



Genetic structure of *Hydrilla verticillata* L.f. Royle in eastern China and the Republic of Korea: Implications for surveys of biological control agents for the invasive monoecious biotype



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ARTICLE INFO

Keywords:

Genetic structure
Biotype matching
Invasive weed
Microsatellite

ABSTRACT

Monoecious and dioecious biotypes of *Hydrilla verticillata* were introduced from Asia into the United States (U.S.). Although biological control agent development has been ongoing for many years to combat this invasive aquatic weed, the focus has now shifted towards the monoecious biotype because of apparent incompatibilities between previously introduced agents and this lineage. To facilitate collection of natural enemies, we surveyed eastern areas of China and all of South Korea (592 samples from 129 sites) to locate geographic source areas with the introduced monoecious biotype. We used both chloroplast and nuclear microsatellite markers to identify genotypes. Eastern China had high genetic diversity and significant genetic structure across river basins, including three previously described chloroplast clades (B, C, D), one of which (B) includes both biotypes that were introduced into the U.S. South Korea had the monoecious biotype from clade B and clade C. South Korea had a subset of the genetic diversity in China, consistent with China being the ancestral region for hydrilla. U.S. introduced monoecious hydrilla had significantly lower diversity than this genotype in China and South Korea. U.S. monoecious microsatellite profiles cluster with samples from both China and South Korea, failing to resolve a clear region of origin. Reproductive strategies for clade B are more variable than in the introduced range with both monoecious and dioecious individuals sharing the same chloroplast haplotypes and microsatellite clusters. The introduced monoecious biotype of hydrilla is becoming a major problem in the U.S., but in the native range it is rare, patchily distributed, and often mixed with individuals from clade C. Current exploration for biological control agents will need to determine the genetic identity of the plants from which potential biological control agents are collected.

1. Introduction

Hydrilla verticillata L.f. Royle (Hydrocharitaceae), is native throughout Asia and the Indo-Pacific (Cook and Lüönd, 1982). Hydrilla is thought to have been introduced into the southern and the north-eastern United States (U.S.) as a result of the aquarium trade (Madeira et al., 2000). These two introductions correspond to genetically and ecologically distinct dioecious and monoecious biotypes that now infest waterbodies in much of the eastern, southern, and far western continental U.S. (Madeira et al., 2000; Balciunas et al., 2002). Hydrilla infestations are currently a major economic concern in the U.S. as they block irrigation systems, clog boat motors, decrease water quality, and cause damage to hydroelectric power plants (Balciunas et al., 2002).

Hydrilla is also an ecological concern since it displaces native vegetation, alters the native ecosystem, and decreases biodiversity (Balciunas et al., 2002).

Hydrilla has been controlled by mechanical, chemical, and biological means. Mechanical control is not cost effective and some populations of dioecious hydrilla have recently become resistant to the herbicide fluridone (Michel et al., 2004). Biological control (biocontrol) offers an attractive alternative or complement to other methods, and *Hydrellia pakistanae* (Diptera: Ephydriidae), a leaf-mining fly from India has provided some control of the dioecious biotype in the United States (Grodowitz et al., 1999, 2003). The U.S. monoecious biotype is minimally affected by *H. pakistanae*, however, due to an apparent conflict of monoecious hydrilla phenology with overwintering requirements of the

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agent (Grodowitz et al., 2010; Harms and Grodowitz, 2011). Current anecdotal evidence suggests that *Hydrellia* spp. are adapted to specific hydrilla biotypes. *Hydrellia pakistanae* was collected from hydrilla in southern India, which was later determined to genetically match the dioecious biotype but not the monoecious biotype (Madeira et al., 1997, 2007; Williams et al. unpublished data). An attempt at introducing another fly, *Hydrellia balciunasi* from Australia onto dioecious hydrilla in the United States also failed, possibly because *H. balciunasi* in Australia originates from hydrilla with a different genotype than the introduced dioecious biotype (Grodowitz et al., 1997, Grodowitz et al., 2013). In South Africa, invasive hydrilla originated from Malaysia and Indonesia (Madeira et al., 2007). The newly described *Hydrellia purcelli* that originates from Singapore has significantly better performance on South African hydrilla than *H. pakistanae* which originated on hydrilla with a different genotype (Grodowitz et al., 1997; Bownes, 2015; Bownes and Deeming, 2016). Both host specificity and efficacy of biocontrol agents should be highest in the source region of the invader and a number of authors have recommended ‘biotype matching’ of target plants and candidate natural enemies (Roderick and Navajas, 2003; Hufbauer and Roderick, 2005; Goolsby et al., 2006; Manrique et al., 2008). Therefore, to apply biological control to invasive monoecious hydrilla it will be important to identify the native source regions with the same genotype as the monoecious hydrilla biotype and collect agents from those plants (Harms et al., 2013).

RAPD (random amplified polymorphic DNA) profiles and sequences from the *trnL-F* chloroplast region have suggested that India and South Korea (Republic of Korea) are the most likely source regions for the U.S. introduced dioecious and monoecious biotypes respectively (Madeira et al., 2000, 2007). The chloroplast genotype of the introduced dioecious biotype is found over a large area in Asia from India, Pakistan, China, Nepal, and northern Vietnam as well as the African Great Lakes region (Madeira et al., 2007; Williams et al. unpublished data). The chloroplast genotype of the introduced monoecious biotype has been found only in South Korea and eastern China (Madeira et al., 2007; Zhu et al. 2015). A past survey for biocontrol agents in South Korea did not produce any suitable candidates (Bennett and Buckingham, 1999), although these studies were not conducted on genetically characterized populations. Only a few samples have ever been genotyped from South Korea, and so it is unknown how hydrilla is genetically structured in South Korea or how common the monoecious biotype is in that region. Zhu et al. (2015) described the phylogeographic structure of hydrilla in China using the *trnL-F* region and in conjunction with the samples described in Madeira et al. (2007), inferred that China was the most likely region of origin for all four major hydrilla clades. If China is the ancestral region for hydrilla, it may be especially important to survey for biocontrol agents for the monoecious biotype in eastern China since there may be older herbivore-host associations and possibly more specific natural enemies.

The invasive U.S. biotypes are genetically distinct and appear to have fixed reproductive morphology. In the U.S., only females have been described for the dioecious biotype and both female and male flowers always co-occur on the monoecious biotype although female flowers can sometimes appear before the male flowers (Ryan et al., 1995; Madeira et al., 2000). The reproductive condition of these biotypes has not been described in the native range and so it is unknown whether they are also always dioecious or monoecious. Sexual strategy could potentially impact biological control agents by affecting phenology and differential herbivory on males and females and so may need to be taken into consideration when surveys are conducted in the native range (e.g. Ågren, 1987; Muenchow and Delesalle, 1992; Watson, 1995; Cornelissen and Stiling, 2005; Vilas and Pannell, 2011).

We expand on earlier studies by characterizing the genetic diversity of hydrilla in eastern China and throughout South Korea using two chloroplast regions and eight nuclear microsatellite loci. These data will identify regions that have native populations of the monoecious biotype that was introduced into the U.S., and will indicate where surveys for

natural enemies should be conducted. We then compare genetic diversity between China and South Korea and compare U.S. monoecious hydrilla to native populations of this biotype. We predicted 1) South Korea would have lower genetic diversity than sites in China if China is the ancestral region, and 2) introduced U.S. monoecious hydrilla would have lower diversity than native populations as expected if it went through an introduction bottleneck. We also ask if reproductive condition in the native range is related to chloroplast and microsatellite genotypes like it is in the introduced range.

2. Materials and methods

2.1. Field collections

We collected hydrilla ($n = 592$ samples) from a total of 66 sites in China ($n = 320$ samples) and 63 sites in South Korea ($n = 276$ samples) during 2013–2015 (Interactive map here, Appendix A). Most sites were identified through examination of herbarium specimens, online database searches (<http://www.cvh.org.cn/>, www.GBIF.org), and contacts with Chinese and Korean scientists. Other site visits were unplanned; if surfaced aquatic vegetation was observed during transit between locations then the site was examined for the presence of hydrilla. Site types were diverse and included urban and rural ponds, lakes and rivers, manmade canals, shallow irrigation ditches and natural wetlands. Twelve monoecious hydrilla samples were also collected in the United States from New York, South Carolina, Kansas, Georgia, and Missouri in 2013.

We collected hydrilla in several ways: from the shore by tossing a double-sided metal rake with a rope handle and snagging plants during the retrieval, by wading and hand collecting, or from a boat by hand or using a rake. Collections were spaced ≥ 2 m apart to limit the likelihood of resampling the same plant. Five cm apical meristems were collected from several plants per site. The number of plants sampled at each site depended on the abundance of hydrilla and ranged from a single sample to ten samples. Samples were placed in individual small re-closable plastic bags with silica gel desiccant. If flowers were present, we classified samples as monoecious (both male and female flowers present) or dioecious (only male or female flowers were present). These plants were not monitored over time however, and so it is possible that some plants categorized as dioecious were in fact monoecious if flowers of one sex appeared before flowers of the other sex.

2.2. Genetic methods

We extracted DNA from all samples using the IBI Scientific MINI Genomic DNA kit (Plants) as per manufacture instructions. We then amplified and sequenced three chloroplast (cpDNA) regions (*trnL* intron, *trnL-F* spacer, using primers *trnFf* – 5'-ATTTGAACTGGTGACAC GAG-3' and *trnFc* – CGAAATCGGTAGACGCTACG and the *psbM-trnD* spacer using primers *psbMF* - AGCAATAAATGCRAGAATATTTACTTC and *trnD_GUC_R* – GGGATTGTAGYTCAATTGGT) for all samples (Shaw et al., 2005). Polymerase chain reactions (PCR) (10 μ L) contained 10–50 ng DNA, 0.5 μ M of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3 mM MgCl₂ pH8.7, and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95 °C for 15 min, followed by 30 cycles of 30s at 94 °C, 90s at 55 °C (for *trnL-F*) or 50 °C (for *psbM-trnD*), 90s at 72 °C, and then a final extension at 72 °C for 5 min. Products were sequenced using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Life Technologies). Sequencing primers for the *trnL* intron, *trnL-F* spacer were the PCR primers and an internal primer developed for this study, *trnFint* – GTTCGGATCCGTCGTTT. The *psbM-trnD* spacer was more difficult to sequence due to apparent secondary structure near the *psbM* region. In addition to the *trnD_GUC_R* PCR primer we used several internal primers for sequencing that were developed for this study, including *trnDinta_R* – TCCTTTATACCTTGATCCATATTTCT, and

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