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Note

Do the U.S. dioecious and monoecious biotypes of *Hydrilla verticillata* hybridize?

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INTRODUCTION

Hydrilla verticillata L.F. Royle (hydrilla; Hydrocharitaceae) is an invasive submersed plant that was introduced into the United States on at least two occasions, from India and South Korea (Madeira et al. 2000). These introductions correspond to genetically and ecologically distinct dioecious and monoecious biotypes (Madeira et al. 2000). The dioecious biotype (pistillate flowers only) is thought to have been first introduced into Florida in the 1950s and has since become highly invasive (Schmitz et al. 1991, Balciunas et al. 2002). The monoecious biotype (pistillate and staminate flowers) is a more recent introduction and was first identified in the Potomac River in 1976 (Steward et al. 1984). The dioecious biotype has been reported principally from East Coast and Gulf Coast states, but also from California and Washington State (Langeland 1996). The monoecious biotype is spreading rapidly and is now found in 22 states, more commonly in the northern United States, though it appears to be increasing in southern states as well (Grodowitz et al. 2010).

Historically, the two hydrilla biotypes were geographically separated but their North American ranges have begun to overlap. Ryan et al. (1995) reported both biotypes from Lake Gaston, North Carolina. Both biotypes are also believed to coexist at Guntersville Reservoir, Alabama (D. Webb, pers. comm.) and Lake J. Strom Thurmond, Georgia (U.S. Army Corps of Engineers 2013). The occurrence of both biotypes within the same water body raises concerns about potential hybridization. Hybridization may lead to novel secondary chemistry combinations (Orians 2000, Whitehead and Bowers 2013) or structural changes (Grosholz 2010) that give the hybrid a competitive advantage in the environment and lead to reduced efficacy of chemical and biological control technologies (Roley and Newman 2006, Blair et al. 2008, Schierenbeck and Ellstrand 2009, LaRue et al. 2013). Hybridization can also increase genetic variation, thereby increasing the chance of adaptive evolution in the introduced range. Experimental crosses have revealed that female dioecious hydrilla plants are potentially fertile and can produce viable seed when pollinated by monoecious and dioecious strains from Asia, though crosses between the U.S. biotypes have not been attempted (Steward 1993). In addition, viable seeds have been identified from U.S. monoecious plants at a North Carolina lake and in cultures maintained in Florida, though productivity and viability were reportedly low (Conant et al. 1984, Langeland and Smith 1988). For a variety of ecological and management reasons, it is important to determine whether 1) hybridization between hydrilla biotypes in the United States can occur under controlled conditions, and 2) whether hybridization is occurring in field populations. This note reports the results of two studies designed to address these questions.

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MATERIALS AND METHODS

Field hybridization studies

In September 2013 Guntersville Reservoir, Alabama, and Lake J. Strom Thurmond, Georgia, were surveyed at areas reported to contain both the dioecious and monoecious hydrilla biotypes. Survey sites were selected based upon visual observation and local expert opinion, where it was assumed both biotypes were growing in the same general vicinity. Plants were collected from a boat by hand or using a rake. Five-centimeter apical meristems were collected $\geq$ 2 m apart to reduce the likelihood of sampling from the same plant. Ten sites (50 apical tips) were sampled in each reservoir. Upon collection, the biotype of each sample was classified by floral examination and general morphological characteristics (e.g., monoecious plants are less robust, have thinner leaves, and are more easily broken at nodes). Plant pieces were placed in silica gel desiccant in 5.1- by 7.6-cm, 4-mil-thick, sealable plastic bags and shipped to Texas Christian University for genotypic analyses. Samples of monoecious hydrilla were also collected from sites in New York (Erie Canal and Lake Cayuga, $n = 8$), North Carolina (Lake Gaston, $n = 1$), Kansas ($n = 1$), and Missouri ($n = 1$), and these were compared to the monoecious hydrilla collected in Guntersville and Lake J. Strom Thurmond.

DNA was extracted from all samples using the IBI Scientific MINI Genomic DNA kit (Plants)[1] as per manufacturer's instructions. The biotype of each sample (as classified in the field) was also checked using modified genetic markers developed by Madeira et al. (2004). The forward primer from Madeira et al. (2004) was used: MadieraF 5′-CCCTCTATCCCCAATAAAAATCC-3′ and a new reverse primer was developed for this study: HvmonoR 5′-ATTTGGAACCACCGATGAAA-3′. Polymerase chain reactions (PCRs) (10 μl) contained 10–50 ng DNA, 0.5 μM of each primer, 1× PCR buffer with 2.5 mM $MgCl_2$ (pH 8.7), 0.2 mM dNTPs, 2× bovine serum albumin, and 0.4 units Taq polymerase. Reactions were cycled in an ABI 2720 thermal cycler.[2] The cycling parameters were one cycle at 94 C for 2 min; followed by 30 cycles of 15 s at 94 C, 15 s at 60 C, and 30 s at 72 C; and then a final extension at 72 C for 5 min. With this marker system the monoecious biotype gives a single 118-bp band visible on an agarose gel while the dioecious biotype does not produce a band. Positive samples were run in each batch to check amplification efficiency.

Samples were also genotyped at eight microsatellite loci in two multiplexes (Grajczyk et al. 2009). PCR (10μl) contained 10–50 ng DNA, 0.5 μM of each primer, 1× Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer[3] with 3mM $MgCl_2$ (pH 8.7), and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95 C for 15 min; followed by 30 cycles of 30 s at 94 C, 90 s at 60 C, and 90 s at 72 C; and then a final extension at 60 C for 30 min. The resulting multiplexes were diluted 20× with dH_2O. For each sample, 0.5 μl diluted product was loaded in 10 μl HIDI formamide with 0.1 μl LIZ-500 size standard[4] and electrophoresed on an ABI 3130XL Genetic Analyzer.[5] Genotypes were then scored using GENEMAPPER v5.0.[6]

Hydrilla in the United States exhibits a variety of ploidy levels from diploid to tetraploid, with triploid individuals being the most common in the dioecious biotype of hydrilla. All individual samples in this study exhibited three microsatellite alleles at two or more of the loci, suggesting they may have been triploid or tetraploid. Since the ploidy levels were unknown for these specimens we transformed all multilocus microsatellite genotypes into dominant markers with samples scored for each potential allele as either present or absent using GENODIVE (Meirmans and Van Tienderen 2004). We then created a distance matrix by calculating the p-distance between all pairs of samples (simply the number of allele differences between individuals) using GENODIVE. This distance matrix was then used to cluster individuals using a principle components analysis (PCA) in GenAlEx v6.5 (Peakall and Smouse 2006). These multilocus profiles were also used to calculate h or haplotype diversity in GenAlEx v6.5, which in this case is simply the probability that two samples taken at random will have different multilocus genotypes.

Greenhouse hybridization

Both hydrilla biotypes used in this study were procured from cultures maintained at the Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX (Denton County). The dioecious hydrilla was originally collected from east Texas in the early 1990s and the monoecious hydrilla was collected from Lake Gaston, North Carolina in 2006 and has been growing in culture in mesocosm tanks (1,850-L capacity) at the LAERF since that time.

This study was conducted in a greenhouse under ambient light conditions beginning May 2014 through February 2015. Twelve 0.9-L plastic containers were filled with commercial topsoil that was amended with 5 g of Miracle-Gro® granules[7]: (10–16–10). Each container was submerged into one of 12 18.9-L plastic buckets placed in a 2.7-m-long by 1.2-m-wide by 0.2-m-deep water bath and then filled with City of Lewisville tap water, treated with Proline® Aqua-Coat[8] per label rate (1.25 ml per 18.9-L bucket) as a dechlorinater, and allowed to sit for 24 h prior to the initial planting. Six containers were then planted with three apical tips (10- to 15-cm length) of dioecious hydrilla, and six with monoecious hydrilla. Planting consisted of gently pressing approximately one-third of each apical fragment into substrates until suitably anchored to prevent floating. Water levels were kept to within 2.54 cm of the container top for consistency between treatments. A regenerative blower was used to deliver air through a single air stone placed into each bucket to circulate water and replenish dissolved carbon during the course of the study. Once flowering occurred, containers of dioecious and monoecious plants were paired in six buckets to enable pollination between biotypes. Observations were made several times weekly to ensure that female surface flowers, free-floating male flowers (postrelease), and pollen were present in the six buckets. Occasionally, free-floating male flowers were transferred by eyedropper from a separate monoecious culture to promote pollination. Approximately 120 female flowers bearing retracted styles of both biotypes were collected between September 2014 and February 2015 for assessment of seed formation. Ovaries were dissected to determine whether or not fertilization (seed development) was evident.

RESULTS AND DISCUSSION

Field hybridization studies

Distinguishing hydrilla biotypes in the field was shown to be inexact. Genetic data indicated that 28% of samples were incorrectly identified at the time of collection: 33% of samples labeled as dioecious were monoecious while 19% of plants identified as monoecious were dioecious. Samples from Guntersville Reservoir, Alabama, had a higher proportion of dioecious (55%) than monoecious (45%), while all samples from J. Strom Thurmond Reservoir were monoecious. Genetic diversity of multilocus microsatellite profiles for the dioecious biotype was similar to the monoecious biotype in these reservoirs ($h = 0.077 \pm 0.02$ SE vs. 0.073 ± 0.02, respectively).

The dioecious biotype contained 11 alleles across six loci that occurred in all samples but were not present in the monoecious biotype, while the monoecious biotype had six alleles across five loci that occurred in all samples but were not present in the dioecious biotype. The PCA clearly

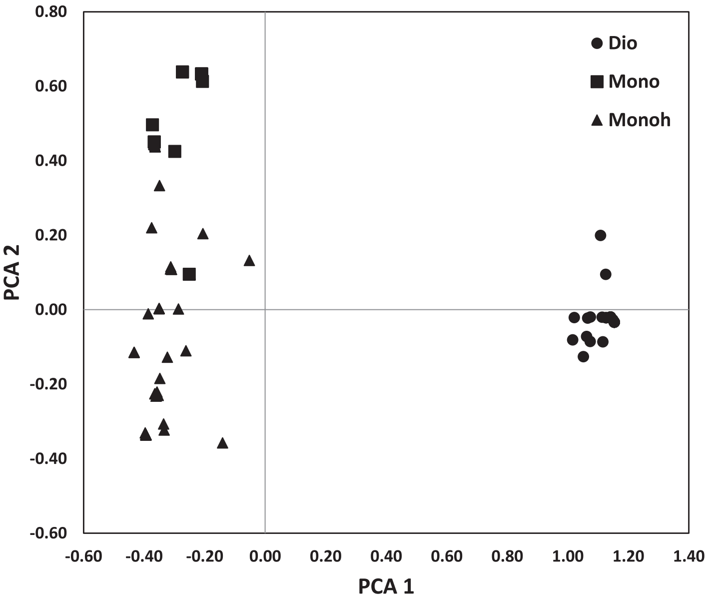

Figure 1. Principle component analysis of multilocus microsatellite genotypes for monoecious hydrilla collected in the northern United States (Mono) and monoecious hydrilla (Monoh) and dioecious hydrilla (Dio), both collected in Guntersville Reservoir and Lake J. Strom Thurmond.

separates the samples into two clusters and these correspond to the two biotypes identified genetically, with the first two axes of the PCA explaining 77% of the genetic variation (Fig. 1). Monoecious samples collected from other areas in the United States cluster with the monoecious samples from the reservoirs and there were no intermediate samples between the two clusters suggesting there were no hybrid individuals. Although our sample size was small, our data suggest that hybridization between the U.S. biotypes has not occurred in Guntersville Reservoir, or, if it has, those hybrids may be at an ecological disadvantage in this area and so are rare.

Greenhouse hybridization studies

Monoecious hydrilla began producing pistillate flowers in August, 12 wk after planting. Staminate floating flowers were observed within several days of the female flowers. Dioecious plants began producing pistillate flowers approximately 3 wk later. Flowering of both biotypes continued through November 2014, and pistillate ovaries remained intact through February 2015. Ovary dissections revealed only ovules in both biotypes, with no seed production found in either through February 2015. Pollination, fertilization, or embryo development did not occur in examined flowers of either biotype in this study.

Summary

Molecular analysis of hydrilla from Guntersville Reservoir, where both biotypes grow in close proximity, indicates that hybridization at this reservoir does not occur or is rare. Attempts to produce hybrids under greenhouse conditions also did not result in fertilization. We used a single monoecious genotype in our experiment that has been maintained in the greenhouse for over 10 yr. It is possible that this particular genotype has low fertility or being maintained in the greenhouse for a number of years has resulted in low fertility. In some ways low fertility was expected because seed production in the U.S. monoecious biotype generally occurs only rarely and in low quantities. Conant et al. (1984) found only one seed per 100 fruits examined in a monoecious biotype collected from Delaware and even fewer seeds (0.45 seeds per 100 fruits) in a North Carolina strain. Up to 50 seeds m^{-2} were collected from samples taken from several North Carolina lakes; however, this seed density is relatively low in comparison to other plant species (Langeland and Smith 1984). Controlled experiments have indicated low viability and seedling vigor for field-collected seeds in the United States, which was attributed to triploidy (Langeland and Smith 1988). In contrast, when the U.S. dioecious biotype and biotypes from across the world were crossed, large numbers of viable seeds were produced (Steward 1993).

These were not exhaustive studies on the occurrence of monoecious/dioecious hybrids in U.S. waters or the potential for U.S. hydrilla biotypes to successfully cross-pollinate, fertilize, and produce viable seeds under greenhouse conditions. Indeed, as the geographic and environmental ranges of both U.S. biotypes increase, there will continue to be sites where they grow side by side. The environmental conditions at Guntersville Reservoir may not have been adequate to encourage cross-pollination and fertilization or the particular genotypes at Guntersville Reservoir and in our greenhouse experiment may not have been very fertile and so our results do not preclude the possibility that hybridization occurs elsewhere. It is prudent to continue this line of research by collecting additional hydrilla samples from areas where both biotypes occur to identify hybrids before they become established and spread. Additionally, more extensive greenhouse experiments should be conducted to better determine whether viable seed production can result from a U.S. monoecious–dioecious cross. These experiments should 1) test multiple genotypes of monoecious hydrilla collected from throughout its U.S. range, 2) conduct a much larger number of pairings to estimate seed production, and 3) use hand-pollination to ensure pollen transfer.

SOURCES OF MATERIALS

[1]IBI Scientific MINI Genomic DNA kit (Plants), IBI Scientific, Peosta, IA 52068.

[2]ABI 2720 thermal cycler, Applied Biosystems, Foster City, CA 94404.

[3]Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer, Qiagen, Valencia, CA 91355.

[4]LIZ-500 size standard, Applied Biosystems, Foster City, CA 94404.

[5]ABI 3130XL Genetic Analyzer, Applied Biosystems, Foster City, CA 94404.

[6]GENEMAPPER v5.0, Applied Biosystems, Foster City, CA 94404.

[7]Miracle-Gro® granules, Scotts Miracle-Gro Company, Marysville, OH 43040.

[8]Proline® Aqua-Coat, Pentair, Sanford, NC 27330.

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