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Hybridization potential of three extant subspecies of *Hydrilla verticillata* in the United States

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ABSTRACT

Manually pollinated controlled crosses were performed between three subspecies of *Hydrilla verticillata* (L.f.) Royle to assess whether viable hybrid offspring could be produced in anticipation of sympatric populations of *H. verticillata* subspecies in the United States. Manual pollination produced 33 fruits from 82 crosses. Seed germination occurred only once (*H. verticillata* subsp. *peregrina* [seed parent] × subsp. *lithuanica* [pollen parent]), leaving hybrid seed viability unknown. Future work should repeat crosses, evaluate viability of different combinations, and investigate the relative competitive ability of hybrid offspring compared to parent subspecies.

Key words: Connecticut River hydrilla, invasive hybridization, invasive aquatic plants, submerged aquatic vegetation

INTRODUCTION

Over 13,000 vascular plant species have escaped their native range and become established in introduced ranges (van Kleunen et al. 2015, 2019). Many become invasive and cause immense economic and ecological damage (Pimentel et al. 2005, Pyšek and Richardson 2010, Vilà et al. 2011). Aquatic invasive plants not only pose a threat to native submerged macrophyte and algal species diversity but can also alter critical abiotic processes such as nutrient cycling, transfer of dissolved gases across the water column, and changes in hydrology, on top of the negative economic impacts (Xu et al. 2006, Wang et al. 2016). In some circumstances, sexual reproduction between sympatric congeners can create novel allelic combinations, leading to unique phenotypes that may increase the adaptive potential of hybrids and confer increased fitness or altered sensitivity to management activities (i.e., hybrid vigor; Abbott 1992, Keller and Taylor 2010). Examples of hybrid vigor in invasive plants are prevalent across functional groups and habitat types (Schierenbeck and Ellstrand 2009). In aquatic invasive plants, hybrid vigor has been observed in several species, including hybrids between *Vallisneria spiralis* L. * *Vallisneria denseserrulata* (Makino) Makino (Hydrocharitaceae) (Wasekura et al. 2016) and hybrid watermilfoil *Myriophyllum spicatum* L. * *Myriophyllum sibiricum* Kom. (Haloragaceae) (Glisson & Larkin 2021). In addition to increased vigor, hybridization of aquatic plants has been linked to select cases of reduced herbicide efficacy (hybrid watermilfoil; Berger et al. 2012, 2015; LaRue et al. 2013) as well as reduced effectiveness of biological control agents (*Tamarix* spp.; Williams et al. 2014).

Hydrilla verticillata (L.f.) Royle is a submersed aquatic plant with a cosmopolitan distribution and is found on every continent except for Antarctica (Cook and Lüönd 1982, Patrick and Florentine 2021). Multiple introductions of distinct *H. verticillata* subspecies throughout the mid- to late 20th century have led to negative invasion-related ecological outcomes across much of the contiguous United States, including native species displacement, alterations to aquatic hydrology, and disruption of trophic networks (Theel et al. 2008, Monterroso et al. 2011). Hydrilla has a variety of characteristics that contribute to its invasive success globally (Cook and Lüönd 1982, Steward and Van 1987, Bowes et al. 2002, True-Meadows et al. 2016). It can outcompete many lower-statured submerged aquatic plants for light and space, has rapid vegetative growth once established, is both low- and high-nutrient adapted, tolerates salinity up to 7 g L^{-1} (ppt), and is a facultative C4 plant (Van et al. 1976, Bowes et al. 1977, Holaday and Bowes 1980, Steward and Van 1987, Reiskind et al. 1997, Van et al. 1999, Wang et al. 2008). Hydrilla has multiple reproductive and dispersal mechanisms, as it can establish new populations through apical shoot fragments as well as persistent axillary and subterranean turions.

In North America, hydrilla is found throughout the East Coast and South-Central regions of the United States (USDA 2025). Populations have also been identified in the American West in parts of California, New Mexico, and Washington State. Recent genetic work has classified three distinct hydrilla subspecies in North America, which have previously been defined by their reproductive characteristics (Tippery 2023). In the Northeast and Mid-Atlantic, *H. verticillata* subsp. *peregrina* (hereafter referred to as monoecious hydrilla) dominates (Langeland 1996, True-Meadows et al. 2016). This subspecies was first observed in the Mid-Atlantic along the Potomac River in Delaware and Washington, DC, in the late 1970s, possibly due to escape from a water garden (Haller

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1982, Steward et al. 1984). Monoecious hydrilla has since spread as far south as Georgia (Madeira et al. 2000, True-Meadows et al. 2016). The subspecies that dominates in the Southeast is *H. verticillata* subsp. *verticillata*, a dioecious subspecies for which only female individuals have been observed in North America (USGS 2025). The first introduction of dioecious hydrilla occurred due to an escape from the aquarium trade in Florida in the 1950s and has since spread throughout the state and northward as far as North Carolina and as far contiguously as Texas (Blackburn et al. 1969, Madeira et al. 2000, USGS 2025). The newest subspecies to colonize North America was first recognized in 2016 in the lower Connecticut River, but now inhabits other watersheds within southern New England, and has been identified as *Hydrilla verticillata* subsp. *lithuanica* (Tippery et al. 2020, Foley et al. 2024). While populations of *Hydrilla verticillata* subsp. *peregrina* in Connecticut have been known for decades, this newly introduced *Hydrilla verticillata* subsp. *lithuanica* is morphologically distinct from *Hydrilla verticillata* subsp. *peregrina*, having a more robust stem, longer internode length between whorls, and a darker green coloration (Tippery et al. 2020; Tippery 2023). Although field observation has found only male or female plants, greenhouse observations have also indicated that *H. verticillata* subsp. *lithuanica* is dioecious (Williams et al. 2018; unpublished observations). Phylogeographic studies suggest that *H. verticillata* subsp. *verticillata* in North America may be composed of multiple genotypes, which suggests escape of multiple individuals and demonstrates previously unrealized genetic diversity in North America (Zhu et al. 2015).

Although seed production in the field has not been observed in North America, fruits and seeds have been observed in wild populations in India and other parts of Southeast Asia (Cook and Lüönd 1982, Steward 1993). In controlled-cross experiments, dioecious hydrilla from Florida was able to create viable seeds with both dioecious and monoecious hydrilla populations from New Zealand and India (likely subsp. *australis* or subsp. *augustifolia*), suggesting that dioecious pistillate flowers are fertile (Steward 1993, Madeira et al. 2007, Zhu et al. 2015, Benoit et al. 2019, Tippery et al. 2020, Tippery 2023). *Hydrilla verticillata* subsp. *peregrina*, subsp. *verticillata*, and subsp. *lithuanica* occurring in North America are thought to originate from Korea, Southeast Asia, and northern Europe to northern China and Siberia, respectively, although the full native range of the latter lineage remains poorly characterized. Sympatry among all three subspecies has been observed in northeast China, and Williams et al. (2018) reported genetic admixture between clade C (*H. verticillata* subsp. *lithuanica*) and the other two subspecies (dioecious and monoecious), as well as between the dioecious and monoecious subspecies (Williams et al. 2018, Tippery 2023). These findings suggest that hybridization among the three extant subspecies in North America is possible. Although *H. verticillata* subspecies in North America generally exhibit largely nonoverlapping distributions, areas of co-occurrence have been documented. Sympatric populations *H. verticillata* subsp. *peregrina* and subsp. *verticillata* have been reported along the Virginia–North Carolina border, as well as within reservoirs of the Tennessee Valley system, although recent observations remain inconclusive (Ryan et al. 1995, Netherland and Greer 2014). More recently, the introduction of *H. verticillata* subsp. *lithuanica* into the lower Connecticut River and its subsequent spread throughout southern New England has resulted in this subspecies occupying areas previously populated by *H. verticillata* subsp. *peregrina*, although evidence of sympatry is not found (Les et al. 1997, Madeira et al. 2000). Thus, understanding the potential for hybridization among introduced *H. verticillata* subspecies is critical for informing the development of effective management strategies as subspecies sympatry increases. Consequently, the objective of this study was to evaluate hybridization potential of the hydrilla subspecies in a controlled-cross experiment.

MATERIALS AND METHODS

Culture stock and materials

Hydrilla verticillata subsp. *verticillata* used in controlled crosses was obtained in autumn 2023 from field-collected apical shoots and greenhouse-cultured apical shoots, both originally derived from the same pond population at the U.S. Army Engineer Research and Development Center's Lewisville Aquatic Ecosystem Restoration Facility (LAERF), Lewisville, TX. Initiation of *H. verticillata* subsp. *verticillata* pistillate flowering commenced in late August 2024 in an outdoor mixed community freshwater pond at LAERF dominated by *H. verticillata* subsp. *verticillata*, watermilfoils (*Myriophyllum* spp.), and pondweeds (*Potomegeton* spp.). In early September, initiation of *H. verticillata* subsp. *verticillata* pistillate flowering occurred in greenhouse cultures at LAERF under ambient conditions and continued flowering through mid-October. *Hydrilla verticillata* subsp. *peregrina* used in the controlled crosses was taken from culture stock, sourced from Guntersville Reservoir in Guntersville, AL. Initiation of *H. verticillata* subsp. *peregrina* pistillate and staminate flowering commenced in early September 2024 in greenhouse cultures. *Hydrilla verticillata* subsp. *lithuanica* was taken from culture stock sourced from the lower Connecticut River in spring of 2023. *Hydrilla verticillata* subsp. *lithuanica* staminate flowers were first observed in June 2024 and continued to flower until early September 2024 and continued through mid-October. Development of pistillate flowers within *H. verticillata* subsp. *lithuanica* cultures was not observed in 2024. All cultures were maintained in a greenhouse at LAERF in cylindrical plastic aquaria (46 cm [Ø] × 92 cm [H]) potted in top soil[1] with a 1-cm-thick sand[2] cap amended as needed with 5 g nutrient tablets[3] under ambient light and temperatures. All cultures were genotyped using chloroplast and microsatellite markers following Williams et al. (2018) before experiments to confirm subspecies genetic identity. In late August 2024, pistillate flowering was observed in *H. verticillata* subsp. *verticillata* under ambient conditions in an outdoor mixed community freshwater pond at LAERF.

Controlled crosses

Apical shoots bearing pistillate flowers were collected, ranging from 30 to 50 cm, then planted in 1 L clear-plastic containers (11.5 cm [Ø] × 14 cm [H]) filled with 2 cm of top

Figure 1. Receptive pistillate *Hydrilla verticillata* flowers pollinated in controlled crosses. (A) *H. verticillata* subsp. *peregrina* pistillate flower pollinated with *H. verticillata* subsp. *lithuanica* pollen; (B) *H. verticillata* subsp. *verticillata* pistillate flower pollinated with *H. verticillata* subsp. *lithuanica* pollen.

soil[4] and a 5-mm sand cap.[2] Pollination of pistillate flowers was achieved by acquiring dehisced-from-bract staminate flowers, which had floated to the water's surface but had not yet explosively dispersed their pollen. Staminate flowers were placed over receptive pistillate flowers on collected *H. verticillata* apical shoots. Forceps were then used to rupture the staminate flowers, releasing pollen directly onto the stigma to ensure pollination. In late August through early September, 47 crosses were established between *H. verticillata* subsp. *lithuanica* (pollen parent) and *H. verticillata* subsp. *verticillata* (seed parent) (Figure 1B; Table 1). We established 14 crosses of *H. verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) between 11 and 16 September 2024 (Figure 1A; Table 1). We further established 21 crosses between culture-sourced *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *peregrina* (pollen parent) between 16 September and 17 October 2024 (Table 1).

Fruit development

All controlled crosses were placed in environmental growth chambers[5] fitted with 17 W fluorescent lights[6] measured to output ~100 µMol photons $m^{-2}s^{-1}$ programmed to 12 : 12 h day : night cycle at 25 C and monitored for fruit development. Conspicuous fruit development was noted using descriptions of successfully ripened fruit described in Lal and Gopal (1993). Fruits of *H. verticillata* subsp. *verticillata* from wild populations in India have been described as cylindrical with long tapered distal ends, measuring 1–1.7 cm in length and approximately 2 mm in diameter (Lal and Gopal 1993). Fruits are either smooth or bear one to eight spiny lateral projections and typically contain three to eight seeds (Lal and Gopal 1993). Fruiting development within each controlled cross was noted, and the number of fruit and the position of the fruits were recorded.

Hydrilla verticillata seeds are dispersed through decay of the pericarp (Lal and Gopal 1993), and naturally reproducing populations of *H. verticillata* subsp. *verticillata* exhibit vivipary, where seeds germinate within the decaying fruit. To mimic natural dispersal and germination dynamics, fruits were left to ripen on the mother shoot for

Table 1. Chi-squared test summary statistics for model testing effect of cross combination of proportion of fruits produced per cross (top). Crosses performed between *Hydrilla verticillata* subspecies displaying pollen donors (pollen parent) and egg donors (seed parent), number of conspicuous fruits developed, proportion of crosses resulting in conspicuous fruit, and Tukey's HSD *post-hoc* test results presented in compact-letter design.

Fixed effects	df, residual df	Deviance	Residual deviance	*P* value
Cross combination	2, 79	8.96	101.58	0.01

Seed parent	Pollen parent	Crosses performed	Fruits developed	Proportion of crosses resulting in fruit	CLD
Hydrilla verticillata subsp. *verticillata*	*Hydrilla verticillata* subsp. *peregrina*	21	6	0.29	ab
Hydrilla verticillata subsp. *verticillata*	*Hydrilla verticillata* subsp. *lithuanica*	47	25	0.53	b
Hydrilla verticillata subsp. *peregrina*	*Hydrilla verticillata* subsp. *lithuanica*	14	2	0.14	a

60 d after fruit development was confirmed. Fruits were then removed and placed in 300 ml of deionized water (with fruits from the same maternal plant when multiple fruits were present) and allowed to decay for an additional 40 d. After 40 d, ungerminated seeds were excised from the fruit for germination trials. Seeds were examined under a dissecting microscope to confirm that they were filled and contained intact endosperm, and any seeds showing signs of decomposition were excluded from subsequent trials.

Seed germination

Although the literature suggests that hydrilla seeds are nondormant (Lal and Gopal 1993), the high-latitude provenance of CT hydrilla and monoecious hydrilla may indicate the need for cold stratification to break dormancy. Seeds that had not germinated in the 40-d period described above were subjected to a standard 8-wk cold-stratification period at 5 C to induce breaking dormancy, these parameters are commonly applied to temperate aquatic plant species that require cold stratification to break (Ferasol et al. 1995; Biber and Caldwell 2008; Baskin and Baskin 2014). Seeds collected from the same mother were placed in aluminum foil-wrapped 50-ml scintillation tubes filled with deionized water and placed in a 5 C refrigerator. Scintillation tubes were manually inverted several times once per week to equilibrate dissolved gases with the headspace prior to venting by briefly loosening the tube caps. After this period, seeds were washed in deionized water and placed in plastic Petri dishes (5 cm [Ø] × 1 cm [H]) filled with 2 ml of deionized water and placed in a growth chamber programmed to 16 h (30 C) : 8 h (24 C) day : night cycle. Germination was recorded daily for 4 wk.

After the 4-wk germination trial, any ungerminated seeds were subject to tetrazolium staining. Tetrazolium staining was used to assess the viability of ungerminated seeds, which appeared not to have rotted and were filled, using methods modified from Meerbeek et al. (2015). As such, we prepared a 1% 2,3,5-triphenyltetrazolium chloride solution with deionized water. Seeds to be assessed were preconditioned at 20 C in distilled water for 24 h, after which they were cut in two longitudinally and placed in the 1% tetrazolium stain for 24 h at 35 C. Seed halves were then rinsed 3× in distilled water before being assessed for viability. Viable seeds are evaluated to be uniformly pink, while unviable seeds are assessed to be white or partially pink in color.

Data analysis

The number of replicate crosses was not determined *a priori* and was constrained by pollen availability and overlapping flowering phenology among the *H. verticillata* subspecies used in this study, resulting in an unbalanced experimental design. To evaluate the statistical power of the experiment, we conducted a simulation-based power analysis in R (v. 4.4.3). Simulated datasets were generated using the *replicate* function in base R, using the observed fruit-set proportions for each controlled cross combination as the expected success probabilities while maintaining the unbalanced number of replicate pollinations among cross types (Table 1; R Foundation for Statistical Computing 2025). For each of 2000 simulations, fruit outcomes were drawn from a binomial distribution using the *rbinom* function and analyzed using a generalized linear model with a binomial error distribution using the *glm* function in the "stats" R package (v. 4.4.3; R Foundation for Statistical Computing 2025). The significance of cross combination was evaluated using a likelihood ratio test using the *drop1* function in the "stats" R package (v. 4.4.3), specifying a chi-squared test (R Foundation for Statistical Computing 2025). Statistical power was estimated as the proportion of simulations in which the treatment effect was detected at $\alpha = 0.05$.

Generalized linear models were used to assess the effect of cross combinations on fruit-set using the *glm* function in the "stats" R package (v. 4.4.3) using a binomial error distribution and logit link function (R Foundation for Statistical Computing 2025). The overall effect of cross combination was evaluated using a likelihood ratio test using the *anova* function in the "stats" R package (v. 4.4.3; R Foundation for Statistical Computing 2025). If significant variation exists within the cross combination levels ($P < 0.05$), estimated marginal means were calculated for each cross combination level using the *lsmeans* function in the "emmeans" R package (v. 1.11.0; Lenth et al. 2025), and significant pairwise differences were then extracted and displayed in compact letter display using the *cld* function in the "multcomp" R package (v. 1.4-29; Hothorn et al. 2025).

RESULTS AND DISCUSSION

Fruit and seed development

The simulation-based power analysis indicated that the unbalanced experimental design provided approximately 0.81 power to detect differences in fruit set among cross combinations, which exceeds the widely accepted 0.8 power threshold across a variety of scientific disciplines (Krebs 1999, Cohen 2013, Serdar et al. 2021).

Of the 47 controlled crosses performed between *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent), 25 produced fruits, corresponding to a fruit-set proportion of 0.53 (Figures 2 and 3; Table 1). Among the 21 crosses between *H. verticillata* subsp. *verticillata* (seed parent), and *H. verticillata* subsp. *peregrina* (pollen parent), six produced conspicuous fruits, yielding a fruit-set proportion of 0.29. In contrast, only two fruits were produced from the 14 crosses between *H. verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent), corresponding to a fruit-set proportion of 0.14. We found moderate evidence that fruit set differed among cross combinations, with *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) crosses (CLD: b) exhibiting 3.8 × higher fruit set than *H. verticillata* subsp. *peregrina* × *H. verticillata* subsp. *lithuanica* crosses (CLD: a) (Table 1; significance levels reported according to Muff et al. 2022).

Of the 25 fruits resulting from *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) crosses, only five of the fruit contained seeds. Among these, three fruits contained two seeds, one fruit contained a single seed, and one fruit contained three seeds. None of the fruits produced from *H. verticillata* subsp. *verticillata* (seed parent)–*H. verticillata* subsp. *peregrina* (pollen parent) crosses contained

Figure 2. *Hydrilla verticillata* subsp. *verticillata* plants bearing fruit (indicated in the red ellipses) pollinated by *H. verticillata* subsp. *lithuanica*.

seeds. One of the two fruits produced from *H. verticillata* subsp. *peregrina* (seed donor)–*H. verticillata* subsp. *lithuanica* (pollen donor) crosses contained two seeds, while the other fruit contained no seeds (Figure 4).

Seed germination

Almost no germination was observed in any of the seeds from any of the fruits, both within and outside of the fruits, even after an 8-wk cold-stratification period. Despite *H. verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) crosses yielding the lowest number and proportion of conspicuous fruit, one seed from this cross combination did germinate (Figure 5). The seed germinated after the 8-wk cold-stratification period, 7 d after seeds were transferred to the growth chamber. The seedling was planted into washed sand[4] in 300 ml of 1% Hoagland solution (0.160 g/L $Ca[NO_3]_2$, 0.052 g/L KH_2PO_4, 0.066 g/L $MgSO_4$, 0.015 g/L Fe, and 0.115 g/L KNO_3) to promote seedling growth. The seedling died within 2 wk of transplanting. All other seeds were subject to 1% tetrazolium staining as per the stated methods, and no viable seeds were detected (Figure 6).

CONCLUSIONS

This study represents a renewed effort to explore the possibility of hybridization between extant hydrilla subspecies in North America. Although over half of the controlled

Figure 3. (A) *Hydrilla verticillata* seed, (B) terminal pore of the seed, and (C) fruit resulting from a controlled cross between *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent).

Figure 4. Seeds resulting from a controlled cross between *Hydrilla verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent).

crosses between *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) produced resultant fruit (Table 1), none of these fruits produced viable seeds, resulting in seed germination. However, this work demonstrated that crosses between *H. verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) can produce fruits that bear viable seeds, although successful germination was observed only after an 8-wk cold-stratification period to break physiological dormancy. The mortality observed in the successfully germinated seedling may not be reflective of all seeds resulting *H. verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) crosses, nor does the lack of viable seeds in other crosses exclude possible hybridization between these subspecies.

Hydrilla verticillata subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) hybridization, as shown in this study, highlights the need for improvements in monitoring and control efforts to limit the spread of *H. verticillata* subspecies within the Connecticut River and Connecticut River adjacent water bodies. Since the introduction of *H. verticillata* subsp. *lithuanica*, it has spread from within the Connecticut River to surrounding water bodies, likely driven by hitchhiking on recreational watercrafts, which has increased the likelihood of sympatry between the two subspecies (Foley et al. 2024). Furthermore, climatic suitability modeling has revealed that *H. verticillata* subspecies expansion is expected to intensify under projected global change, creating areas of potential sympatry and increasing the likelihood of hybridization events (Paul et al. 2026). Thus, without proper management, continued range expansion will likely increase sympatry between subspecies and the opportunity for hybridization.

Vivipary observed in naturally reproducing *H. verticillata* populations was not observed in this study. Only a single hybrid seed germinated, and this occurred only after an 8-wk cold-stratification period. This result suggests that successful germination of hybrid seeds may require dormancy-breaking conditions not immediately encountered following fruit decay. In addition to the low proportion of fruits producing viable seeds, the requirement for cold stratification and the generally low germination success observed here indicate that multiple barriers may exist between pollination and successful seedling recruitment. Hybrid seed germination dynamics in *H. verticillata* may shift under ongoing climate change and changing species distributions, potentially influencing regional rates and success of hybridization (Paul et al. 2026). Such barriers may limit realized hybridization rates despite potential geographic and phenological overlap between subspecies.

Phenological characterization of *H. verticillata* subsp. *peregrina*, along with unpublished observations of flowering phenology in *H. verticillata* subsp. *lithuanica*, indicates substantial overlap in floral initiation (True-Meadows et al. 2016, B. Sperry, unpub. data, 2025). This overlap greatly increases the likelihood of hybridization events in the field. In addition, viable *H. verticillata* pollen can remain afloat on the water surface for more than a day, enhancing opportunities for pollination between segregated populations of the two subspecies (Tanaka 2000, Zhang et al. 2020). Additionally, the tidal nature of the Connecticut River estuary and other tidal systems in the northeastern United States may increase hydrophily and propagule dispersal, thereby enhancing pollination and subsequent sexual/asexual propagule dispersal (Ackerman 2000, Tanaka 2000). Consequently, hybridization in natural systems

Figure 5. (A) Seedling, (B) apical tip of coleoptile, (C) midstem of coleoptile, and (D) caryopsis from a post-cold-stratification germinated seedling resulting from a controlled cross between *Hydrilla verticillata* subsp. *peregrina* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent).

may occur at a spatial scale far exceeding the limited number of controlled pollinations demonstrated in this study, potentially facilitating the establishment and spread of hybrid populations.

Although most of the fruits and seeds produced resulted from crosses between *H. verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent), no viable seeds were produced during the experiment. This suggests that interspecific reproductive barriers (IRBs), particularly post-zygotic barriers affecting embryogenesis or seed development, may explain the lack of seed viability despite substantial fruit production. Post-zygotic IRBs have been described in a variety of natural and agricultural species complexes, including *Mimulus* spp. (Oneal et al. 2016), *Solanum* spp. (Florez-Rueda et al. 2016), and *Capsella* spp. (Rebernig et al. 2015). While our results suggest that IRBs may contribute to the low or absent seed viability observed here, further research is needed to determine whether these barriers occur consistently across genotypes and to identify the mechanisms underlying them.

Although modest in scale, our study represents the first successful documented viable hybrid between *H. verticillata* subsp. *peregrina* and *H. verticillata* subsp. *lithuanica* in North America. Given this result, larger scale controlled-cross studies using multiple genotypes are needed to produce larger seed sets and holistically assess the hybridization potential of hydrilla subspecies across populations in the invasive range in North America. Furthermore, performance comparisons among hybrids, extant subspecies, and co-occurring native aquatic plants must be evaluated to determine whether hybrid vigor occurs and how hybridization may influence the future spread and ecological impact of *H. verticillata* in North America.

SOURCES OF MATERIALS

[1]Top soil, Denton Sand & Gravel Inc., Sanger, TX.
[2]Quickcrete Play Sand, The Quickcrete Companies, Atlanta, GA.
[3]Planting tablets, 20-10-5, Forestry Suppliers, Jackson, MS.
[4]Timberline Top Soil, Timberline (Oldcastle), Atlanta, GA.
[5]Percival E-36L1C8 Growth Chamber, Percival Scientific, Perry, IA.
[6]Ushio F17T8/840 fluorescent lightbulb, Ushio America, Cypress, CA.

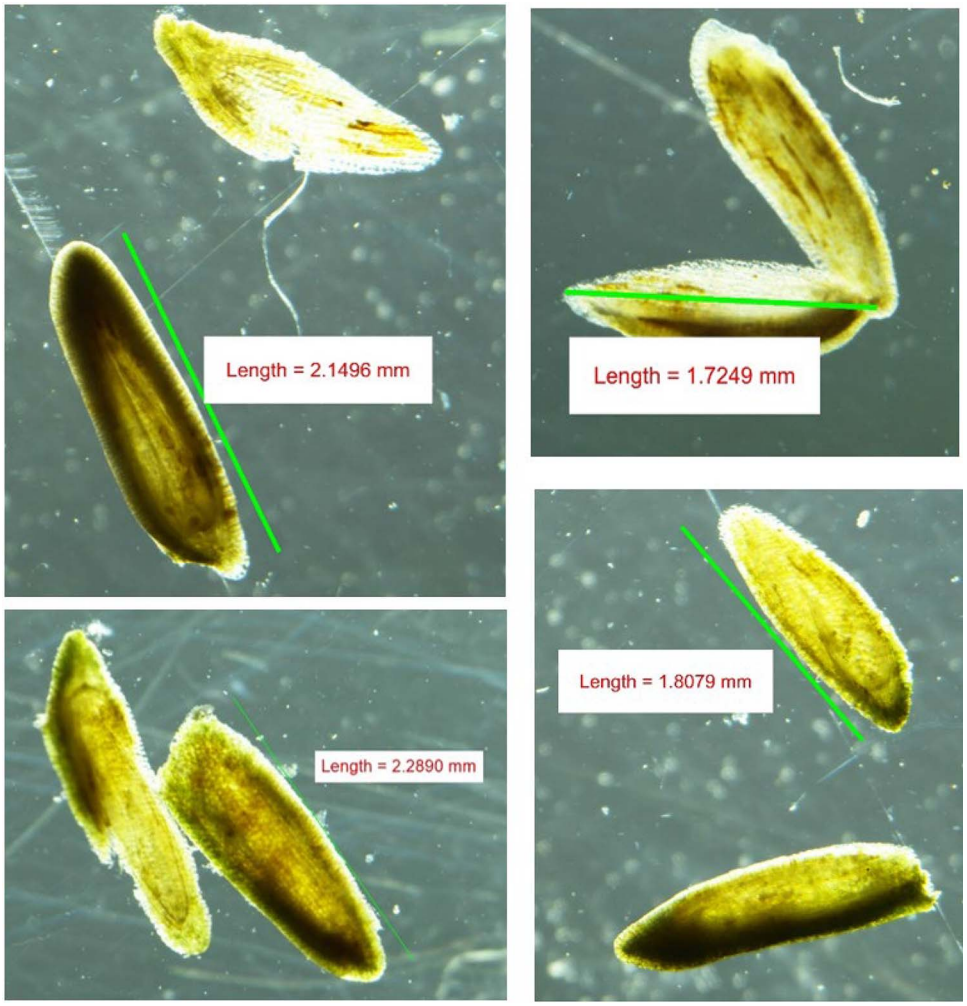

Figure 6. Seeds resulting from crosses between *Hydrilla verticillata* subsp. *verticillata* (seed parent) and *H. verticillata* subsp. *lithuanica* (pollen parent) following tetrazolium staining. A uniform pink hue indicates viable tissue, whereas white or unstained tissue indicates nonviable tissue. None of the seeds exhibited staining, indicating that all seeds were nonviable.

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