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Genetic Relationships among Invasive Hydrilla (*Hydrilla verticillata* L.f. Royle) Biotypes in the US and Their Implications for Management

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INTRODUCTION: *Hydrilla verticillata* L.f. Royle (hydrilla; Hydrocharitaceae), an invasive submersed plant native to Southeast Asia and Australia, was first introduced into the US through the aquarium trade in Florida in the 1950s (Schmitz et al. 1991). Hydrilla impacts freshwater resources in the US by its aggressive, canopy-forming growth that degrades aquatic ecosystems, limits recreation, fouls boat motors, and clogs irrigation and hydroelectric plants.

In the US there are two recognized hydrilla biotypes: the dioecious biotype is generally found in the southeastern and south central US; whereas the monoecious biotype is mostly found in the central Atlantic and northeastern US (Madeira et al. 2000). The dioecious biotype has been present, and spreading, in the US since the first introduction over 50 years ago. In contrast, monoecious hydrilla is widely recognized as a relatively recent introduction, first identified in the northeastern United States in 1982 (Steward et al. 1984). Since the initial identification, monoecious hydrilla has been reported in Connecticut, Massachusetts, Maine, New York, and as far south as Georgia and Alabama. Additional populations of the monoecious biotype are known to be found in Washington and California (Madeira et al. 2000).

Management of hydrilla is typically accomplished through mechanical, chemical, and biological means. Mechanical harvesting of hydrilla has been attempted over the years but it is typically cost prohibitive and results in production of additional plant propagules through fragmentation. Chemical applications include complexed copper, diquat, endothall, fluridone, and — more recently — imazamox, penoxsulam, bispyribac-sodium, and flumioxazin (Gallagher and Haller 1990, Netherland 2009). In the 1970s and 1980s, partly because of the successes of the *Alternanthera philoxeroides* (Mart.) Griseb. (alligatorweed) and the *Eichhornia crassipes* Kunth (water hyacinth) biological control programs, the United States Department of Agriculture (USDA) and the United States Army Corps of Engineers (USACE) began overseas searches for insect biological control agents of hydrilla in its native range (Bennett and Buckingham 2000). These surveys and subsequent host-specificity testing culminated in the release of four insect agents, *Hydrellia pakistanae* Deonier (Diptera: Ephydriidae), *H. balciunasi* Bock, *Bagous affinis* Hustache (Coleoptera: Curculionidae), and *B. hydrillae* O'Brien in the late 1980s and early 1990s (Buckingham and Balciunas 1994). Because the initial foreign surveys for biological control agents were conducted before it was known that multiple biotypes were present in the US, it is not clear whether the surveyed plants in the native range were dioecious (present, at the

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time, in the US) or monoecious (not yet introduced into the US). This question has been raised by various authors in recent years (Grodowitz et al. 1997, Benoit 2011), and could partially explain the mixed results (i.e., some successes and some failures) in the hydrilla biocontrol program (Grodowitz et al. 2010). While recent foreign explorations of herbivores for dioecious hydrilla have taken place in Africa and southern China (Copeland et al. 2011, Copeland et al. 2012, Zhang et al. 2012), examination of monoecious plants for natural enemies has yet to take place.

It has recently become apparent that monoecious hydrilla may be minimally affected by the current insect biocontrol agents available on dioecious hydrilla (Grodowitz et al. 2010), due to an apparent clash of monoecious hydrilla phenology (winter senescence and lack of water-column biomass) with overwintering requirements of the agents (*H. pakistanae* larvae utilize available water-column plant material to survive winter months). Because of this, it is important to begin searches for additional agents. A number of authors have suggested that both host specificity and efficacy of biocontrol agents should be highest in the source region of the invader and have recommended “biotype matching” of target plants and candidate natural enemies (e.g., Roderick and Navajas 2003, Wardill et al. 2005, Goolsby et al. 2006, Manrique et al. 2008). This strategy is predicated on the idea that host-specific natural enemies will be locally adapted to their hosts and that local adaptation of the herbivore will result in higher population growth rates and greater damage to their hosts (Hufbauer and Roderick 2005). For this reason, it is important to identify the geographic source of monoecious hydrilla.

With refinement of molecular techniques, the genetic variability between and within hydrilla populations has been examined by various researchers and has led to a clearer understanding of the geographic origin of both hydrilla biotypes. The goal of this technical note is to summarize and report the most recent information available on the geographic origin of both hydrilla biotypes and discuss the implications of genetic differences with regard to management of hydrilla in the US.

DISCUSSION

Phylogenetic Relationships within the Genus *Hydrilla*. *Hydrilla* is native to the Old World and has a very broad geographic distribution in Asia and Australasia (Cook and Lüönd 1982). Disjunct populations also exist in the East African Great Lakes, Europe, Brazil, and Central America. Morphological differences exist throughout this large distribution, although they may be due to environmentally induced plasticity, rather than being indicative of taxonomic differences (Cook and Lüönd 1982). *Hydrilla* also varies with respect to ploidy (diploids, triploids, and tetraploids) and sexuality (monoecious and dioecious). At present, there do not appear to be clear geographic or ecological correlates with ploidy or sexuality or between ploidy and sexuality (Chaudhuri and Sharma 1978, Cook and Lüönd 1982, Nakamura and Kadono 1993).

Early work with isoenzymes revealed some genetic differences among populations of hydrilla, including differences between US monoecious and dioecious biotypes (Verkleij et al. 1983a, b, Verkleij and Pieterse 1991, Pieterse et al. 1985). Madeira et al. (1997, 2004, 2007) used random amplified polymorphic DNA (RAPD; Welsh and McClelland 1990) and chloroplast (cpDNA) sequences to determine the source of introduced US hydrilla. Their data revealed significant genetic diversity and divergence in worldwide collections of hydrilla, and identified India as the most likely source region for the dioecious biotype and South Korea as the most likely source region for the monoecious biotype. These studies also revealed that monoecious and dioecious biotypes are genetically distinct from each other and occur in separate genetic clusters.

Benoit (2011) built upon these early studies and conducted a formal cladistic analysis of hydrilla, utilizing the cpDNA region (*trnL-F*) developed by Madeira et al. (2007) and two nuclear regions (a section of the phytoene desaturase *pds* gene and the nuclear ribosomal internal transcribed spacer (nrITS) region). Benoit's analyses suggest that the monotypic genus *Hydrilla* may contain up to three cryptic species based on the presence of three monophyletic clades that approximately correspond to three geographic lineages: Indonesia/Australia, Japan/Korea/Europe, and India/Nepal. China has a high diversity of cpDNA haplotypes (D.A. Williams's unpublished data), suggesting that it is the center of origin for the genus; however, the nuclear sequence data of Benoit (2011) suggest some of this diversity may have been introduced into China from elsewhere. More extensive collections in China will be needed in order to determine which genetic diversity may be unique to China. China has been poorly sampled genetically, especially in the more northern areas.

The chloroplast and nuclear sequence data indicate the origin of the US dioecious biotype is India, as seen in the earlier work of Madeira et al. (1997, 2007). The US monoecious biotype was only found in South Korea, which was also supported by the earlier work of Madeira et al. (1997, 2007). The nuclear data, however, indicate that the monoecious biotype is an inter-clade hybrid which likely arose from parental types in the Indonesia/Australia and India/Nepal hydrilla lineages. The nuclear and cpDNA phylogenetic trees in Benoit (2011) show general agreement regarding the three major clades; although some of the clades have fairly low bootstrap support, topological differences occur between nuclear sequence data sets, and all trees contain some unresolved taxa. These issues may result in part from recombination and hybridization between and within the three major hydrilla lineages (Benoit 2011). Nevertheless, even with some uncertainties in the phylogenetic relationships, the US dioecious and monoecious biotypes belong to separate genetic lineages and have their origin in ecologically different areas. It is therefore likely that biological control agents will perform differently on the two biotypes.

Origin of US *Hydrilla*. Although early reports claimed that US dioecious hydrilla was introduced from Sri Lanka (Schmitz 1991), the genetic origin is almost certainly India (Madeira et al. 1997, 2007, Benoit 2011). Because of close geographic proximity between Sri Lanka and India, it is possible that the plants were originally collected from mainland India and then cultured in Sri Lanka for the commercial aquatic plant trade. The genetic origin of US monoecious hydrilla is probably South Korea (Madeira et al. 1997, 2007); however, recent data from Benoit (2011) suggests that this biotype is a hybrid that was itself introduced into South Korea, although the timing of this introduction is uncertain. Exact genetic matches between the nuclear genes of the parental types of this hybrid have not been found in South Korea or elsewhere, suggesting that the parental sequences may have diverged from their original state and are no longer recognizable as such (Benoit 2011). If this scenario is correct, then the hybridization event (and presumably the introduction event) was very old. Alternatively, the extent of genetic sampling in Asia may not have been sufficient to find these parental genotypes.

Genetic Relationships between *Hydrilla* Populations in the US. There are some general morphological differences between the US monoecious and dioecious plants (see Ryan et al. 1995 for illustration); however, these characteristics are not always reliable and therefore molecular techniques were developed to differentiate these biotypes (Madeira et al. 2004). Early work with isoenzymes and RAPDs could distinguish the biotypes in the US (Verkleij et al. 1983b, Ryan et al. 1995, Madeira et al. 1997), although one drawback of these techniques was the need for reference specimens to compare with unknown samples. More recently, Madeira et al. (2004) developed a modified technique based on the polymerase chain reaction (PCR) that amplifies a portion of the DNA sequence in dioecious — but

not in monoecious — hydrilla. This modified technique does not require reference samples and the results are easily replicated between independent molecular laboratories (Madeira et al. 2004).

Both the dioecious and monoecious biotypes appear to spread through vegetative means in the United States; only female plants of the dioecious biotype have been found in the field and viable seedlings have not been detected in the monoecious biotype (Steward 1993), though Langeland and Smith (1984) reported that monoecious plants can produce viable seed in the field. Due to the lack of genetic diversity in US monoecious hydrilla populations, all current US populations likely trace their origin to a single introduction or multiple introductions from the same source area (Benoit 2011). The dioecious biotype, on the other hand, has a surprisingly high level of genetic diversity for a plant that spreads clonally, which suggests that either a large number of diverse clonal lineages were introduced, or that this plant has high levels of somatic mutation (Grajczyk 2009).

There does not appear to have been hybridization between monoecious and dioecious hydrilla populations in the US thus far, although this has not been extensively tested using molecular markers (Benoit 2011). Experimental crosses have revealed that female dioecious plants in Florida are potentially fertile and can produce viable seed when pollinated by monoecious and dioecious strains from Asia (Steward 1993). Although Benoit (2011) was not convinced that sympatric populations of both biotypes occur in the US, Ryan et al. (1995) reported both biotypes in Lake Gaston, NC. Ryan et al. (1995) were able to induce flowering in both biotypes (only female flowers were present in the dioecious form) and provided additional evidence of separate biotypes based on growth and reproductive parameters. In addition, plant material from at least two sites where both biotypes are believed to exist — Guntersville Reservoir, AL and Lake J. Strom Thurmond, GA — has not yet been subject to genetic examination.

Implications for *Hydrilla* Management. Genetic considerations should play a large role in devising effective management strategies for invasive hydrilla populations. These considerations fall into three main areas: 1) matching biological control agents with the appropriate hydrilla biotype; 2) understanding the role of somatic mutation in the development of herbicide resistance; and 3) understanding the implications of hybridization and genetic mutations for biological control efforts and the development of increased invasiveness.

The potential success of biological control efforts is predicated on the evolved relationship between the invasive plant and its putative control agent. These relationships may develop over tens of thousands of years and, depending on the taxa involved, are quite conservative in nature (i.e., herbivorous taxa tend to feed on similar species within a group; Van Driesche et al. 2008). Because of the close, and often dependent (insect herbivores may not be able to complete their life cycle on another plant species, or even another subpopulation of a species, for example) relationship between biocontrol agents and their host plant, it can be important to match herbivores and plants from the same geographic region (e.g., Goolsby et al. 2006, Manrique et al. 2008).

Researchers have noted for at least 30 years that geographically separated strains of hydrilla inhabit very different aquatic habitats and display considerable variation in morphology and phenology (Cook and Lüönd 1982, Verkleij et al. 1983a). Several examples illustrate the importance of matching the US hydrilla biotypes with biocontrol agents from their population of origin. For instance, many European and north Asian hydrilla populations persist throughout winter (Verkleij et al. 1983a); whereas other populations senesce during winter months (Madsen and Owens 1998). The ability or lack of

overwintering capability can have a profound effect on the success of biocontrol agents. For example, the apparent incompatibility of the leaf-mining fly, *H. pakistanae*, with the monoecious hydrilla biotype can be traced, in part, to overwintering habitat requirements of the fly. In this case, *H. pakistanae* appears to require some intact stem tissue to survive winter (Harms and Grodowitz 2011), and monoecious plants tend to senesce completely in most US locations, leaving no aboveground biomass during the winter months (Sutton et al. 1992). This incompatibility seemingly limits the effectiveness of *H. pakistanae* as a biocontrol agent for the monoecious biotype.

The ecology of the various hydrilla strains has also had historical implications for management. Failure to establish *Bagous affinis* from India on US hydrilla populations has often been attributed to specific environmental requirements of the weevil that are not commonly found at field sites in the US; i.e., drying periods in which the weevil pupates in dry soil along the shore (Buckingham and Bennett 1998, Buckingham and Grodowitz 2004).

Somatic mutation has been implicated in the development of fluridone (a phytoene desaturase inhibitor) resistance in hydrilla from Florida (Michel et al. 2004). Fluridone-resistant hydrilla has been reported from numerous large Florida lakes and, in areas where herbicide application is the primary management method for hydrilla, increased resistance is to be expected. Thus far, only the dioecious biotype has developed resistance to fluridone (Michel et al. 2004, Benoit 2011). The genetic changes in populations of hydrilla resulting from persistent application of herbicides have increased interest in integrating weed management practices that may limit further development of resistance and improve efficacy of treatments. This may include application of herbicides with multiple target site mutations or an increased application of biocontrol.

Hybridization between species or between genetically differentiated populations of the same species is of particular concern for the efficacy of biological control agents since hybrids represent a novel host that does not exist in the native range (Moody and Les 2002, Blair et al. 2008). Hybridization may produce novel, secondary chemical compounds (Orians 2000), or structural changes (Grosholz 2010) that can provide defenses against natural enemies and give hybrids a competitive edge in their new environment. Hybridization can also increase genetic variation, thereby increasing the chance of adaptive evolution in the introduced range. There are now a number of examples of hybridization that have led to increased invasiveness (reviewed in Schierenbeck and Ellstrand 2009). It is not possible to know for certain whether a hybrid of the monoecious and dioecious biotypes would be more invasive than either one is alone; however, enough examples of the increased invasiveness of hybrids have accumulated that concern is warranted. An additional concern would be the potential transfer of fluridone resistance to the resulting hybrid.

Future Directions. Based on our current understanding of hydrilla genetics, there is a critical need to 1) reexamine the location of natural enemy surveys of dioecious hydrilla; 2) develop a strategy for overseas exploration of monoecious populations; and 3) determine if hybridization is occurring between US monoecious and dioecious biotypes in regions where they overlap.

Surveys of hydrilla herbivores have been undertaken in India, Pakistan, Malaysia, Korea, Japan, Thailand, Vietnam, Africa, and Australia (Bennett and Buckingham 2000). Currently, areas in Southern China are being explored to identify additional agents for the dioecious biotype (Ding et al. 2011). Results of these most recent surveys have been promising, such as the identification of an aquatic weevil (Curculionidae: *Bagous* sp. 2; Zhang et al. 2012) that completes its entire life cycle on

submersed hydrilla. Current surveys are focused on the dioecious biotype; however, it is unclear from the reports of early surveys whether the monoecious or dioecious biotype was examined at each site. Without having definitive information, it may be assumed that the dioecious biotype was more heavily surveyed because it is the more common biotype.

Because biological control agents may be adapted to a particular biotype, future surveys of natural enemies of hydrilla should focus on each of the two US biotypes individually. Although re-examining results from previous surveys of hydrilla may turn up insect candidates that are compatible with the monoecious biotype, new surveys should be planned and executed in which the US monoecious type is sought out and rigorously sampled. In addition to identifying plants in the field based on morphology, specimens should be retained for genetic analyses to confirm the biotype. Genetically testing all sampled populations is important because dioecious or monoecious designations are not enough to determine whether the plants are closely related to the US biotypes. Both dioecious and monoecious plants potentially occur across the different genetic clades identified by Madeira et al. (1997, 2007) and Benoit (2011) and so their sexual designation does not reflect their exact genetic identity. For instance, dioecious plants have been collected in India, China, and New Zealand, while monoecious plants have been collected in Nepal, Pakistan, Taiwan, South Korea, New Guinea, Australia, Java, and Malaysia (Steward 1993).

US monoecious hydrilla plants likely originated in Korea (Madeira et al. 2007, Benoit 2011); therefore, natural enemy surveys should concentrate there and in regions near South Korea. It is unknown whether the hybrid nature of this monoecious biotype will affect its suitability as a host for potential biological control agents. If this hybrid has been present for a long time in South Korea, then presumably herbivores in that region have become adapted to utilize this biotype. If the geographic origins of the parental genotypes can be found, then these areas may also contain potentially useful control agents. Surveys in the northeastern provinces of China may be useful, since this is a region that has not been previously surveyed and it is relatively near Korea, geographically. Because of this, northeastern China may contain one or both of the parental types for the hybrid South Korean monoecious biotype.

Hybridization can potentially increase invasiveness or decrease the efficacy of biological control agents. Therefore, suspected or known regions of overlap between the biotypes in the United States should be 1) genetically tested to confirm the presence of each biotype, and 2) systematically screened using genetic markers to test for potential hybridization. If hybrids are confirmed, then steps should be taken to control and limit the dispersal of these populations and candidate biological control agents should also be tested on these hybrids.

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