An Improved Molecular Tool for Distinguishing Monoecious and Dioecious Hydrilla

PAUL T. MADEIRA, THAI K. VAN, AND TED D. CENTER[1]

ABSTRACT

Two biotypes of hydrilla [*Hydrilla verticillata* (L.f.) Royle] occur in the United States, a dioecious type centered in the southeast and a monoecious type in the central Atlantic and northeastern states. Ecosystem managers need tools to distinguish the types as the ranges of each type expand and begin to overlap. A molecular tool using the randomly amplified polymorphic DNA (RAPD) procedure is available but its use is limited by a need for reference samples. We describe an alternative molecular tool which uses "universal primers" to sequence the trnL intron and trnL-F intergenic spacer of the chloroplast genome. This sequence yields three differences between the biotypes (two gaps and one single nucleotide polymorphism). A primer has been designed which ends in a gap that shows up only in the dioecious plant. A polymerase chain reaction (PCR) using this primer produces a product for the monoecious but not the dioecious plant.

INTRODUCTION

Hydrilla, a submersed aquatic plant belonging to the monocot family Hydrocharitaceae, is widely distributed throughout Asia and many of the Pacific Islands from Japan to New Zealand. Localized and disjunct populations also occur in Africa and Europe (Cook and Lüönd 1982, Pieterse 1981). In the early 1950s a dioecious female biotype was introduced from Sri Lanka to Florida by a tropical fish and plant dealer (Schmitz et al. 1990) and was subsequently identified in 1959 (Blackburn et al. 1969). The dioecious plant has spread throughout the south as far west as Texas with separate distributions in California (Yeo and McHenry 1977, Yeo et al. 1984). In 1976 a second, monoecious plant was discovered from Delaware and, in 1980, in the Potomac river (Haller 1982, Steward et al. 1984). The monoecious plant has spread through the Atlantic states with populations occurring as far south as Georgia and with northern populations reported in Pennsylvania, Connecticut (Madeira et al. 2000), Massachusetts and Maine (NAS database[2]). Separate and disjunct monoecious populations also occur in California (Ryan and Hommberg 1994) and Washington State (Anderson 1996). Both dioecious and monoecious populations occur within the same or adjacent USGS Hydrological Units (HUC) in North Carolina (Ryan et al. 1995), South Carolina and Georgia (Madeira et al. 2000).

Although biotype cannot be definitively identified without flowering, a reasonable guess may be made by observing growth habit. The dioecious plant is generally more robust, produces smaller numbers of subterranean turions (commonly known as tubers) of greater size, and produces them only under short-day conditions as compared to the monoecious biotype. Shoots of sprouting dioecious tubers grow vertically towards the surface rather than laterally like the monoecious form (Van 1989). This growth habit may correlate with it's likely origin on the Indian subcontinent (Madeira et al. 1997) and adaption to deep waters which can fluctuate rapidly during monsoon season. Tubers of the monoecious biotype sprout at lower temperatures (Steward and Van 1987), after which their stems grow laterally along the soil surface, producing more new root crowns and higher shoot densities than dioecious hydrilla (Van 1989). Tubers are also produced by the monoecious biotypes under long-day photoperiods during the summer (Van 1989). In the fall under short-day conditions, an induced flush of both axillary turions and tubers occurs as the monoecious hydrilla mat declines. The mat then breaks loose from the substrate, and the axillary turion laden fragments drift downstream (Steward and Van 1987). This growth habit suggests a temperate plant, which is consistent with the probable Korean origin (Madeira et al. 1997). Langeland (1996) and Madeira et al. (2000) discuss the management implications of these biotype differences.

As hydrilla spreads and the biotype ranges begin to intersperse, the need for resource management tools to keep track of infestation locality and biotype has increased. The United States Geological Survey (USGS) offers a repository for geographic accounts of nonindigenous aquatic organisms called the Nonindigenous Aquatic Species (NAS) database, which includes the distribution of hydrilla in U.S. watersheds (Madeira et al. 2000). Staff scientists assemble and evaluate spatial information from many sources including literature, monitoring programs, museum accessions, professional communications and a web site[2] reporting form. Spatial information is geo-referenced to drainage basin or watershed according to USGS HUC. The data is used to produce dynamic distribution maps available to resource managers and the public through internet access[2]. Because hydrilla's growth habit displays a great deal of environmental plasticity, environmental managers have sought a more definitive tool for rapid biotype identification of plants without flowers. The first definitive tool made available was the use of isozymes (Verkleij et al. 1983, Ryan 1988). However, isozyme procedures require a good deal of experience to implement and are seldom easily accessible to managers. Ryan and Holmberg (1994) and Ryan et al. (1995) presented an inexpensive and easily run molecular assay using random amplified polymorphic DNA (RAPD) and primer Operon G17. However, RAPDs are notoriously difficult to replicate between labs so identification is definitive only when the reaction is run with both monoecious and dioecious positive controls (Madeira et al. 2000).

This report presents the discovery of an additional molecular tool useful for distinguishing monoecious and dioecious hydrilla. This procedure is inexpensive, can be quickly run by most molecular laboratories, is reproducible, and requires no positive hydrilla controls.

MATERIALS AND METHODS

Plant material and DNA extraction. Dioecious samples originated from Ft. Lauderdale, Florida (26N, 80W); Martin Creek L., Rusk Co., Texas (32N, 94W); Rodemacher L., Louisiana (31N, 93W) and Spruce Lateral 4, Brawley, California (33N, 116W). Monoecious samples originated from Lake Anne, North Carolina (36N, 79W), Trapp Pond, Delaware (38N, 75W), Schuylkill River, Philadelphia, Pennsylvania (40N, 70W), Lucerne/Pipe Lakes complex, Washington State (47N, 122W) and Mystic, Connecticut (41N, 72W). Bangalore, India (13N, 77E) and Seoul, Korea (37N, 127E), "nearest neighbor" plants determined most similar genetically to the biotypes presently found in the U.S. according to the RAPD analysis of Madeira et al. (1997) were also selected. Most of the plants analyzed here are identical to those presented in Madeira et al. (2000) where more details are available. Sample apical stem fragments were thoroughly rinsed in a jet of deionized water and blotted dry. Approximately 50 mg wet weight of leaves were then placed into a 1.5-ml microcentrifuge tube and stored in an ultracold freezer at -80 C. Total DNA was extracted using the "micro" method of Van and Madeira (1998). The DNA solution was quantified using fluorometry and stored at 4 C.

PCR & Sequencing. PCR amplification reactions contained 1× reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 1% Triton X-100), 0.5 mM Betaine, 0.2 mM dNTPs, 0.5 μM each primer and 0.04 U/μl Taq polymerase (New England Biolabs[3]). PCR products for sequencing were generated using the "c" (CGAAATCGGTA-

GACGCTACG) and "f" (ATTTGAACTGGTGACACGAG) universal primers located on the trnL (UAA) 5' exon and the trnF (GAA) gene, respectively (Taberlet et al. 1991). The PCR product covers two non-coding regions of chloroplast DNA, the trnL intron and the trnL-F intergenic spacer. Non-coding regions usually display greater variation than coding regions and are therefore useful regions to look for intra-specific variation. Reaction tubes were added to a preheated block held at 94 C, incubated at 94 C for 3 min, then cycled 35 times (denaturation: 94 C, 1 min; annealing: 55 C, 1 min; extension: 72 C, 3 min) followed by a final extension of 5 min at 72 C. Amplification products were electrophoresed on 1.4% agarose gels and visualized with ethidium bromide to check for amplification. PCR products were purified using the Qiaprep 96 Turbo Kit (Qiagen[3]) using a Bio-Robot 9600 (Qiagen[3]). Approximately 25 ng of purified PCR products was used as template for sequencing. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems[3]) in a 10 mL reaction volume. In addition to primers "c" and "f" the internal reverse primer "d" (GGGGATAGAGGGACTTGAAC) and forward primer "e" (GGTTCAAGTCCCTCTATCCC), both located on the conserved trn L (UAA) 3' exon, were used as sequencing primers (Taberlet et al 1991). Cycle-sequencing products were precipitated using four volumes of 70% iso-propanol for 30 min, pelleted, washed with 70% ethanol, re-centrifuged, dried, and the pellet re-suspended in 15 µL of sterile water. The product was then loaded onto a DNA analyzer 3700 (Applied Biosystems[3]). Sequences were edited and aligned using Sequencher 4.1.4 (Gene Codes[3]) then further viewed using Bioedit (Hall 1999).

RESULTS AND DISCUSSION

All monoecious samples produced identical sequences. All dioecious samples also yielded identical sequences that differed from the monoecious samples. The Seoul, Korea sample produced a sequence that was identical to the monoecious plants from the U.S. The Bangalore, India sample was identical to the dioecious plants from the U.S. All sequences are accessible at GenBank as "tRNA-Leu (trnL) gene, partial sequence; trnL-trnF intergenic spacer, complete sequence" and as "tRNA-Phe (trnF) gene, partial sequence". The monoecious read available for all sequences and submitted to GenBank was 1146 base pairs (b.p.) in length while the dioecious was 1132. Some sequences generated longer reads producing short additional leading and trailing sequences which extend into the primer "c" and "f" regions (Figure 1). The PCR product size using the "c" and "f" primers can be calculated for the monoecious plant as 1191 b.p. and for the dioecious plant as 1177 b.p. The PCR product using the "c" and "d" primers (TrnL) is 686 b.p. for monoecious and 685 b.p. for dioecious while the product using the "e" and "f" primers (TrnL-F) is 526 b.p. for monoecious and 513 b.p. for dioecious.

The monoecious and dioecious sequences differ in three places; two are gaps and one is a single nucleotide polymorphism (SNP). Figure 1 presents the variable regions as well as the regions where the primers anneal. The sequences are

```
          * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | *      * | * * * * | * * * * | * * * * | * * * * | *
                      10          20          30       130         140         150
monoecious                            CTTGATTGG  TTTTGAAAAAAAAAAACAAAAAA
dioecious                             CTTGATTGG  TTTTGAAAAAAAAA.CAAAAAA
leading sequence              TACGGA
primer c          CGAAATCGGTAGACGCTACG ------->


          | * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | *
                      670         680         690         700         710
monoecious        GGGTTCAAGTCCCTCTATCCCCAATAAAAATCCCCAATAAAATAAAA
dioecious         GGGTTCAAGTCCCTCTATCCCCAAT.............AAAATAAAA
primer d          <----- CAAGTTCAGGGAGATAGGGG
primer e              GGTTCAAGTCCCTCTATCCC ------->
biotype gap primer           CCCTCTATCCCCAATAAAAATCC ------->


          * | * * * * | * * * * | * *      * | * * * * | * * * * | * * * * | * * * * | * * * * | * * * * | *
          1120        1130       1160        1170        1180        1190
monoecious  AAAAGAGTAGTCGA    GAGGACTGAA
dioecious   AAAAGGGTAGTCGA    GAGGACTGAA
trailing sequence                         AATCCTCGTG
primer f                          <----- GAGCACAGTGGTCAAGTTT
```

Figure 1. Sequence differences between monoecious and dioecious hydrilla for the chloroplast trnL intron and trnL-F intergenic spacer: position 144—a single nucleotide (A) gap; position 690—a 13 base gap; position 1124—a single nucleotide polymorphism (SNP) where the base is "A" for the monoecious type and "T" for the dioecious type. The leading and trailing sequences represent regions of poor sequence quality near to the PCR primers which were not readable in all the samples. Primers "c" and "d" produce the trnL sequence while "e" and "f" produce the trnL-F sequence. The biotype gap primer, designated primer "h" in the text, is presented with it's trailing edge positioned relative to the annealing site on the monoecious plant and the gap in the dioecious plant.
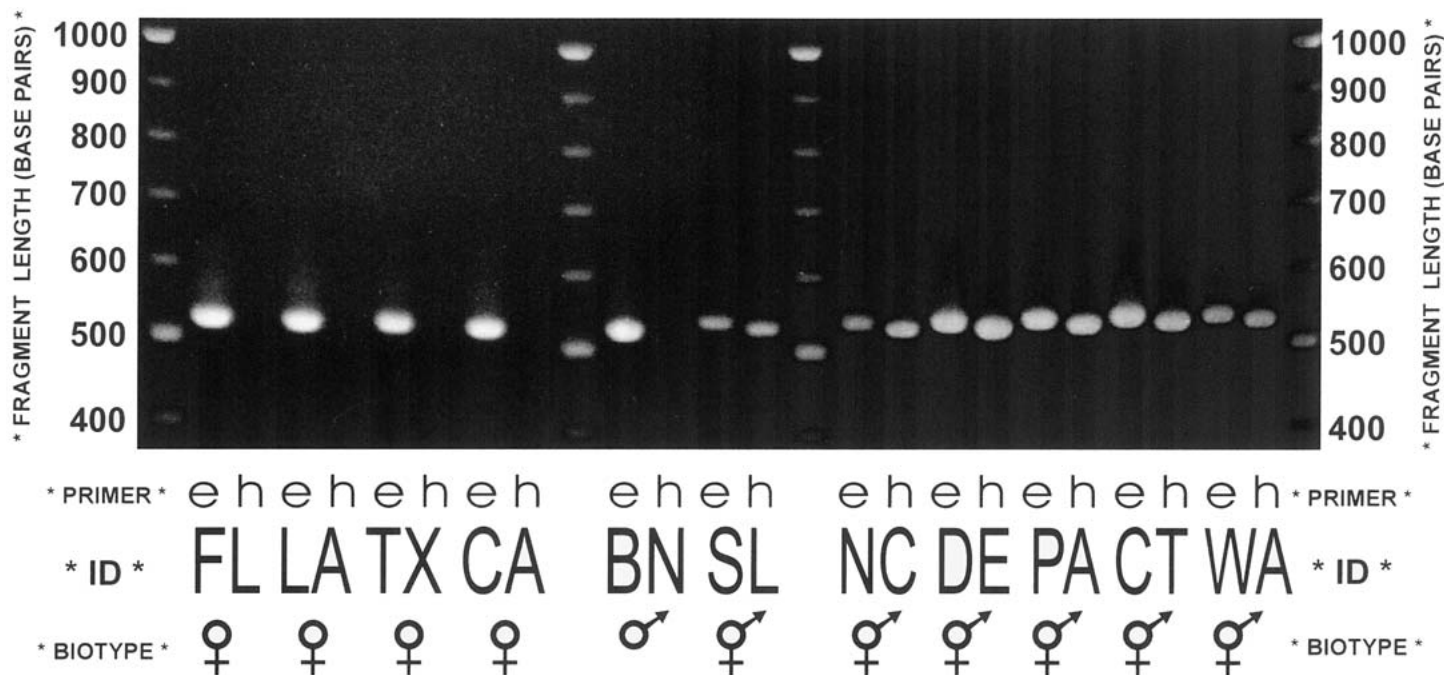
Figure 2. Results of the hydrilla gap "h" primer reaction diagnostic for the U.S. monoecious (⚥) and dioecious (♀) biotypes. Note the absence of "h" product for the dioecious plants. Results of the "e" primer reaction are also presented as positive controls and appear in both types. Each primer was spotted independently in tubes and master mix containing the reverse primer "f" added to each tube. Monoecious plants shown are North Carolina (NC), Delaware (DE), Pennsylvania (PA), Connecticut (CT), and Washington State (WA). Dioecious plants include Florida (FL), Louisiana (LA), Texas (TX) and California (CA). Nearest neighbor plants representing the probable origins of the domestic plants include a dioecious male plant (♂) from Bangalore, India (BN) and a monoecious plant from Seoul, Korea (SL).

presented as they appear in GenBank and are aligned with the primer sequences and with the probable leading and trailing sequences. Arrows indicate the direction of replication from each primer. The first difference is a single nucleotide gap in a series of "A" repeats which appears at position 144. This is the only difference within the TrnL intron. The second gap is 13 bases long and appears 5 bases beyond primer "e" at position 690. The final change is a SNP at position 1124 where the base is "A" for the monoecious type and "T" for the dioecious type.

As a practical matter, managers who wish to identify the biotype of a sample can now enlist any molecular lab with sequencing capability to extract the DNA, perform the PCR, cleanup and sequencing. No reference material is necessary and repeatability is not an issue, as is the case with the RAPD procedure (Madeira et al. 2000, Ryan et al. 1995).

In some situations small molecular labs may not have ready access to sequencing or, alternatively, population analysis requiring large numbers of samples may make the cost of sequencing high. An alternative approach has been developed which uses a "biotype gap primer" [CCCTCTATC-CCCAATAAAAATCC] designed to end inside the 13 b.p. gap which appears near primer "e" (see Figure 1). This primer, designated "h", in combination with primer "f", amplifies only monoecious samples when stringent conditions (an annealing temperature of 66 C) are used. In contrast, primers "e" and "f" generate a product for both biotypes. Therefore, genomic DNA may be added to paired reaction tubes, primer "e" spotted in one, the "h" primer spotted in the other, a simple master mix (1× reaction buffer, 0.2 mM dNTPs, 0.5 μM primer "f"

and 0.04U/μl Taq polymerase) added to each tube, and both tubes placed in the thermal cycler (40 cycles). If the sample produces product in both combinations it is monoecious while if it appears only with the "e" primer it is dioecious. Note that while other conditions of the thermal cycling are the same as presented in Materials and Methods, the annealing temperature must be at least 65 C or mis-priming may occur producing product in dioecious samples as well. Figure 2 presents an agarose gel with this reaction for the samples also sequenced in this study. The monoecious samples from North Carolina, Delaware, Pennsylvania, Connecticut, and Washington State along with the "nearest neighbor" plant from Seoul, South Korea show amplification products for both the "e" and "h" primers, while the dioecious samples from Florida, Louisiana, Texas, and California, along with the "nearest neighbor" plant from Bangalore, India show amplification products for only the "e" primer. The "e" primer therefore serves as a positive control assuring the quality of the DNA template and of the reaction mixture. It is nevertheless suggested that if the "h" primer indicates the introduction of a biotype to a new region that this be confirmed by sequencing.

of the USDA Horticultural Research Laboratory in Ft. Pierce, Florida for the assistance of their Sequencing Laboratory.

LITERATURE CITED

Anderson, L. 1996. Eradicating California's hydrilla. Aquatic Nuisance Species Digest 1(3): 1,29,31-33.

Blackburn, R. D., L. W. Weldon, R. R. Yeo and T. M. Taylor. 1969. Identification and distribution of certain similar-appearing submersed aquatic weeds in Florida. Hyacinth Control J. 8:17-23.

Cook, C. D. K. and R. Lüönd, 1982. A revision of the genus *Hydrilla* (Hydrocharitaceae). Aquat. Bot. 13:485-504.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Haller, W. T. 1982. Hydrilla goes to Washington. Aquatics 4(4):6-7.

Langeland, K. A. 1996. *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), "The perfect aquatic weed." Castanea 61:293-304.

Madeira, P. T., T. K. Van, K. K. Steward and R. J. Schnell. 1997. Random amplified polymorphic DNA analysis of the phenetic relationships among world-wide accessions of *Hydrilla verticillata*. Aquat. Bot. 59:217-236.

Madeira, P. T., C. C. Jacono and T. K. Van. 2000. Monitoring hydrilla using two RAPD procedures and the nonindigenous aquatic species database. J. Aquat. Plant Manage. 38: 33-40.

Pieterse, A. H. 1981. *Hydrilla verticillata*—a review. Abstracts on Tropical Agriculture. Vol. 7, Royal Tropical Institute, Amsterdam, pp. 9-34.

Ryan, F. J. 1988. Isoenzymes of turions of *Hydrilla verticillata* (L.f.) Royle from the USA. Aquat. Bot. 31: 337-345.

Ryan, F. J., C. R. Coley and S. H. Kay. 1995. Coexistence of monoecious and dioecious hydrilla in Lake Gaston, North Carolina and Virginia. J. Aquat. Plant Manage. 33:8-12.

Ryan, F. J. and D. L. Holmberg. 1994. Keeping track of hydrilla. Aquatics 16 (2):14-20.

Schmitz, D. C., B. V. Nelson, L. E. Nall and J. D. Schardt. 1990. Exotic aquatic plants in Florida: a historical perspective and review of the present aquatic plant regulation program. Proc. Symp. on Exotic Pest Plants, Univ. Miami, Nov. 2-3, 1988, Miami, FL, pp. 303-326.

Steward, K. K., T. K. Van, C. Carter and A. H. Pieterse. 1984. Hydrilla invades Washington, DC, and the Potomac. Am. J. Bot. 71: 162-163.

Steward, K. K. and T. K. Van, 1987. Comparative studies of monoecious and dioecious hydrilla (*Hydrilla verticillata*) biotypes. Weed Sci. 35:204-210.

Taberlet, P., L. Geilly, G. Pantou and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17:1105-1109.

Van, T. K. 1989. Differential responses to photoperiods in monoecious and dioecious *Hydrilla verticillata*. Weed Sci. 37:552-556.

Van, T. K. and P. T. Madeira. 1998. Random amplified polymorphic DNA analysis of water spinach (*Ipomoea aquatica*) in Florida. J. Aquat. Plant Manage. 36:107-111.

Verkleij, J. A. C., A. H. Pieterse, H. P. M. Staphorst and K. K. Steward, 1983. Identification of the two different genotypes of *Hydrilla verticillata* (L.f.) Royle in the U.S.A. by means of isoenzyme studies. Proc. Int. Symp. Aquat. Macrophytes. Nijmegen, The Netherlands, pp. 251-261.

Yeo, R. R. and W. H. McHenry. 1977. Hydrilla, a new noxious aquatic weed in California. Calif. Agric. 31:4-5.

Yeo, R. R., R. H. Faulk and J. R. Thurston. 1984. The morphology of hydrilla [*Hydrilla verticillata* (L.f.) Royle]. J. Aquat. Plant Manage. 22:1-17.