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Evidence for a genetically distinct strain of introduced *Hydrilla verticillata* (Hydrocharitaceae) in North America

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ABSTRACT

The invasive aquatic weed hydrilla [*Hydrilla verticillata* (L.f.) Royle] exists in North America as two genetically and morphologically distinct strains, with the dioecious strain mostly found in the southern United States and the monoecious strain being more northern, including previously known sites in Connecticut. In 2016 an additional hydrilla population was located in a portion of the Connecticut River in Hartford County, Connecticut, with unusual morphological features relative to other Connecticut populations. Hydrilla plants from this population were subjected to genetic testing, and their molecular sequences for one chloroplast (*trnL-F*) and two nuclear gene regions (internal transcribed spacer and phytoene desaturase) were compared against published data. The Connecticut River hydrilla plants are distinct from all known North American plants, representing a novel introduction, likely from northern Eurasia. The genetic novelty of this recent introduction may present additional ecological and management challenges beyond what has been encountered for hydrilla to date.

Key words: aquatic plants, invasive species, ITS, molecular phylogenetics, *PDS*.

INTRODUCTION

Hydrilla verticillata (L.f.) Royle ("hydrilla") is a submersed aquatic angiosperm of ecological and economic importance. Globally it is among the most noxious invasive aquatic plants because of its ability to adapt to a variety of environments and outcompete native vegetation (Langeland 1996, Haller 2014). In North America, hydrilla consists of two "strains," or "biotypes": a monoecious strain and a dioecious strain, the latter comprising only female individuals in the introduced range (Ryan et al. 1995). Phylogenetic evidence from chloroplast (*trnL-F* region) and nuclear gene regions (internal transcribed spacer [ITS] and phytoene desaturase [*PDS*]) has demonstrated that the introduced hydrilla strains in North America were derived from two distinct sources. The monoecious strain most closely matches hydrilla plants that are native to Korea, whereas the dioecious strain resembles plants from India (Madeira et al. 1997, 2007, Benoit et al. 2019) and also matches plants more recently introduced to South America (L. C. Lucio, unpub. data; Zhu et al. 2017, Benoit et al. 2019).

Molecular data from the chloroplast *trnL-F* marker initially established the phylogenetic distinctness of monoecious and dioecious strains in North America (Madeira et al. 1997, Madeira et al. 2007). Subsequent data from the nuclear ITS and *PDS* regions additionally documented the existence of widespread hybridization among hydrilla lineages worldwide, including many native populations in Eurasia and Australia (Benoit et al. 2019). Combined nuclear and chloroplast data present clear evidence that hydrilla plants worldwide harbor considerable genetic variation, which is correlated to some extent with biogeography (Benoit et al. 2019). Both the monoecious and dioecious strains in North America are predominantly triploid (Harlan et al. 1984, Langeland 1989), and their molecular sequences likely reflect a past hybridization event involving parental lineages native to Asia (Benoit et al. 2019).

Specimen collection data support a native range for hydrilla that extends from Eurasia to Australia, and samples from these regions are genetically distinct and diverse (Pieterse et al. 1984, Madeira et al. 1997, Madeira et al. 2007, Zhu et al. 2015, Efremov et al. 2017, Zhu et al. 2017, Williams et al. 2018, Benoit et al. 2019). Isolated but long-established populations apparently also are native in central Africa, Ireland, and central Europe (Cook and Lüönd 1982, Madeira et al. 2007). In contrast, plants introduced to North and South America, South Africa, and New Zealand have been documented fairly recently, with each introduction having minimal genetic variation and a distinct phylogenetic placement (Cook and Lüönd 1982, Cook 1985, Schmitz et al. 1991, Madeira et al. 2007, Sousa et al. 2009, Benoit et al. 2019).

In Connecticut, the first confirmed occurrence of hydrilla came from a pond at Mystic Seaport (New London County) in 1989 (Les et al. 1997). Initial testing suggested the population to be dioecious (Les et al. 1997), but later testing of hydrilla from the same pond (Madeira et al. 1997, Madeira et al. 2000, Madeira et al. 2004) confirmed the plants to be monoecious. More recently, hydrilla has been documented in several other Connecticut water bodies including Held Pond (= Crystal Lake) and Silvermine River (Fairfield County), Mason's Island (New London County), and Wangumbaug Lake (= Coventry Lake, Tolland County) (CAES IAPP 2019). Genetic testing has verified all of these

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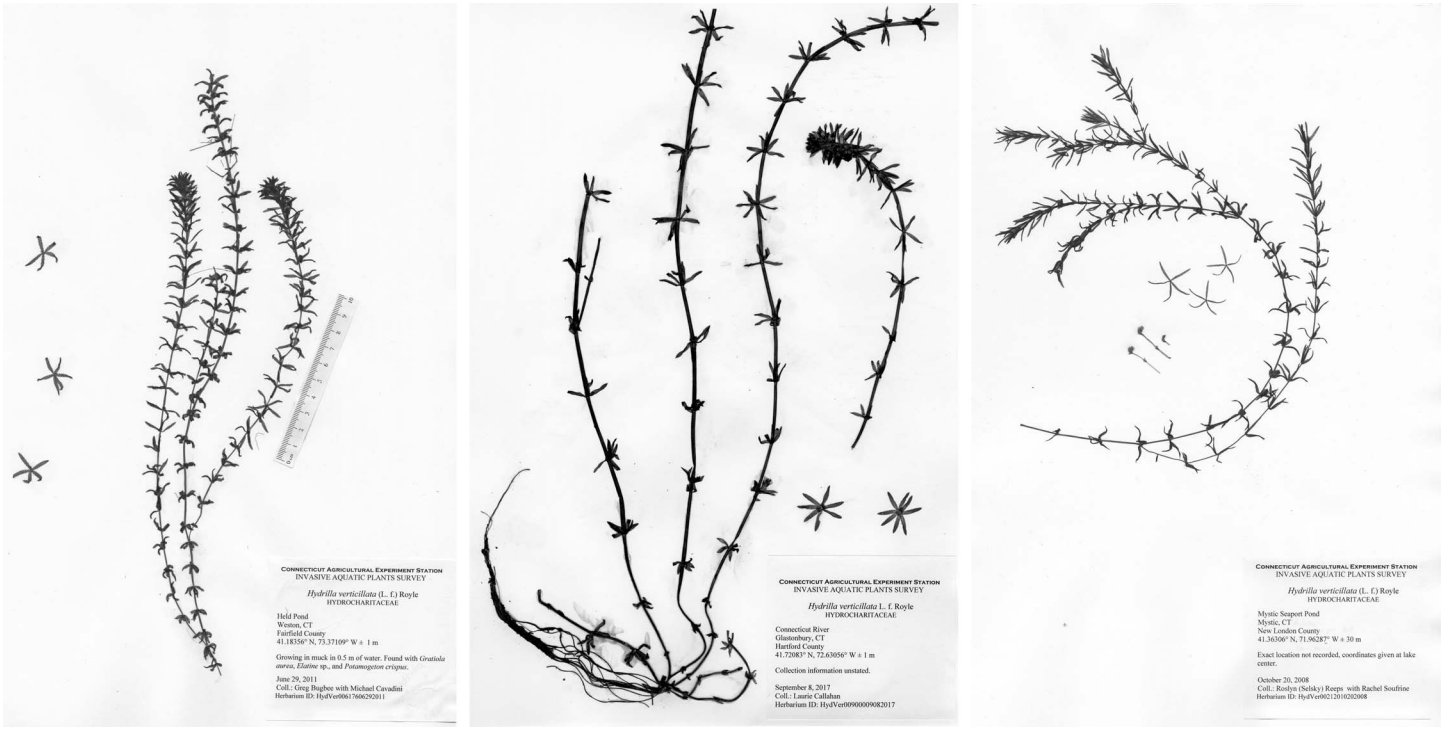

Figure 1. Comparison of the phenology of the Connecticut River hydrilla (center) with hydrilla from two Connecticut ponds (left and right). Leaves from previous Connecticut collections occur in whorls of five or six, whereas the Connecticut River plants exhibit whorls with six to eight or more leaves each. Connecticut River plants also differ by having wider leaves.

populations as monoecious hydrilla (Madeira et al. 2007, King and Les 2016, Benoit et al. 2019).

Winding 660 km from the Canadian border through New Hampshire, Vermont, Massachusetts, and Connecticut, the Connecticut River empties into Long Island Sound. The river and its 38 main tributaries drain a 2.9 Mha watershed that provides recreation, wild places, and working lands critical to New England's identity (Mullens and Bristow 2003, Marshall and Randhir 2008). Its water sustains terrestrial and aquatic ecosystems, farms, industry, and the domestic water needs of 2.3 million watershed residents (Clay et al. 2006). Hydrilla was discovered in the lower Connecticut River in 2016. Initial examination of Connecticut River specimens revealed morphological features that differed from hydrilla samples previously encountered in the state (Figure 1). These included a more robust nature, widely spaced whorls often of 5–10 leaves, and a darker color, and prompted concern that the Connecticut River hydrilla might be genetically different from the strains that are currently known to exist in North America.

Populations in a section of the Connecticut River in Glastonbury (Hartford County) have been known since 2016 (*Hagstrom s.n. 26 Sep 2016* [CONN00214689], *Lech s.n. 4 Jun 2016* [CONN00209603]), but their existence in a long, contiguous water body caused us to treat them with greater scrutiny. We obtained DNA sequence data from these hydrilla populations to compare them against the known genetic diversity of North American invasive hydrilla as well as native populations worldwide.

MATERIALS AND METHODS

In response to the confirmation of hydrilla in the Connecticut River in 2016 and further confirmed samples in 2017, a multistate task force was organized by the Silvio O. Conte National Fish Wildlife Refuge and Northeast Aquatic Nuisance Species Panel in 2018. The task force's goal was to survey the Connecticut River from New Hampshire/Vermont through Massachusetts and Connecticut, record hydrilla population locations using global positioning systems, and collect samples for genetic testing. Survey teams checked approximately 13 km (3%) of the 443 km of the New Hampshire/Vermont section, the entire 111 km (100%) of the Massachusetts section, and 86 km (81%) of the 106-km Connecticut section. The southernmost portion of the river was not surveyed due to the onset of cold weather. Surveyors located hydrilla both visually at the water surface and by using rakes and grapples to obtain more deeply submersed material. With the exception of the Connecticut Agricultural Experiment Station Invasive Aquatic Plant Program (CAES IAPP) team, all surveyors marked hydrilla location with point features using a handheld global positioning system (GPS) or cell phone GPS. The CAES IAPP team used a handheld GPS[1] and marked hydrilla locations less than 3 m in diameter with point features and those larger than 3 m in diameter with polygon features. Maps were produced using geographic information system software.[2,3] Eighteen hydrilla samples from distinct localities were collected, by hand or with a rake or grapple, for genetic testing (Figure 2).

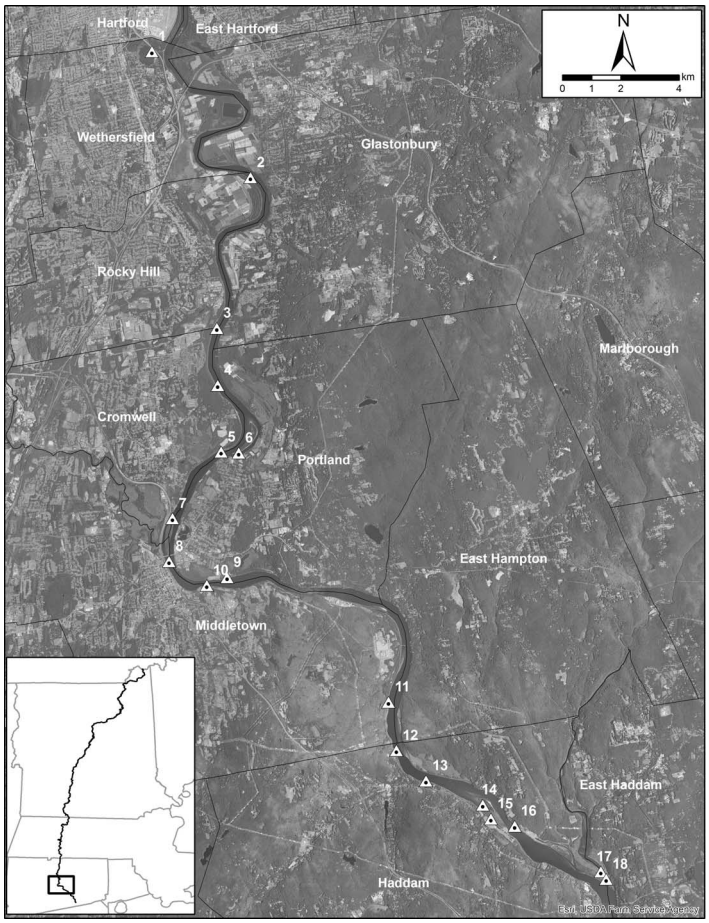

Figure 2. Localities in the section of the Connecticut River from which hydrilla plants were collected for genetic testing.

Genomic DNA was extracted using a modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987), quantified using a spectrophotometer,[4] and diluted to a concentration of 10 ng/µl. Polymerase chain reaction (PCR) was conducted to amplify the ITS region using the ITS-A and ITS-B primers (Blattner 1999) and a 55 C annealing temperature, the *PDS* gene using the 793F and 1208R primers (Benoit and Les 2013) and a 55 C annealing temperature, and the *trnL-F* region, using the c and f primers (Taberlet et al. 1991) and a 60 C annealing temperature. PCR reactions consisted of 2.5 ng genomic DNA, 0.5 µM of each primer, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1× buffer, 0.5 µl hot-start DNA polymerase,[5] and water to a final volume of 25 µl. PCR was conducted using a thermal cycler[6] program of 98 C for 30 s; then 35 cycles of 98 C for 5 s, annealing temperature for 5 s, and 72 C for 20 s; ending with 1 min at 72 C.

PCR products were cleaned by ethanol precipitation (final concentration of 70% ethanol and 0.3 M sodium acetate) and quantified using a spectrophotometer.[4] Sequencing reactions were conducted using the same primers that were used for PCR, in a reaction with 50 ng DNA, 5 pmol of primer, 1× buffer, 2 µl of BigDye,[7] and water to a final volume of 20 µl. Sequencing amplification was conducted using a thermal cycler[6] program of 96 C for 2 min; then 35 cycles of 96 C for 10 s, 52 C for 15 s, and 60 C for 3 min; ending with 1 min at 72 C. Amplified products were analyzed on a DNA analyzer.[8] Amplicons that were found to be polymorphic were subcloned into bacterial vectors,[9] followed by amplification and sequencing as above.

Sequence chromatograms were evaluated using the program 4Peaks ver. 1.8 (Griekspoor and Groothuis 2005) and exported as text files. Newly acquired sequences were combined with previously published sequences, selected from across the established diversity of sequences (Madeira et al. 2007, Zhu et al. 2015, Benoit et al. 2019), and aligned manually in the program Mesquite ver. 3.6 (Maddison and Maddison 2018). Insertions/deletions (indels) were scored for the *trnL-F* and *PDS* alignments using simple indel coding (Simmons and Ochoterena 2000) implemented with the program SeqState ver. 1.4.1 (Müller 2005). With the exception of invasive U.S. populations, previously studied accessions with polymorphic ITS or *PDS* sequences were not included for simplicity. Phylogenetic analyses were conducted in BEAST ver. 1.10.2 (Drummond and Rambaut 2007) using the Hasegawa-Kishino-Yano (HKY) model of evolution (Hasegawa et al. 1985), following model selection using jModelTest ver. 2.1.10 (Guindon and Gascuel 2003, Darriba et al. 2012). Summary trees were rooted according to previously determined phylogenetic relationships for hydrilla (Benoit et al. 2019).

RESULTS

No hydrilla was found from the New Hampshire/Vermont sections of the Connecticut River southward into lower Massachusetts. Approximately 2 km north of the Connecticut border, the first population of hydrilla was found. From this point south hydrilla sightings became more frequent. From Hartford to approximately 20 km from the river's outlet into Long Island Sound, hydrilla sightings were common, with coverages ranging from single plants to large dense patches. The largest patch recorded by the CAES IAPP covered 4.4 ha and was located in a shallow cove near Hurd State Park in the town of East Hampton. Plants were found in 0 to 2 m of water. Because the Connecticut River is tidal where the hydrilla was found (with a tidal range of 1 m or less; NOAA 2019), some of the plants were exposed briefly at low tide. No flowers, turions, or tubers were observed, although detailed inspections for tubers were not performed. Fragmentation was extensive and floating plant parts were common.

We obtained sequence data from 18 Connecticut River hydrilla collections (Figure 2). Sequences for all three gene regions (ITS, *PDS*, *trnL-F*) were identical across all specimens (Appendix 1). Sequences for the ITS and *trnL-F* regions of the Connecticut River hydrilla were monomorphic and most similar to sequences obtained from Ireland, Japan, Latvia, and South Korea (Figure 3; Benoit et al. 2019). The plastid *trnL-F* sequences for the Connecticut River hydrilla had greatest similarity to the previously identified haplotype H8/H9, representing "clade C" (Zhu et al. 2015, Benoit

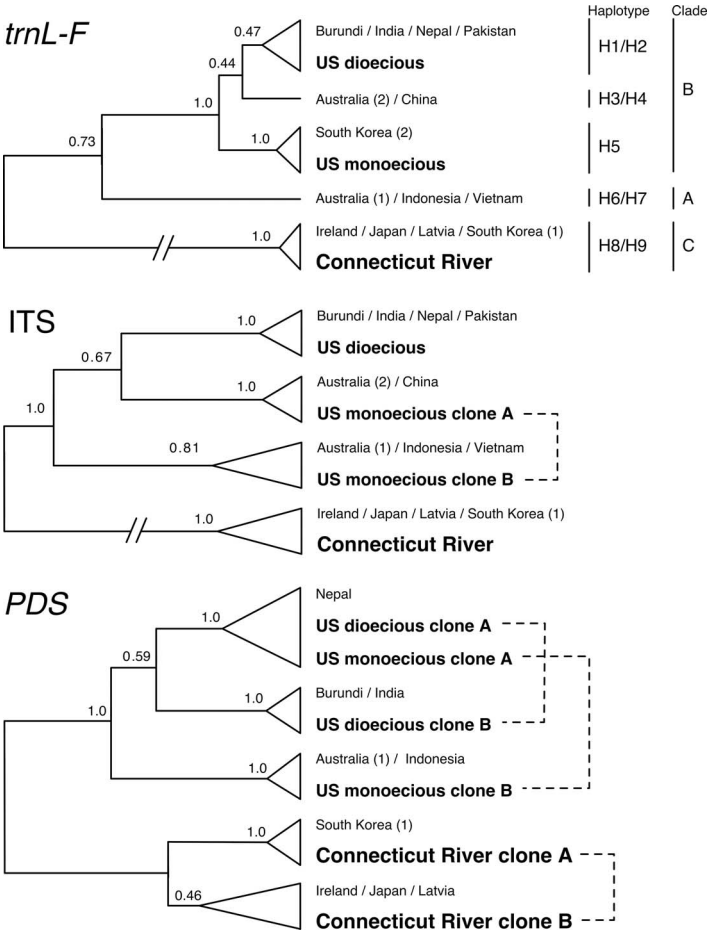

Figure 3. Phylogenetic relationships among hydrilla lineages, determined by analyzing the plastid *trnL-F* region and the nuclear ITS and *PDS* regions. Polymorphic sequences for the same accession, where present, are connected with dotted lines. Groups of closely related sequences are collapsed, and nodal support values indicate Bayesian posterior probability.

et al. 2019). The *PDS* sequences for the Connecticut River hydrilla were polymorphic, with elements that were most similar to sequences obtained from Ireland, Japan, Latvia, and Korea (Benoit et al. 2019).

DISCUSSION

Until recently, the *trnL-F* region was used exclusively to evaluate diversity and phylogenetic relationships among hydrilla plants worldwide. Phylogenetic evidence from *trnL-F* has strongly supported the inference of two independent origins for monoecious and dioecious strains in North America, which were both different from the origin of invasive plants in South Africa (Madeira et al. 2007). More recent evidence from the nuclear ITS and *PDS* regions also supported the genetic distinctness of introduced hydrilla strains and additionally presented evidence that some populations, both native and introduced, represent genetic combinations (i.e., intraspecific hybrids), potentially from distant geographic areas (Benoit et al. 2019). Both the monoecious and dioecious strains in North America have at least one nuclear locus with such genetic combinations. Introduced hydrilla from South Africa, by contrast, have monomorphic sequences at both nuclear loci, with consistent affinity to plants growing natively in Indonesia (Benoit et al. 2019).

The plants we collected from the lower Connecticut River are genetically distinct from all known introduced hydrilla, with respect to all three loci that we sequenced (ITS, *PDS*, and *trnL-F*). They most closely match plants from northern Eurasia, where the species is sparsely distributed but likely native (Cook and Lüönd 1982, Efremov et al. 2017). Plants from northern Eurasia (Ireland, Japan, Latvia, and South Korea) show consistent similarity across plastid and nuclear phylogenetic analyses, and they are genetically distinct from plants found in more tropical latitudes (Benoit et al. 2019). Although the lower Connecticut River hydrilla plants are most similar to plants from Eurasia, they are not absolutely identical to any previously sequenced accessions. The sequences we obtained for *trnL-F* and ITS matched a variety of Eurasian plants, but the *PDS* sequences were polymorphic, containing a combination of genetic elements that matched plants from Ireland, Japan, Latvia, and South Korea. The polymorphic *PDS* sequences from Connecticut River plants may have resulted from an intraclade hybridization event, i.e., genetic recombination between lineages that are genetically distinct at the *PDS* locus but nevertheless share the same plastid haplotype. A similar polymorphic pattern for *PDS* exists in plants from South Korea (*Na* 90155-3 [CONN00225401]); however, these plants also are polymorphic for the ITS region (Benoit et al. 2019).

Hydrilla plants have a wide native range, a large portion of which has been sampled for molecular sequence data (Madeira et al. 2007, Zhu et al. 2015, Benoit et al. 2019). Nonetheless, there may be genetic elements in the native range that have not been identified yet. For example, hydrilla plants in China exhibit a wide variety of plastid haplotypes, some with close affinity to the Connecticut River plants (Zhu et al. 2015). Moreover, hydrilla exists widely in cultivation, where the plant is desirable for aquaria. Cultivated plants worldwide likely have multiple geographic origins, potentially with no traceable record of their original collection sites.

Besides being informative for phylogenetic reconstruction, variation in the coding region of *PDS* sequences also has been shown to correlate strongly with resistance to the herbicide fluridone, which is widely used to treat hydrilla (Michel et al. 2004). Plants with the "wild-type" sequence at a specific codon position are susceptible to fluridone, whereas nonsynonymous mutations at the codon position confer some degree of fluridone resistance (Michel et al. 2004). *PDS* sequences from Connecticut River hydrilla plants showed the wild-type CGT codon and presumably would be vulnerable to fluridone treatment (Michel et al. 2004, Benoit et al. 2019).

The high degree of genetic variation among native hydrilla populations and their pattern of interbreeding makes it rather difficult to ascribe a single locality of origin for the Connecticut River plants. Potential candidate regions include Ireland, northern Europe, China, Japan, and Korea. Regardless of their geographic origin, it is clear

that these plants represent a unique genetic element in North America and one that should be monitored carefully. In particular, the more northern distribution of putative source localities may give Connecticut River plants an adaptive edge in northern regions of the invasive range. Ecological differences are known to exist between the established monoecious and dioecious invasive strains: the monoecious strain (distributed more in temperate regions) undergoes winter senescence via turion production, whereas the dioecious strain (predominantly subtropical) grows more consistently throughout the year (True-Meadows et al. 2016, Jacono et al. 2019a, Jacono et al. 2019b). As a heretofore unknown ecological entity, the newly discovered hydrilla strain potentially could have a unique invasion strategy requiring novel management approaches.

SOURCES OF MATERIALS

[1]R1 GNSS®, Trimble Inc., 935 Stewart Dr., Sunnyvale, CA 94085.

[2]Pathfinder® 5.85, Trimble Inc., 935 Stewart Dr., Sunnyvale, CA 94085.

[3]ArcGIS Desktop 10.6.1®, ESRI Corp., 380 New York St., Redlands, CA 92373.

[4]NanoDrop™ 2000 spectrophotometer, Thermo Fisher Scientific, Inc., 168 3rd Ave., Waltham, MA 02451.

[5]Phire Hot Start II DNA Polymerase, Thermo Fisher Scientific, Inc., Waltham, MA 02451.

[6]Bio-Rad S1000™ Thermal Cycler, Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Dr., Hercules, CA 94547.

[7]BigDye™ v.3.1, Thermo Fisher Scientific, Inc., Waltham, MA 02451.

[8]Applied Biosystems™ 3730xl DNA Analyzer, Thermo Fisher Scientific, Inc., Waltham, MA 02451.

[9]pGEM®-T Easy Vector System, Promega Corporation, 2800 Woods Hollow Rd., Fitchburg, WI 53711.

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APPENDIX 1

GenBank accession numbers (ITS, *PDS*, *trnL-F*) for sequences from representative populations that were used to construct the backbone hydrilla phylogeny. Multiple cloned sequences were obtained from U.S. plants for ITS (monoecious only) and *PDS* (monoecious and dioecious). Dashes (—) indicate sequences that were not available. Voucher information is given for newly reported sequences, which are indicated with asterisks (*).

AUSTRALIA: (1) MK819362, MN013215, MN013318; (2) MK819334, —, MN013326; **BURUNDI:** MK819293, MN013267, EF458070; **CHINA:** MK819342, —, MN013327; **INDIA:** MK819301, MN013269, EF458065; **INDONESIA:** MK819339, MN013224, EF458056; **IRELAND:** MK819396, MN013259, MN013305; **JAPAN:** MK819403, MN013257, EF458053; **LATVIA:** MK819397, MN013260, MN013313; **NEPAL:** MK819305, MN013300, EF458066; **PAKISTAN:** MK819306, —, EF458063; **SOUTH KOREA:** (1) MK819405, MN013200, MN013312; (2) —, —, AY496144; **United States: Connecticut (monoecious biotype):** MK819349 / MK819374, MN013220 / MN013272, MN013332; **Connecticut River, *Stebbins s.n. 1 Oct 2018* (UWW):** MN176615*, MN180201*/MN180202*, MN180203*; **Florida (dioecious biotype):** MK819313, MN013245/MN013291, MN013333; **VIETNAM:** MK819387, —, EF458059.