and EWM6 in the accompanying figure), which suggests movement of Eurasian watermilfoil among these two lakes. The mechanism of movement – human versus natural – is unclear. We found 22 distinct hybrid genotypes among our 28 hybrid samples for which we collected microsatellite data. Only one of these hybrid genotypes was found multiple times (HWM25), whereas the rest were found only once each (i.e., each black circle in the accompanying figure is a unique genotype). Noxon Reservoir is therefore by far the most genetically diverse population of watermilfoil in this study. It is unclear why Noxon Reservoir hybrids are so much more genetically diverse than other lakes, but may be due to their recent



formation in the lake. Hybrids were not found in a 2008 survey of Noxon Reservoir, and the Reservoir was first managed in 2009. Therefore, it seems likely that hybrids have formed in the lake in the past several years. It is possible that this extensive genetic diversity is coincident with ecological diversity in growth and herbicide response. The diversity in Noxon Reservoir is of interest and potential importance, as it is located upstream of Idaho waterbodies (e.g., Noxon shares Eurasian watermilfoil genotypes with CDA Lake). As with all water bodies, we recommend continued genetic monitoring and quantitative studies of growth and herbicide control efficacy. My laboratory is currently collaborating with the Sanders County Invasive Aquatic Plant Task Force, and Montana's Department of Natural Resources and Conservation, to quantitatively evaluate the growth, spread, and herbicide efficacy of pure versus hybrid watermilfoils in this waterbody.

Conclusions and Recommendations

The genetic analysis had the following key results –

1. "Eurasian watermilfoil" in Idaho waterbodies currently consist of a mixture of pure and hybrid Eurasian watermilfoil genotypes.
2. Two different biotypes of pure Eurasian watermilfoil can be distinguished from one another genetically, and both kinds of Eurasian watermilfoil occur in Idaho.
3. Hybrid watermilfoils are genetically diverse. I.e., there is not one widespread hybrid watermilfoil present in Idaho. Rather, it is most likely that Eurasian and northern watermilfoil have independently hybridized on numerous occasions in numerous locations to create a large amount of genetic diversity.
4. However, hybrid watermilfoils from different lakes are genetically distinct from one another. (The only microsatellite genotypes that were shared among lakes were two Eurasian watermilfoil genotypes in both Noxon Reservoir and CDA Lake.)
5. Lakes with hybrid watermilfoil were generally dominated by a widespread microsatellite genotype ("clone"), with the exception of Noxon Reservoir, which had a highly diverse set of hybrid watermilfoil genotypes.

Taken together, these results clearly illustrate a high genetic diversity among watermilfoils in Idaho. We found both pure and hybrid Eurasian watermilfoil in Idaho waterbodies, and the proportions of the two vary among lakes. For example, Hayden Lake samples were dominated by hybrid watermilfoil, while Pend Oreille samples were dominated by pure Eurasian watermilfoil, and Coeur d'Alene Lake and Noxon Reservoir samples were roughly an even mixture of pure and hybrid Eurasian watermilfoil. It is unclear whether these proportions remain stable over time, or whether there are predictable factors that govern the dynamics of these proportions over time. Previous research comparing pure and hybrid Eurasian watermilfoil suggests that hybrids are commonly more vigorous and invasive (LaRue et al. 2013). Thus, it is possible that populations may predictably come to be dominated by more vigorous and invasive hybrid genotypes over time. In fact, we have recently documented a relative increase in hybrid watermilfoil genotypes compared to pure Eurasian watermilfoil genotypes following treatment with auxinic herbicides (2,4-D and triclopyr) in a northern Michigan Lake (Parks et al. 2016). It isn't clear if current management approaches may speed up the rate at which more invasive hybrid genotypes displace pure Eurasian and/or native watermilfoils. **As such, the proportion of pure versus hybrid Eurasian watermilfoil in different Idaho waterbodies should be carefully monitored over time –**

especially in relation to management activities – and complemented with experimental laboratory studies of growth patterns and herbicide response.

That hybrid genotypes are different among lakes suggests that hybridization occurs locally and frequently. However, it is unclear why some lakes contain numerous hybrid genotypes (e.g., Noxon Reservoir) whereas other lakes are clearly dominated by a single genotype (e.g., Hayden Lake). We hypothesize that widespread hybridization among invading Eurasian watermilfoil and resident northern watermilfoil leads to an initially genetically diverse hybrid population, but that subsequent selection for important traits such as vegetative growth rate and/or herbicide response reduces overall diversity. In extreme cases, a single, highly-fit and invasive genotype may be all that remains after strong selection. Such a model may explain why Hayden Lake is dominated by a single microsatellite genotype that exhibits unusually high vegetative growth rate and tolerance to auxinic herbicides. However, genetic diversity may also be low in a given lake as a result of genetic drift resulting from demographic bottlenecks associated with active management and control programs and/or colonization of a water body by one or a small number of genotypes.

In addition, we found a variety of different genotypes of pure and hybrid watermilfoil. Two different pure Eurasian watermilfoil types can be distinguished genetically, and we found evidence that both types of Eurasian watermilfoil have hybridized with northern watermilfoil in Idaho. It is clear that the hybridization event forming hybrids in Coeur d'Alene Lake is distinct from the event(s) forming them in other lakes, since hybrids from Coeur d'Alene are clearly in a genetically distinct group from hybrids in the other lakes. However, because the specific hybrid genotypes present in each lake are distinct from hybrid genotypes in other lakes, it seems most likely that hybridization has occurred independently in each waterbody and/or that hybrid genotypes from different sources have been introduced to different waterbodies. It is generally known that different hybrid genotypes can exhibit different patterns of growth and herbicide response (Thum et al. 2012; Berger et al. 2012; Taylor et al., submitted). Therefore, even within lakes dominated by hybrids, there is the potential for changes in the composition of genotypes that are generated through sexual reproduction of hybrids and/or introduction from other waterbodies. It is unclear whether the specific distinct genotypes found within and among Idaho lakes will exhibit similar or different patterns of growth, spread, and herbicide response, and genetic analysis should be explicitly integrated into quantitative monitoring efforts, such as quantitative efficacy evaluations following specific control techniques. Laboratory study of different genotypes to compare their vegetative growth and responses to a variety of potential herbicide control techniques would also shed important insight into whether different management strategies should be employed on genetically distinct populations.

So, what does all of this mean for Eurasian watermilfoil management? Most Eurasian watermilfoil projects will require multiple years of control and management to accomplish long-term management goals of either eradicating or reducing populations to acceptable levels. Even when acceptable levels are reached, some level of maintenance management is likely. Given that there is considerable genetic diversity of pure and hybrid Eurasian watermilfoils, and the potential for the genetic composition of populations to change over time, we recommend the following procedures for adaptively managing watermilfoil populations:

1) A detailed genetic survey should be conducted for each water body to identify how many, and which, genotypes are present in the water body. The genotypes can be mapped in relation to any

available treatment history data to develop initial hypotheses regarding which genotype(s) may be the most worrisome. For example, the current study shows a clear association between a specific hybrid genotype and treatment history in CDA Lake. Such genotypes can be specifically targeted for growth and herbicide response studies.

2) Whenever possible, conduct focused growth and herbicide response studies to identify whether genotypes present are unusually tolerant to proposed herbicide(s). Current laboratory studies are logistically limited to studying a small number of genotypes and herbicide treatments. However, continued development of methods by several labs should increase the scale of these experiments in the future.

3) Quantitative monitoring of operational control measures. The scale at which field evaluations can be conducted will vary from project to project, so field sampling must be tailored to the specific project needs and budget. However, some level of field monitoring of efficacy and regrowth/re-establishment of plants in treated areas is a critical part of an adaptive management program, because herbicide effects and regrowth may occur at time scales that are not easily captured in small-scale laboratory studies.

4) Genetic monitoring pre- and post-treatment to determine whether genotypes are changing in relative abundance. Changes in relative abundance may signal dynamic changes in genetic composition that are associated with selection for faster growth and spread ("weediness") and/or more tolerant genotypes (i.e., herbicide resistance evolution).

5) Repeat steps 1-4 as necessary. Preferably, this would be done in detail continuously until the management goals were achieved, and then done at a "maintenance" level to stay out ahead of any potential changes that may lead to a resurgence of watermilfoil to nuisance levels.

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