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Inter- and intraspecific hybridization affects germination and vegetative growth in Eurasian watermilfoil

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ABSTRACT

We used artificial crosses to compare the development and vegetative growth of hybrid versus parental crosses of northern watermilfoil (*Myriophyllum sibiricum* Komarov) and two genetically distinct biotypes of Eurasian watermilfoil (*Myriophyllum spicatum* L). These crosses simulated the different opportunities for sexual reproduction encountered by a newly introduced Eurasian watermilfoil population: 1) habitats where native northern watermilfoil is present, 2) habitats where a genetically distinct biotype of Eurasian watermilfoil is present, and 3) habitats where only closely related individuals of the same biotype are present. In two separate experiments using different parental Eurasian and northern genotypes, we found a clear trend of interspecific (Eurasian × northern) and intraspecific (hybridization between two Eurasian biotypes) hybrid vigor for vegetative growth traits, and germination percentages and rates were generally higher for hybrid compared with parental crosses. Although variation in watermilfoil growth is undoubtedly influenced by numerous environmental and genetic factors, our results suggest that differences in the opportunities for hybridization with either northern watermilfoil or other Eurasian watermilfoil biotypes may underlie some of the variation in vegetative growth observed among populations identified as invasive Eurasian watermilfoil; specifically, that first-generation hybrids are likely to have higher vegetative growth than parental genotypes. Therefore, aquatic plant scientists and managers should consider the potential for genetic composition and dynamics to affect the potential for establishment, spread, impact, and control when designing and assessing Eurasian watermilfoil management plans.

Key words: heterosis, *Myriophyllum sibiricum*, *Myriophyllum spicatum*, northern watermilfoil.

INTRODUCTION

Populations of an invasive species can differ in important properties such as dispersal ability, potential for establishment, rate of proliferation, and impacts on invaded systems (Reichard et al. 2015). Differences in environmental factors may explain part of this variation, but genetic and phenotypic variation may also contribute. For invaders capable of sexual reproduction, one specific factor that may contribute to variation among populations is the relative opportunities for sexual reproduction with genetically similar versus distinct individuals. For example, numerous examples of inter- and intraspecific hybridization preceding the evolution of invasiveness have now been documented (Ellstrand and Schierenbeck 2000; Schierenbeck and Ellstrand 2009), suggesting that hybridization frequently leads to increased fitness relative to pure or inbred parental lines. Therefore, different opportunities for invasive species to hybridize with native species (interspecific hybridization) and/or genetically distinct lineages (biotypes) that were historically isolated from one another (intraspecific hybridization) may provide fitness boosts that lead to variation in invasiveness between hybridized versus nonhybridized lineages.

It is increasingly clear that the widely managed invasive Eurasian watermilfoil (*Myriophyllum spicatum* L. *sensu lato*) is genetically diverse, and that populations and genotypes can differ in their vegetative growth, potential for spread, and response to management (Glomski and Netherland 2010, Berger et al. 2012, Thum et al. 2012, LaRue et al. 2013a, Berger et al. 2015, Netherland and Willey 2017, Taylor et al. 2017). Although Eurasian watermilfoil reproduces extensively through vegetative propagation (stolons and fragmentation), it also flowers prolifically and can produce viable seed (Aiken et al. 1979, Madsen and Smith 1997). That stigmas ripen in advance of stamens has been suggested to favor outcrossing (Aiken et al. 1979), but the actual degree of selfing versus outcrossing is unknown. Since many flowering spikes are often produced by the same individual plant, selfing is possible despite the difference in ripening of stamens and stigmas. In our lab, we have successfully selfed individuals, but we have not quantitatively compared selfing versus outcrossing rates or success. However, genetic data reveal a surprising amount of variation that suggests that sexual reproduction is more common than previously thought for a plant taxon with such extensive vegetative reproductive capacity (Zuellig and Thum 2012, LaRue et al. 2013a,b). Specifically, genetic analyses indicate that extensive and frequent hybridization has occurred between introduced Eurasian watermilfoil and its native sister species, northern watermilfoil (*Myriophyllum sibiricum* Komarov; Moody and Les 2002, Moody and Les 2007, Sturtevant et al. 2009, Zuellig and Thum 2012, LaRue et al. 2013a). Additionally, molecular markers have revealed

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two genetically distinct biotypes of Eurasian watermilfoil in North America (denoted EWM1 and EWM2; Zuellig and Thum 2012). It is unclear whether these exhibit morphological or phenotypic differences, but they do possibly represent two distinct introductions. Thus, it stands to reason that different populations of introduced Eurasian watermilfoil have different opportunities for sexual reproduction with genetically similar versus distinct individuals; in some populations, only one biotype with limited genetic diversity may be present, whereas other populations may contain native northern watermilfoil and/or a genetically distinct Eurasian watermilfoil biotype.

Evidence from two previous studies suggests that hybridization may lead to an increase in vegetative growth for Eurasian watermilfoil, which would likely influence the potential for establishment, spread, and impacts. LaRue et al. (2013a) found that hybrid watermilfoils collected from natural populations grew faster on average than pure Eurasian watermilfoil. Taylor et al. (2017) found significant variation in vegetative growth rate among 10 distinct hybrid genotypes; though there were exceptions, hybrid genotypes tended to have faster vegetative growth rates compared with reference Eurasian watermilfoil. These studies corroborate anecdotal reports by aquatic plant managers that hybrid watermilfoils are more invasive and difficult to control than parental Eurasian watermilfoil. However, it is unclear whether hybridization leads to an immediate boost in fitness via hybrid vigor that is shared by distinct parental crosses, or whether invasive hybrid genotypes found in natural populations represent genetic combinations that have been selected for from a diverse array of genotypes exhibiting wide variation in fitness (see Hovick and Whitney 2014). Furthermore, the potential for intraspecific hybridization among distinct Eurasian watermilfoil biotypes is unknown, including whether intraspecific hybrids will exhibit greater invasiveness relative to plants from within-biotype crosses.

In this study, we use artificial crosses to test whether interspecific (Eurasian × northern) and intraspecific (distinct Eurasian biotypes) hybridizations produce progeny with higher vegetative growth rates than progeny from within-biotype crosses of Eurasian and northern watermilfoil.

MATERIALS AND METHODS

Our study was designed to simulate the different opportunities for sexual reproduction encountered by a newly introduced Eurasian watermilfoil population: 1) habitats where native northern watermilfoil is present, 2) habitats where a genetically distinct biotype of Eurasian watermilfoil is present, and 3) habitats where only closely related individuals of the same biotype are present (i.e., neither northern watermilfoil nor genetically distinct biotypes of Eurasian watermilfoil are present).

Table 1. Descriptive statistics for the two experiments. Locations of the parental populations are available from the authors upon request.

Cross Type[1]	Parental Populations	Seeds	Plants Measured	Experiment
EWM1 × NWM	Sawyer × Robinson	35	35	1
EWM2 × NWM	Spring × Hanbury	30	26	2
EWM1 × EWM2	Sawyer × Spring	35	33	1
EWM1 × EWM2	Wolf × Spring	24	24	2
NWM × NWM	Robinson × Robinson	35	11	1
NWM × NWM	Rose × Rose	31	12	2
EWM1 × EWM1	Sawyer × Sawyer	35	24	1
EWM1 × EWM1	Wolf × Wolf	29	25	2

[1] EWM1 and EWM2 refer to the two distinct biotypes of Eurasian watermilfoil. NWM refers to northern watermilfoil.

We performed five types of crosses using two genetically distinct biotypes of Eurasian watermilfoil (EWM1 and EWM2) and northern watermilfoil (NWM) plants from source populations in six different lakes (Table 1). Two cross types simulate sexual reproduction among closely related genotypes of the same species and biotype (NWM × NWM; EWM1 × EWM1). Two other cross types simulate sexual reproduction between introduced Eurasian watermilfoil and native northern watermilfoil (EWM1 × NWM; EWM2 × NWM). The final cross type simulates sexual reproduction between the two distinct Eurasian biotypes (EWM1 × EWM2). We repeated these cross types for two different sets of populations. In total, this included northern watermilfoil from three different lakes (in Experiment 2, we used a different parental NWM population in the interspecific versus within-population crosses, because of limited flowering by NWM at the time the crosses were performed), EWM1 from two different lakes, and EWM2 from one lake (see Table 1).

We chose the six lakes from which we collected parental watermilfoils on the basis of information from previous genetic analyses and logistical convenience. We collected plants from scattered locations throughout each lake using a rake. We verified the taxonomic identify of each population by conducting genetic analyses of 5 to 10 stems from each collection using an internal transcribed spacer (ITS) restriction analysis (Thum et al. 2006, Grafé et al. 2015). Similarly, we confirmed the taxonomic identities of approximately three to five offspring from the crosses.

We established vegetative cultures of each of the parental populations in 568-L tanks located outdoors (water depth ~75 cm) and in 1,136-L (water depth ~90 cm) tanks located indoors at the Robert B. Annis Water Resources Center in Muskegon, MI. Briefly, approximately 30 plants from the collections were planted into 19-L containers within the larger indoor and outdoor tanks. Containers were filled with potting soil supplemented with 2.2 mL kg^{-1} Osmocote[1] (19–6–12 N–P–K). Tanks were filled with water from Muskegon Lake. Indoor tanks were lit with full-spectrum sodium lamps (Sylvania M1000/U M47/S Metalarc) on a 16 : 8 h light : dark cycle. These cultures were maintained and monitored for the production of flowers.

All crosses were performed by hand pollination. Pollen from the target male was thoroughly dusted onto the target female by gently rubbing dehiscent anthers against the pistils when the female flowers opened and were receptive to pollen. We ensured that female flowers wouldn't self-pollinate by removing the male flowers (which occur above female flowers on the inflorescence) before the female flowers opened. In addition, we ensured that female flowers wouldn't be pollinated by any male flowers in their vicinity by transferring them into the laboratory and placing each in an individual Erlenmeyer flask shortly before opening. All crosses used between two and four pollinated females, and crosses were performed in both directions (reciprocal crosses) for each cross type.

After hand pollinating, each female flower was placed in an individual container filled with Muskegon Lake water and monitored for fruit development. Fruits were allowed to mature on the stem until they were loose enough to be gently removed. Fruits were then stored on a damp paper towel in a plastic bag at 4 C for 6 mo to 1 yr. Within each cross type, the seeds from reciprocal crosses were combined, so that the direction of the cross was a random effect in our germination study.

All seeds were surface sterilized before using them in the germination and growth experiments. Batches of 25 seeds from each cross were sterilized with a solution of 3% bleach and 0.01% Tween for 20 to 25 min, vortexing occasionally, and then rinsed with sterile distilled water for several minutes.

Each seed was placed in an individual cone-tainer[2] containing potting soil capped with sand, with the seed resting on top of the sand so it would remain visible for monitoring. The cone-tainers were randomly distributed in a 1,136-L indoor tank filled with filtered Muskegon Lake water (water depth ~90 cm) and lit with a full-spectrum sodium lamp (Sylvania M1000/U M47/S Metalarc) on a 16 : 8 h light : dark cycle with water temperature ranging between 21 and 24 C throughout all studies. Seeds were monitored every day to record the date at which different life stages occurred: germination, cotyledons, first pair of true leaves, first occurrence of branching, and number of days for the plant to reach the water surface of the tank. Monitoring occurred for 5 wk, after which we measured the number and total length of all branches and the wet mass of shoots and roots (after blotting dry with a paper towel).

The traits we measured were intended to capture features that reflect the perception of "nuisance growth" by lake residents and managers (Tavalire et al. 2012). They include the temporal pattern of seed germination, total plant length, wet mass, and number of branches. The temporal pattern of seed germination (germination times and percentages) is important in determining the likelihood of successful sexual reproduction, which may be important in the colonization of new habitats or the re-establishment of watermilfoil populations after removal through management techniques such as herbicides. Sexual reproduction is also important in generating genetic variation through recombination and hybridization. Plant length is a relevant trait because plants that grow longer reach the surface sooner and are visibly recognizable as nuisance plants that impede navigation, swimming, and aesthetics. Wet mass is a useful measure of overall plant size. Although dry mass is a better measure of overall plant growth, we have found that wet mass is highly correlated (E. A. LaRue and R. A. Thum, unpub. data), and does not require destructive sampling. Because we used the plants generated in this study for additional breeding experiments, we preferred wet mass over dry mass. Finally, the number of branches is important because plants with more branches may have higher potential for spread via asexual fragmentation and are more likely to form nuisance mats at the water's surface.

Data from Experiments 1 and 2 were analyzed separately. Patterns of germination time were compared statistically using Kaplan–Meier "survival" curves, which are nonparametric estimates of the probability $S(t)$ that a seed has not yet germinated, as a function of incubation time t. Patterns of germination time for seeds from different crosses were compared statistically using pairwise nonparametric log-rank tests (McNair et al. 2012), adjusting the P values with Holm's correction to control experiment-wise error. For visual comparisons, we plotted germination curves, which are the complements $1 - S(t)$ of the Kaplan–Meier survival curves. Growth data initially were analyzed using one-way ANOVA with cross as a fixed factor, but clear violations of the assumption of Gaussian residuals could not be resolved. We therefore compared growth properties of progeny from each pair of crosses using a bootstrap test based on the two-sample t statistic for potentially unequal samples sizes and variances (Davison and Hinkley 1997), again adjusting the P values with Holm's correction. All statistical analyses were conducted in R (R Development Core Team 2014).

RESULTS AND DISCUSSION

Pairwise log-rank tests based on the Kaplan–Meier survival curves showed that in most cases there were statistically significant differences between the germination patterns of seeds from hybrid crosses (parents from different species or biotypes) and seeds from within-biotype crosses. Examination of the germination curves (Figure 1) suggests that these differences were due to a variable combination of higher germination percentage and shorter germination time in hybrid crosses, though these properties also differed for seeds from different within-biotype crosses. We do not know the underlying physiological causes of the observed differences in germination patterns (e.g., different germination requirements, differences in seed abortion rates, etc.). However, all else being equal, the differences in germination could possibly translate into higher potential for colonization of new habitats, or recolonization of managed habitats, via recruitment from a seed bank for inter- and intraspecific hybrid crosses compared with situations where the seedbank consists only of propagules produced from genetically similar individuals.

We observed a general pattern of hybrid crosses having greater vegetative growth traits compared with the parental crosses, with some differences in statistical significance across traits and experiments. Offspring from both interspecific (EWM1 × NWM) and intraspecific (EWM1 × EWM2) hybrid crosses had significantly greater mean total lengths than offspring from parental crosses, but the mean total lengths of offspring from interspecific crosses were significantly greater than the mean total lengths of offspring from intraspecific crosses (Figure 2). Similarly, mean wet masses of offspring from both interspecific (EWM1 × NWM) and intraspecific (EWM1 × EWM2) hybrid crosses were significantly greater than mean wet masses of offspring from parental crosses, and offspring from interspecific crosses

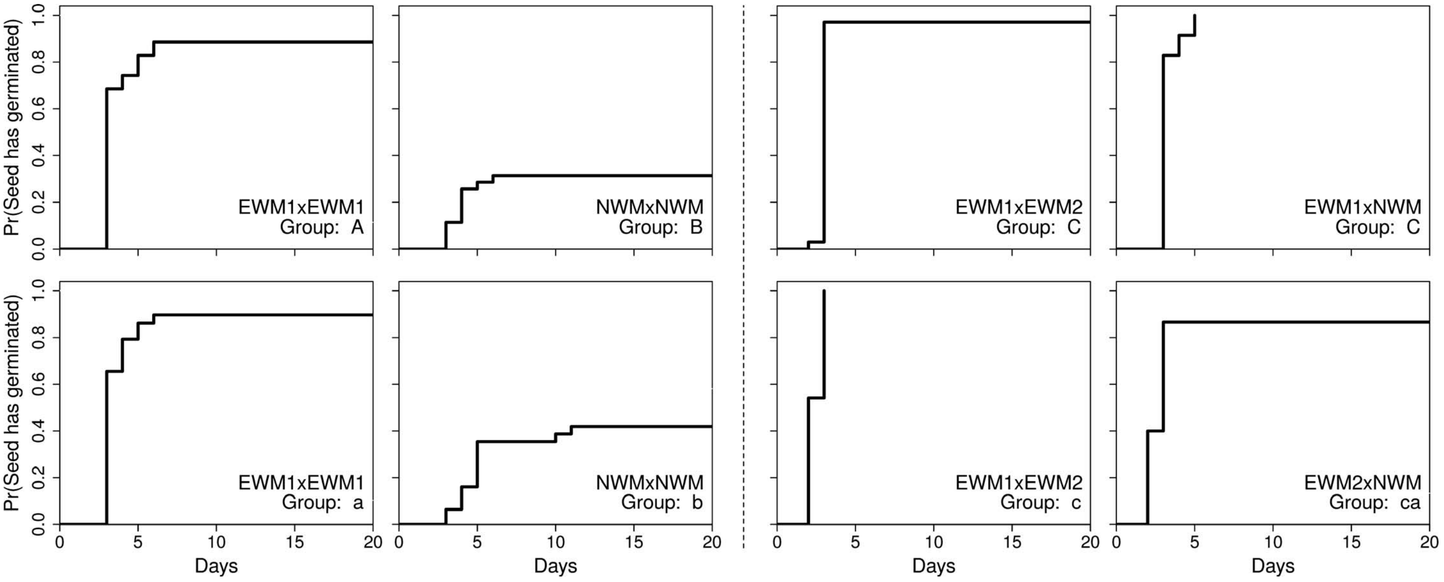

Figure 1. Germination curves for Experiments 1 (top row) and 2 (bottom row). Each curve shows the estimated probability that a seed will germinate by the corresponding number of days on the horizontal axis. Groups of significantly different germination curves were determined separately for Experiments 1 and 2 using pairwise log-rank tests with Holm-corrected *P* values. These groups are shown in the lower right corners of the panels (capital letters for Experiment 1, lowercase for Experiment 2). The vertical dashed line separates results for seeds from within-species/biotype crosses (left) and results for hybrid seeds from between-species/biotype crosses (right). NWM = northern watermilfoil; EWM = Eurasian watermilfoil.

had significantly greater mean wet masses than offspring from intraspecific crosses, although this difference was only significant for Experiment 2 (Figure 3). Finally, the mean numbers of branches of offspring from both interspecific (EWM1 × NWM) and intraspecific (EWM1 × EWM2) hybrid crosses were significantly greater than for offspring from parental crosses, and the means for offspring from interspecific crosses were significantly greater than for offspring from intraspecific crosses (Figure 4). These results therefore suggest that the rate of nuisance watermilfoil development and growth in a particular lake may depend in part on whether there were opportunities for sexual reproduction with northern watermilfoil and/or other Eurasian watermilfoil biotypes after the initial introduction of Eurasian watermilfoil.

For example, if interspecific hybridization commonly leads to increased vigor for key growth traits, as we observed here, then introduction of Eurasian watermilfoil into a

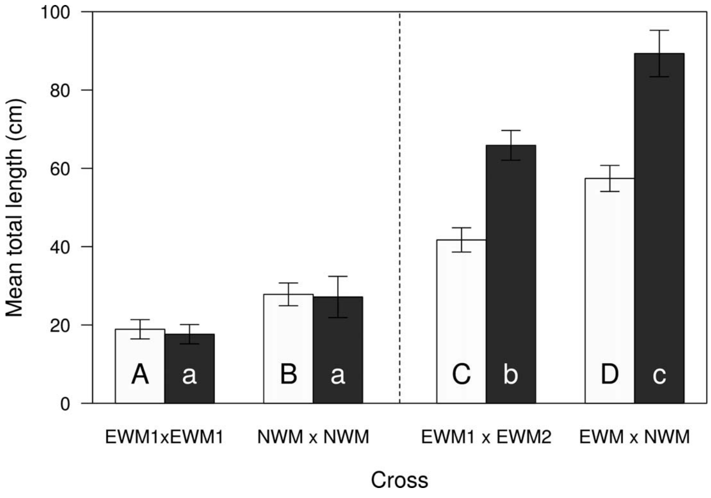

Figure 2. Mean total length (length of all branches) for different cross types (mean ± 1 standard error) after 5 wk. Results from Experiments 1 and 2 are shown side by side (light and dark gray bars, respectively). Multiple comparisons were performed separately for Experiments 1 and 2 using bootstrapped two-sample *t* tests with Holm-corrected *P* values. Pairwise significant differences are indicated by different capital letters (Experiment 1) or lowercase letters (Experiment 2). NWM = northern watermilfoil; EWM = Eurasian watermilfoil.

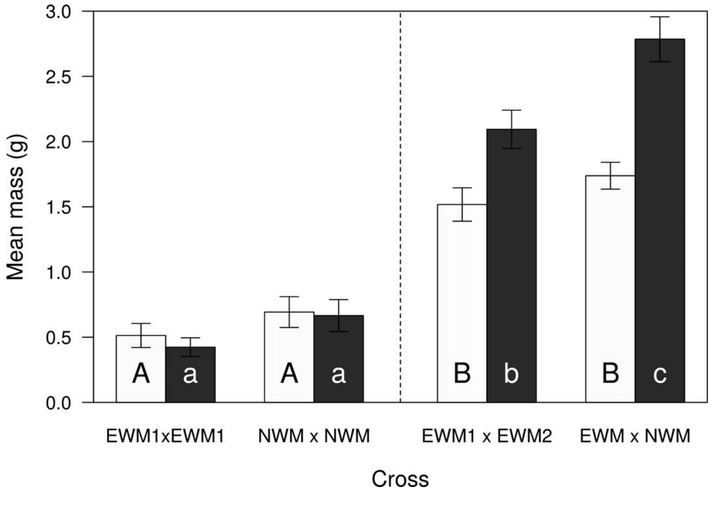

Figure 3. Final wet mass for different cross types (mean ± 1 standard error) after 5 wk. Results from Experiments 1 and 2 shown side by side (light and dark gray bars, respectively). Multiple comparisons were performed separately for Experiments 1 and 2 using bootstrapped two-sample *t* tests with Holm-corrected *P* values. Pairwise significant differences are indicated by different capital letters (Experiment 1) or lowercase letters (Experiment 2). NWM = northern watermilfoil; EWM = Eurasian watermilfoil.

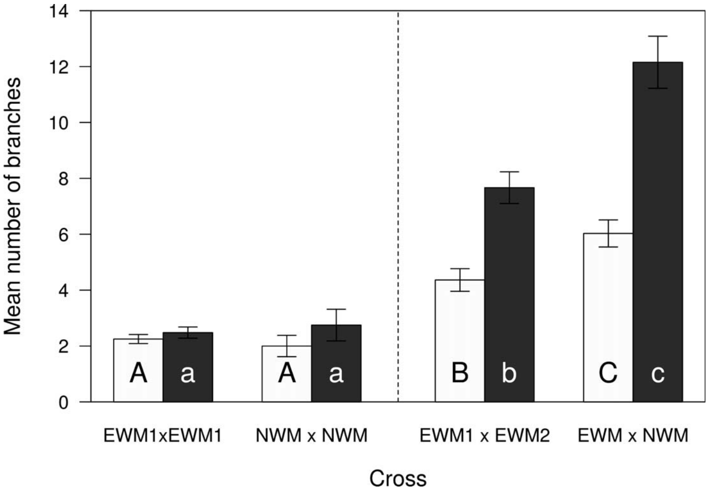

Figure 4. Number of branches for different cross types (mean ± 1 standard error) after 5 wk. Results from Experiments 1 and 2 shown side by side (light and dark black bars, respectively). Multiple comparisons were performed separately for Experiments 1 and 2 using bootstrapped two-sample *t* tests with Holm-corrected *P* values. Pairwise significant differences are indicated by different capital letters (Experiment 1) or lowercase letters (Experiment 2). NWM = northern watermilfoil; EWM = Eurasian watermilfoil.

water body with a resident population of northern watermilfoil poses the greatest risk for nuisance growth if the two species hybridize. However, we also found intraspecific hybrid vigor for crosses between two genetically distinct biotypes of introduced Eurasian watermilfoil. Therefore, introduction of Eurasian watermilfoil into a water body with a resident population of a different Eurasian watermilfoil biotype may also lead to a greater degree of nuisance growth than if the resident population was the same biotype. In addition to the faster vegetative growth of hybrids, seeds from hybrid crosses generally had higher germination percentages and shorter germination times. Thus, for managed populations with successful control, recolonization from a seed source may be more likely if the seed source contained seeds from hybrid crosses compared with seeds generated from sexual reproduction within closely related Eurasian or northern watermilfoil parents. These differences in germination and vegetative growth may therefore result in displacement of pure parental lineages by hybrid lineages within lakes over time. They may also affect long-term control efforts by decreasing the overall persistency of a seed bank.

Before this study, genetic analyses revealed considerable genetic variation among hybrid watermilfoil genotypes collected from different populations, suggesting that hybridization occurs frequently between Eurasian and northern watermilfoil (Zuellig and Thum 2012). Furthermore, two laboratory studies of distinct hybrid genotypes and populations found higher vegetative growth for hybrids compared with pure Eurasian watermilfoil (LaRue et al. 2013a, Taylor et al. 2017). Our experimental results using artificial crosses to generate known first-generation hybrids are consistent with these previous results in that progeny from hybrid crosses had higher vegetative growth rates compared with progeny from pure parental crosses. Taken together, these studies suggest that hybrid genotypes may be more likely to exhibit relatively faster vegetative growth rates than parental Eurasian or northern genotypes where they co-occur, although additional field and laboratory studies of a larger number of genotypes are warranted to determine how commonly this is the case, and how environmental factors may influence relative growth of different genotypes.

We also found clear evidence for intraspecific hybrid vigor in crosses between two genetically distinguishable forms of introduced Eurasian watermilfoil (EWM1 × EWM2). However, nothing is currently known about the frequency with which they co-occur and hybridize in natural populations. The two biotypes can be distinguished using ITS DNA sequences and amplified fragment length polymorphisms (Zuellig and Thum 2012). However, these data are not routinely collected as part of survey and monitoring efforts associated with lake management plans. Given the intraspecific hybrid vigor identified in our study, we recommend that watermilfoil managers invest in genetic surveys to determine whether apparent variation in treatment efficacy among pure Eurasian populations is associated with intraspecific hybridization.

An important limitation of our study is that we were unable to determine how much inbreeding depression might contribute to the observed differences between hybrid and parental crosses, which we interpret as hybrid vigor. This is because we only compared interspecific and intraspecific hybrids with pure parental genotypes that were produced via sexual reproduction from crosses among parents collected from the same populations, as opposed to comparing hybrid offspring with asexually reproducing parental genotypes. For example, northern watermilfoil exhibited the lowest germination, and it is possible that this results from inbreeding depression and/or mechanisms for self-incompatibility. It is also possible that naturally occurring genotypes of pure Eurasian watermilfoil represent a subset of relatively high-fitness genotypes that reproduce primarily through vegetative propagation instead of via sexual reproduction because of high inbreeding depression. Future studies should address this limitation. Nevertheless, the substantial amount of genetic variation observed in parental and hybrid Eurasian watermilfoil (Zuellig and Thum 2012, LaRue et al. 2013a,b) suggests that sexual reproduction can and does occur. Therefore, our study provides compelling evidence for interspecific and intraspecific hybrid vigor for key growth traits in situations where propagules originate via sexual reproduction. In addition, we generally observed a significantly more vigorous vegetative growth rate for interspecific hybrid crosses compared with crosses between EWM biotypes, which essentially serve as controls for the effect of inbreeding depression since they are outcrossed.

Another limitation of our study is that we only compared first-generation hybrids with parental crosses, and furthermore that these comparisons were done in a single, controlled environment. The fitness of hybrids relative to nonhybrids can vary according to hybrid generation (F_1, F_2, backcross, etc.), genetic background of parents creating hybrid offspring, and habitat (Arnold and Hodges 1995, Arnold and Martin 2010, Hovick and Whitney 2014). Thus, although we observed hybrid vigor in our F_1s, it is possible that subsequent sexual reproduction by these hybrids would lead to lower fitness ("hybrid breakdown"). Similarly, although we observed hybrid vigor across two independent sets of crosses using different EWM and NWM genotypes, it is possible that hybrids from different EWM and NWM parents would not exhibit hybrid vigor, and future studies should examine a larger number of parental genotypes. It is also possible that specific hybrid genotypes would have higher fitness relative to nonhybrid genotypes in some habitats, but lower fitness in others. Very little is currently known about the fitness of different watermilfoil genotypes in different environments, with the exception of laboratory comparisons of growth when exposed to different herbicides. From these studies, it is clear that different hybrid genotypes vary in important traits related to growth and herbicide response (e.g., Glomski and Netherland 2010, Berger et al. 2012, Thum et al. 2012, LaRue et al. 2013a, Berger et al. 2015, Netherland and Willey 2017, Taylor et al. 2017). What is not clear is the relative extent to which variation among hybrid genotypes results from hybrid class (F_1, F_2, backcross, etc.) versus differences in parental genotypes across different hybridization events. Future studies should seek to comprehensively address the influences of genetic and environmental factors on hybrid fitness.

Aquatic plant managers increasingly recognize that different populations of invasive aquatic plants can vary considerably in their potential for nuisance growth, spread, impacts, and response to control efforts. Population-centered, as opposed to species-centered, approaches to understanding invasive aquatic plants are therefore important, because populations are ultimately the unit of management for most invasive species (see also Reichard et al. 2015). Therefore, understanding the factors affecting variation among introduced populations can help tailor management approaches to different populations. Although numerous environmental factors may influence the variation among populations, we have provided empirical evidence that variation can result from intrinsic properties of populations, such as the genetic composition of Eurasian watermilfoil populations that may arise through different opportunities for sexual reproduction with genetically similar versus distinct individuals and taxa. Aquatic plant managers should therefore strive to take such factors into account, especially for taxa that are capable of both sexual and asexual reproduction in their introduced ranges.

SOURCES OF MATERIALS

[1]Osmocote (19–6–12), Scotts Miracle-Gro Company, 14111 Scottslawn Road, Marysville, OH 43041.

[2]Cone-tainer, Ray Leach Cone-tainers™ through Stuewe & Sons, Inc., 31933 Rolland Drive, Tangent, OR 97389.

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