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Rapid Identification and Molecular Characterization of Phytoene Desaturase Mutations in Fluridone-Resistant Hydrilla (*Hydrilla verticillata*)

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Florida hydrilla populations have shown an alarming increase in resistance to fluridone, an herbicide used extensively for controlling invasive US hydrilla populations. A rapid PCR and sequencing method was developed to identify and screen hydrilla genomic DNA for three previously identified phytoene desaturase (*pds*) gene mutations that confer resistance to fluridone. Ninety hydrilla accessions were screened for fluridone resistant genotypes including 46 accessions from the US and 44 accessions from 15 other countries. In Florida, hydrilla from five of nine sites tested was heterozygous for wild-type and herbicide-resistant alleles. Additionally, a new resistant population was identified from Lake Seminole in Georgia, the first genetically confirmed strain of resistant hydrilla outside of Florida. All resistance-conferring mutations were located on the same homologous haplotype of US dioecious hydrilla. All other hydrilla samples tested possessed only wild type alleles, including monoecious strains that had been exposed to fluridone. Management implications are discussed.

Nomenclature: Fluridone; hydrilla, *Hydrilla verticillata* (L.f.) Royle HYLLI.

Key words: Aquatic weed, herbicide resistance, invasive species, phytoene desaturase gene, polymerase chain reaction PCR, sequencing, cloning.

The majority of herbicide-resistant weeds occur in agricultural systems, whereas only two out of 210 herbicide-resistant weed species (Heap 2012) have been documented in aquatic habitats. The approval of herbicides for use in aquatic environments is limited by toxicity to biota (e.g., fish, birds, amphibians, nontarget native plant species) (Koschnick et al. 2006) as well as by health concerns for water bodies used for swimming, fishing, and drinking water reservoirs (Brooker and Edwards 1975; Murphy 1988). Consequently, few herbicides have been approved for use in waterways of the United States (US).

The herbicide fluridone was approved in 1986 by the US Environmental Protection Agency for use in lakes. Fluridone is a noncompetitive inhibitor of the enzyme phytoene desaturase (PDS), a nuclear-encoded chloroplast protein that catalyzes a critical step in carotenoid biosynthesis (Bartley et al. 1991). PDS converts the colorless precursor phytoene to the colored carotenoid zeta-carotene, a rate-limiting step in the pathway (Chamovitz et al. 1993). Carotenoids quench excess excitation energy during photosynthesis, protecting the chlorophyll apparatus from oxidative damage. When fluridone prevents PDS activity, the substrate phytoene accumulates while carotenoids decrease (Doong et al. 1993; Puri et al. 2006), thus leading to destruction of chlorophyll in growing shoots, a characteristic "bleaching" of cells, and eventual plant death.

Since its introduction in 1986, fluridone has been used to provide effective, whole-lake control of hydrilla, *Hydrilla verticillata* (L.f.) Royle, a nonindigenous invasive aquatic plant (Pons 2005). Two "strains" or "biotypes" of hydrilla (i.e., dioecious, monoecious) have been recognized (Cook and Luond 1982), and both have been introduced into the United States. Populations throughout states of the Southeast and Gulf Coast, California, Tennessee (Madeira et al. 2000) and Idaho (T. Woolf personal communication) consist only of female plants and are believed to represent a single introduction of the dioecious form, which has spread asexually after its initial introduction. Monoecious plants (i.e., male and female flowers on the same plant) were discovered in Delaware in 1976, and in Kenilworth Aquatic Gardens, Washington D.C. and the Potomac River just south of Alexandria, Virginia in 1982 (Steward et al. 1984). Subsequently, monoecious hydrilla has spread through the Atlantic coastal states from Georgia to Maine (with the exception of New Hampshire and Rhode Island), as well as to California, Indiana, Ohio, Pennsylvania, Washington State (Les et al. 1997; Madeira et al. 2000, EDDMapS 2012), and Wisconsin (Asplund 2007).

Although the contact herbicides copper, endothall and diquat also are used to control hydrilla, fluridone has become the treatment of choice for managing infestations of this aquatic weed (Dayan and Netherland 2005). At low doses, fluridone has limited effects on nontarget plants (Netherland et al. 1997), has low toxicity to invertebrates and fish (Hamelink et al. 1986), and is considered cost-effective (Fox et al. 1994). In Florida, where herbicide use has been extensive, hydrilla populations have shown an increased incidence of herbicide resistance. Evolution of resistance in asexual populations consisting of only female plants was unexpected. Generally, it is assumed that herbicide resistance would evolve only in sexually-reproducing species since the heritable genetic diversity in a population is generated by recombination and mutation during meiosis. Hydrilla reproduces asexually through fragments, turions, and underground tubers. Therefore, a somatic mutation of sufficient phenotypic effect can respond to natural selection and pass on an adaptive allele via clonal reproduction. Michel et al. (2004) determined that hydrilla plants, whose *pds* gene contains the "wild-type" sequence for arginine (CGT) at codon 304, produce a phytoene desaturase enzyme that is very susceptible to the herbicide fluridone. They determined further that the presence of any of three somatic mutations that substitute AGT (serine), TGT (cysteine) or CAT (histidine) at this same codon conferred low, intermediate and high herbicide resistance respectively (Michel et al. 2004).

To date, herbicide-resistant hydrilla has evolved only in female dioecious plants located in Florida waterways (Koschnick et al. 2006), and there is a lack of current information on the existence of resistant populations in other states and in monoecious hydrilla populations. Fluridone-resistant biotypes

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from Florida may spread easily to other parts of the country as hydrilla fragments and vegetative propagules (i.e. turions, tubers) are dispersed. The continued use of fluridone in regions outside of Florida also has the potential to select for new resistant populations. For these reasons, it is evident that the ability to implement a relatively rapid screening test for fluridone resistance in hydrilla should provide an important tool for those involved in the management of waterways.

Current methods to identify fluridone-resistant hydrilla include a complex genetic protocol (Michel et al. 2004) consisting of mRNA isolation, reverse transcription to cDNA, and cloning and sequencing the entire *pds* coding region. In a second, nongenetic method, herbicide resistance is assayed directly by applying fluridone to hydrilla shoots in vitro to evaluate relative degrees of resistance. This time-consuming process can take three to four weeks to collect plant samples, set up aquaria and wait for fluridone, a slow-acting herbicide, to take effect (S. Miller, personal communication). Consequently, we reasoned that hydrilla plants could be screened effectively for fluridone resistance by developing a simpler approach to elucidate the codon 304 genotype by PCR amplifying genomic DNA and then sequencing this critical region directly. This modified procedure (described below) was used to screen multiple accessions of US dioecious and monoecious hydrilla, and hydrilla DNA from plants previously collected around the world, for fluridone resistant genotypes at the *pds* locus. Following identification of resistant and susceptible genotypes for each US locality, fluridone use data were collected for ten water bodies in order to identify possible associations between fluridone usage and occurrence of resistance, geographic area, and biotype.

Additionally, the sequence data were used for further molecular genetic characterization of this important *pds* gene region in hydrilla. A region of the hydrilla *pds* gene is described, including intron and exon boundaries, and molecular genetic characteristics of haplotypes from fluridone susceptible and resistant US accessions.

Materials and Methods

Plant Material. Ninety *Hydrilla verticillata* accessions were screened for fluridone-resistant genotypes. This material (Appendix 1) represented fresh or CTAB-preserved specimens (Rogstad 1992), and included 46 accessions from 37 localities in 18 different political subdivisions in the United States (Arkansas, California, Connecticut, Delaware, District of Columbia, Florida, Georgia, Idaho, Louisiana, Maine, Maryland, Massachusetts, New York, North Carolina, South Carolina, Texas, Virginia, and Wisconsin) as well as 44 representatives from 38 localities in 15 other countries (Australia, Burundi, China, India, Indonesia, Ireland, Japan, Latvia, Malaysia, North Vietnam, Pakistan, South Africa, South Korea, Taiwan, and Thailand).

Molecular Genetic Analyses. Total genomic DNA (gDNA) was extracted either from fresh hydrilla plants, or samples preserved in silica or CTAB using a standard CTAB DNA extraction protocol (Doyle and Doyle 1987).

To identify potentially herbicide-resistant genotypes in the hydrilla populations, an ~880 base pair (bp) region of the *pds* gene was amplified and sequenced; this contained the critical codon 304 that had been implicated by Michel et al. (2004) as conveying differential fluridone resistance responses. Genomic DNA was amplified by polymerase chain reaction (PCR) using a custom-designed "PDS-819F" forward primer (5′-TAA ACC CTG ATG AAC TTT CAA TGC-3′) and "PDS-1219R" reverse primer (5′-GTG TTC TTC AAC TTC CTA TCA A-3′). The nucleotide sequences of these primers were based on degenerate primers to a *pds* gene region conserved among cyanobacteria and higher plants that had been used previously for generating hydrilla *pds* cDNA (Michel et al. 2004). The degenerate primer sequences were modified to match the GenBank sequence for hydrilla *pds* cDNA (accession number AY639658). All primers were assessed for secondary structure stability and rated using NetPrimer (Premier Biosoft International, Palo Alto, California). Because the amplicons consistently had multiple bands, they could not be sequenced directly. Instead, PCR reactions were cleaned with a QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, California) and cloned using a TopoTA cloning kit (Invitrogen Corp., Carlsbad, California). Amplified products ranged from ~300 to 900 bp. Sequences of cloned fragments revealed that the smaller fragments (less than ~800 bp) contained no identifiable *pds* sequence. Eight to twelve positive clones of the appropriate size ~800 to 900 bp product were amplified from each accession and sequenced using the PDS-819F and PDS-1219R primers. All sequencing reactions were analyzed on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, California).

From the initial results, sequences of the codon of interest (CGT/codon 304) were unreadable or not present in greater than 50% of the sequences because of the proximity of the forward primer to the codon 304 sequence. Furthermore, the reverse sequences did not extend to, or were unreadable at, the site of interest. To better target codon 304, new primers were designed (PDS-793F: 5′-GCA ATG TCA AAG GCT CTT AAC-3′; PDS-1208R: 5′-TAC ATT TAT CAC AGG TAC GCC-3′), which shifted the amplified region towards the 5′ direction, yet remained within the conserved coding region. PCR amplifications of genomic DNA were carried out in a 25 μl reaction volume containing 1X Titanium buffer (Clontech Laboratories, Inc., Mountain View, California) final concentration of 1.5 mM $MgCl_2$, 150 μM each dNTP, 0.5 μM each primer, and 1 unit Titanium *Taq* DNA polymerase (Clontech Laboratories, Inc.). PCR conditions included an initial denaturation step (94 C for 3 min.) followed by 30 cycles of denaturation, annealing and elongation (94 C for 40 sec, 54 C for 40 sec, 72 C for 50 sec) and a final elongation step (72 C for 5 min). The amplified product appeared as a single band on a 1% agarose gel. Again, all sequencing reactions were analyzed on an ABI 3100 automated sequencer.

Polymorphic sequences showing single nucleotide polymorphisms and indel-induced artifacts were cloned using the TopoTA cloning kit (Invitrogen Corp.). Eight to twelve positive clones per accession were selected and PCR amplified with the PDS-793F and PDS-1208R primers and sequenced as described above. All US monoecious and dioecious hydrilla accessions were polymorphic so a subset of all US hydrilla gDNA was cloned and sequenced. The biotype or sexuality (monoecious, dioecious) of US accessions was determined by amplifying and sequencing the *trnL-F* chloroplast region, which differs consistently between the two biotypes (Madeira et al. 2004). Biotype could not be determined on plant specimens from outside of the US since an association between sexuality and sequence variations in the *trnL-F* chloroplast region from global hydrilla accessions has not been made.

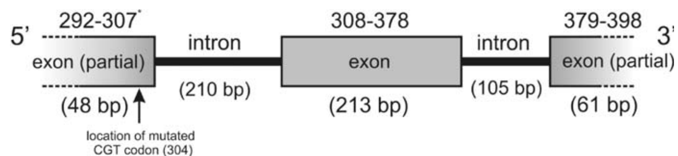

Figure 1. A 637 bp partial phytoene desaturase (*pds*) gene sequence for hydrilla. Sizes (number of bp) of exons and introns are shown in parentheses.
* Range indicates codons numbered relative to the start codon (ATG) of cDNA (after Michel et al. 2004).

To determine the number and types of haplotypes for hydrilla PDS clones, identical sequences were grouped together. Single base pair changes observed in only one haplotype sequence were assumed to be cloning artifacts and were excluded from the consensus sequence. The specific DNA sequence at codon 304 was recorded for each accession as a proxy for their potential fluridone resistance. All resulting DNA sequences were aligned manually using the computer program MacClade 4 (Maddison and Maddison 2000). The program PAUP* 4.0b10 (Swofford 2002) was used to conduct a neighbor-joining analysis of a subset of nonpolymorphic sequences and haplotype sequences from cloning.

To confirm results when a mutated codon 304 was identified, PCR, sequencing, and in some cases cloning, were repeated. Three accessions collected from West Lake Tohopekaliga ("Lake Toho"), Florida were cloned and sequenced. Additionally, a fresh sample of hydrilla, originally collected from Lake Toho in 2000, and grown continuously in culture since then (W. Haller, personal communication), was tested as a positive control for the CAT mutation (Puri et al. 2007). This cultivated sample also was amplified, cloned and sequenced using the new PDS primers. Four Lake Okahumpka, Florida accessions were amplified and sequenced with the modified PDS primers, and two of those were cloned. For other localities possessing a codon 304 mutation, testing was repeated on the same genomic DNA as these sites were represented by only one sample collection.

Lake Management History. Lake management records, including fluridone treatments, were collected from state, federal, and private agencies, and summarized for ten US localities. These localities included three water bodies with herbicide-resistant dioecious hydrilla, two with susceptible dioecious hydrilla, and five with susceptible monoecious hydrilla. It was not possible to obtain complete records of application rates per acre and total acres treated for very many US lakes in this study. For consistency across lakes, we reported the numbers of years of fluridone treatment as this metric was available for the greatest number of study sites.

Results and Discussion

A molecular tool for determining the sequence of the hydrilla *pds* gene at codon 304 was developed to identify mutations that confer fluridone resistance. The PCR amplification of hydrilla genomic DNA using the PDS-793F and PDS-1208R primers produced a single band. Amplicon sizes ranged from 637–646 bp among accessions because of insertions/deletions (indels) in noncoding regions. The amplified region of the *pds* gene included codons 292 to 398 (after Michel et al. 2004, GenBank AY639658.1). The amplified region spanned the 3′ end of one exon, a 210 bp intron located between codons 307 and 308, a 213 bp exon, a 105 bp intron located between codons 378 and 379, and the 5′ end of another exon (Figure 1).

Table 1. Summary of accessions screened for codon 304 mutations of the phytoene desaturase (*pds*) gene of hydrilla genomic DNA.

Location	No. samples	Sites	Wild type	*pds* Mutation
US (monoecious)				
ME	1	1	1	0
MA	1	1	1	0
CT	5	3	3	0
NY	1	1	1	0
DE	4	4	4	0
DC	1	1	1	0
MD	2	2	2	0
VA	3	3	3	0
NC	4	1	1	0
GA	1	1	1	0
WI	1	1	1	0
Total	24	19	19	0
US (dioecious)				
SC	2	2	2	0
GA	1	1	0	1
FL	13	9	4	5
LA	2	2	2	0
AR	1	1	1	0
TX	1	1	1	0
CA	1	1	1	0
ID	1	1	1	0
Total	22	18	12	6
International				
Australia	8	7	7	0
Burundi	1	1	1	0
China	7	7	7	0
India	3	3	3	0
Indonesia	2	2	2	0
Ireland	3	2	2	0
Japan	1	1	1	0
Latvia	4	3	3	0
Malaysia	1	1	1	0
North Vietnam	1	1	1	0
Pakistan	1	1	1	0
South Africa	1	1	1	0
South Korea	8	5	5	0
Taiwan	1	1	1	0
Thailand	2	2	2	0
Total	44	38	38	0
TOTAL	90	75	69	6

Using reverse transcription of mRNA to cDNA, and sequencing the full length coding region of 1743 nucleotides, as previously reported by Michel et al. (2004) and by Puri et al. (2007), is an inefficient and expensive method if the sole goal is to identify codon 304 mutations. Here we describe a procedure that can be completed in any properly-equipped laboratory within the matter of a few days: genomic DNA extraction, PCR amplification using the modified PDS-793F and PDS-1208R primers, and sequencing of a relatively small ~640 nt product.

Nonsynonymous DNA substitutions in codon 304 of the hydrilla *pds* gene, representing all three previously reported resistance-conferring genotypes (Michel et al. 2004), were detected at six US localities. Screening of ninety hydrilla accessions for mutations at codon 304 revealed that hydrilla from five sites in Florida and one in Georgia had a mutation at the codon of interest (Table 1). In these accessions, polymorphisms at the first or second position of codon 304 indicated a heterozygous condition where both wild type

Table 2. Herbicide and physical removal treatment methods for a subset of US hydrilla populations; D = dioecious; M = monoecious.

Locality	Fluridone use	Alternate herbicides used	Physical removal	Fluridone resistant	Biotype
	yrs				
Lake Okahumpka, FL	8	0	No	Yes	D
West Lake Tohopekaliga, FL	8	0	No	Yes	D
Sneads Landing, Lake Seminole, FL	0	?	?	No	D
Wingates Landing, Lake Seminole, GA	10	0	No	Yes	D
Leathersville Ramp, J. Strom Thurmond Lake, SC	0	1	No	No	D
Lake Gaston, NC & VA	6	?	grass carp	No	M
Haven Lake, DE	2	1	mechanical harvester	No	M
Concord Pond, DE	2	0	mechanical harvester	No	M
Long Pond, MA	8	0	No	No	M
Mason's Island Pond, CT	4	0	No	No	M

and mutated alleles were present. These polymorphisms were clearly visible in the genomic DNA sequence chromatograms. For future screening, cloning would be unnecessary in most cases because a codon 304 mutation can be determined from the polymorphism (or lack of it), directly from the DNA sequence chromatogram. In only one instance was the location of an A/G polymorphism ambiguous such that it was difficult to determine whether the A (adenine) peak on the chromatogram was polymorphic with the C (cytosine) in the first position of codon 304 or with the G (guanine) in the second position. Cloning and sequencing the haplotypes confirmed that the A nucleotide was in the second position. Hydrilla samples from other localities, including all monoecious hydrilla and accessions from other countries, possessed only wild type (CGT) alleles. Sonar© (fluridone) has not been marketed in other countries, with the exception of a brief introduction in Brazil (S. Miller, personal communication); therefore, the inability to detect codon 304 mutations outside the US was expected.

Of the five Florida water bodies with resistant hydrilla, three (Lake Toho, Lake Howard, and creek near Lake Lulu) had hydrilla with the genotype AGT (serine), one (Lake Okahumpka) with TGT (cysteine) and one (Lake Rochelle), with CAT (histidine). Hydrilla from the Lake Seminole, Georgia population possessed the CAT (histidine) mutation. The PDS codon 304 mutations were identified originally from hydrilla populations in Florida (Michel et al. 2004), where the use of aquatic herbicides has been extensive (Hoyer et al. 2005). The discovery of a water body in Georgia with one of the mutations that confer herbicide resistance to fluridone is the first confirmed report of an herbicide-resistant mutation occurring outside of the state of Florida. Interestingly, Lake Seminole spans the border between Florida and Georgia, with only the Georgia plants possessing the mutation; a plant tested from Florida was of the wild type. Herbicide application records reveal a pattern of continuous annual use of Sonar© (fluridone) on the Georgia side of the Lake (Table 2). In the Lake Seminole, Florida location, however, fluridone has not been used at the site where a sample was collected (B. Mortimer, personal communication).

Both US dioecious and monoecious accessions possessed three each of distinct haplotypes, labeled clones "A", "B", and "C", which differed between the two biotypes. In fluridone resistant genotypes, two wild type haplotypes and one resistant haplotype containing a codon 304 mutation were identified for each individual accession in all cases except one.

Three Lake Toho, Florida samples collected in 2010 (two accessions of FL 65) and in 2000 (FL W Lake Toho cultivated, one accession) lacked the C haplotype. We had insufficient clone numbers for a fourth Lake Toho accession (FL 19) to confirm presence or absence of haplotype C. It is likely that haplotype C is present in the genome of Lake Toho hydrilla but may not PCR amplify because of one or more unidentified mutations in the primer-binding regions. The Lake Seminole, Georgia specimen possessed the A and C haplotypes, but the presumed B haplotype had nucleotide differences at some of the polymorphic sites that made it more similar to the C haplotype, and therefore aligned near the C haplotype in the neighbor-joining tree (Figure 2). The numbers of different haplotypes ranged from two to four for all other hydrilla samples (data not shown).

A neighbor-joining (NJ) tree (Figure 2) showed that all haplotypes with a codon 304 mutation (FL 13clnA, FL 14clnA, FL 15clnA, FL 65clnA, FL 66clnA, and GA 22clnA) clustered together and therefore represent the same (homologous) allele. An alignment of these mutated *pds* clones showed that they were identical except for the type of point mutation (bp 37 and bp 38 in the aligned sequences) at codon 304. Additionally, the sequences of these clnA haplotypes, with the exception of the codon 304 mutations, were identical with all fluridone-susceptible US dioecious clnA haplotypes, and with two nonpolymorphic sequences, India 1 and Burundi. The B and C haplotypes, from both resistant and susceptible hydrilla, always possessed the wild-type allele CGT at codon 304.

All codon 304 mutations were detected only in US dioecious hydrilla, and they all were located on the same homologous haplotype. The development of herbicide resistance in only the dioecious biotype may be a result of both the repeated, long-term use of the systemic herbicide fluridone in the southeastern US, where the dioecious biotype is located, and the occurrence of a unique US dioecious haplotype that may have a higher rate of mutation, for as yet undetermined reasons, at the first and second positions of codon 304. This haplotype A on the NJ tree (Figure 2) may be the repository of the low level variability observed at codon 304 in US dioecious hydrilla. Fluridone then acts as a selection agent for this haplotype, effecting a rapid increase in the frequency of a population.

It is possible that codon 304 point mutations reside as a component of natural genetic variation existing at low levels in hydrilla populations. However, this study failed to detect any of these single nucleotide polymorphisms outside of herbicide-resistant populations of the southeastern US (i.e., Florida and Georgia). Mutations may arise infrequently in populations and, in the absence of a selective force such as an herbicide, are lost over time by chance events, or remain at very low frequencies. Mutation rates at the codon 304 locus in hydrilla have not been estimated, but per nucleotide mutation rates for acetolactate synthase (ALS) loci that confer resistance

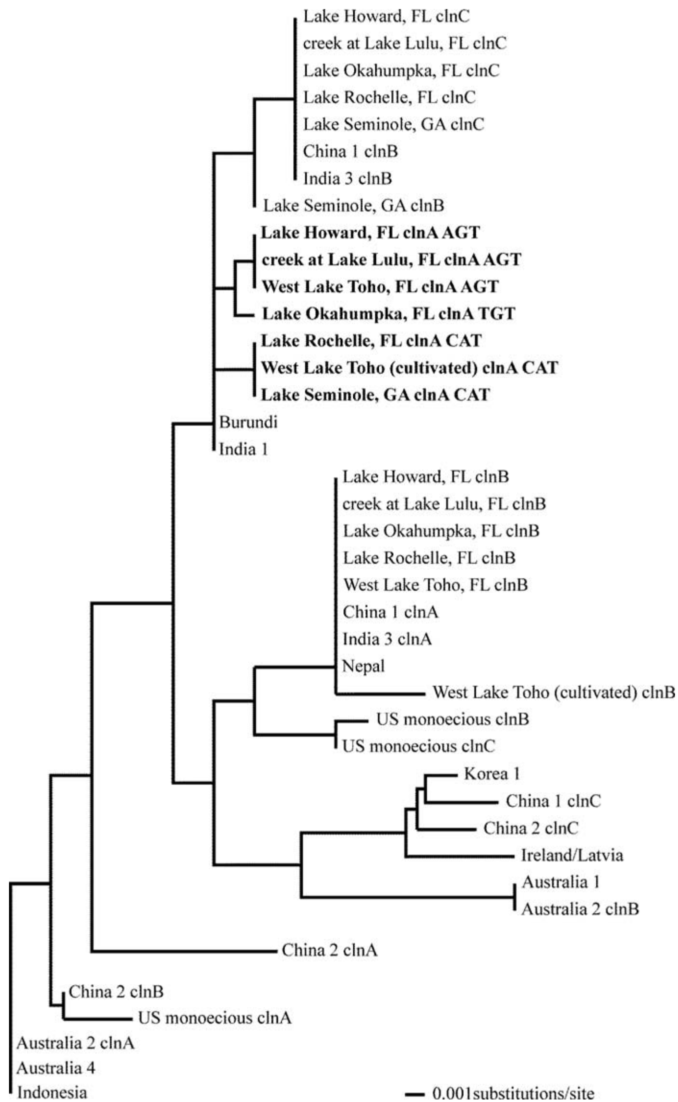

Figure 2. Neighbor-joining tree of genomic DNA and haplotype clones of fluridone susceptible and resistant hydrilla *pds* sequences. The cluster containing all haplotypes with a codon 304 mutation is shown in bold.

have been estimated at 1×10^{-9} in *Arabidopsis thaliana* (Saari et al. 1993). Kovalchuk et al. (2000) estimated 1×10^{-6} to 1×10^{-7} mutations per base pair for a single copy gene in *A. thaliana*. Applying this predicted rate to the single (or low) copy hydrilla *pds* gene, Arias et al. (2005) calculated 1.7×10^{-3} to 1×10^{-4} mutation events for the 1.7-kb hydrilla gene. Tests of weeds collected from untreated populations showed gene frequencies of herbicide-resistant alleles that ranged from 1×10^{-4} to 1×10^{-3} (Darmency and Gasquez 1990). Population genetic models of evolution of herbicide resistance use estimates of 1×10^{-5} to 1×10^{-6} for allele frequencies (Jasieniuk et al. 1996). Following selection by an herbicide, however, the frequency of the wild type allele will decrease rapidly while the frequency of the mutated allele, which confers some degree of resistance to the herbicide, will increase. The rate of this herbicide-resistance evolution depends on the initial frequency of the mutated allele in the population (Diggle and Neve 1998). In this study, the failure to detect existing genetic variation in hydrilla *pds* nucleotides coding for amino acid 304, outside of fluridone resistant populations, is not surprising, given the low probability of finding such rare alleles.

Amplicons of US monoecious and dioecious genomic DNA differed at 16 nucleotide positions. US monoecious hydrilla had 11 polymorphic nucleotide positions and one nucleotide insertion/deletion (indel), whereas US dioecious hydrilla had seven polymorphisms and one indel. Both indels were located in introns. The two US biotypes shared two of the same polymorphisms, an A/C in the silent third position of codon 294 (serine), and a C/T polymorphism in the 210 bp intron separating codons 307 and 308. In both biotypes, most of the DNA substitutions were synonymous, resulting in no change to the amino acid sequence, or located in noncoding regions (introns). However, three polymorphic sites (one in monoecious and two in dioecious) had nonsynonymous substitutions located in the 213 bp middle exon.

The US monoecious biotype had an A/C polymorphism in the first position of codon 339 that resulted in the amino acid substitution Gln^{339}Lys. The US dioecious biotype had an A/C polymorphism at the second position of codon 331 giving an Ala^{331}Asp substitution, and an A/G polymorphism in the first position of codon 341 for an Ile^{341}Val substitution. Within the full length *pds* coding region of dioecious hydrilla, Michel et al. (2004) identified numerous synonymous (silent) mutations in both resistant and susceptible dioecious hydrilla, and two nonsynonymous mutations, including the one identified here at codon 341. Puri et al. (2007) also identified a number of mutations that varied among resistant strains. Because of the phenomenon of PCR and cloning artifacts, where DNA polymerase makes errors when copying the template DNA, greater than 14% of cloned sequences contain point mutations that are not present in the original template DNA (Keohavong and Thilly 1989; Speksnijder et al. 2001). To account for this phenomenon, multiple positive clones per individual should be sequenced, as described previously, and base changes appearing in only one clone are considered artifacts and excluded from further analyses. These steps to identify and exclude artifactual sequences were not described in a previous study (Puri et al. 2007), and thus further confirmation of mutations reported earlier is warranted. However, if the same point mutations consistently are found in multiple individuals, as Michel et al. (2004) reported for 200 susceptible and resistant hydrilla samples, then these mutations likely do reflect the original DNA sequence.

Michel et al. (2004) tested polymorphisms, other than the one at codon 304, in dioecious hydrilla, but those did not affect the level of resistance. Similarly, in the acetolactate synthase gene of weedy rice (*Oryza sativa* L.) accessions, polymorphism patterns did not relate to levels of tolerance to the herbicide imazethapyr (Shivrain et al. 2010). However, Puri et al. (2006, 2007) detected variation in the level of fluridone resistance in hydrilla accessions possessing the same AGT mutation at codon 304. Therefore, there may be uncharacterized changes in *pds* gene regulation mechanisms (e.g. promoters, transcription factors), or altered epigenetic interactions, which may play a role in the degree of resistance. For example, the codon 304 substitutions at the first and second positions break up a potential CpG methylation site, which could have implications for regulation of gene expression (Vaillant and Paszkowski 2007). We observed the same set of point mutations in all fluridone-resistant and susceptible dioecious hydrilla, with the exception of Lake Toho hydrilla that lacked a third allele, and Lake Seminole (Georgia) hydrilla that had one haplotype with a slightly

different combination of nucleotides at polymorphic sites. Therefore, it is unlikely that any of these single nucleotide polymorphisms contribute to herbicide resistance. Similarly, all point mutations in monoecious hydrilla were universal in that biotype. Such polymorphisms suggest an intraspecific, or interspecific, hybrid origin for triploid dioecious and monoecious hydrilla (Benoit 2011). Further research of *pds* gene regulatory mechanisms, including epigenetic factors such as changes in methylation, needs to be conducted.

The efficiencies of the PDS enzymes with codon 304 mutations, relative to the wild type, have been characterized as "similar" (Michel et al. 2004); however, they are not identical. PDS enzymes with the low (AGT), medium (TGT), and high (CAT) resistance genotypes had specific activities (μg of zeta-carotene produced per mg enzyme per hour), as a percent of the wild-type genotype CGT specific activity, of 74, 66, and 95%, respectively (after Michel et al. 2004). Therefore, levels of resistance for each genotype may not correlate with the relative efficiencies of the altered enzymes, or with fitness relative to the wild type. Therefore, other, as yet unidentified, factors may affect PDS enzyme efficiency and fitness of plants, in addition to the mutations in codon 304. In order to better understand the relationship between codon 304 mutations, specific activity of *pds* genotypes, and relative fitness of individuals, tests of specific activities should be repeated for each genotype and from multiple locations.

Hydrilla that was collected originally from Lake Toho in 2000, and grown continuously in aquaria (W. Haller, personal communication), possessed the CAT allele, as reported previously by Puri et al. (2006, 2007). Yet three samples we collected from Lake Toho (one in 2006 and two in 2010), had the AGT allele. Similarly, four samples collected in 2010 from Lake Okahumpka all possessed the TGT (cysteine) allele; whereas, Michel et al. (2004) reported previously the CAT (histidine) allele from this lake. Spontaneous mutations from CAT to TGT or AGT are unlikely in such a short time period, given that two point mutations are necessary in each case. Data from limited sample analysis per lake in this study, and by Michel et al. (2004), suggested that only one genotype was present in each lake. However, greater sampling effort across time and space has revealed that multiple genotypes coexist in Lake Toho (M. Shaner, personal communication). No additional data are available, at this time, regarding the co-occurrence of multiple *pds* genotypes in Lake Okahumpka. Two possible scenarios can explain the co-occurrence of resistant genotypes. Either a single resistant genotype evolved per lake, followed by an unintentional introduction of a second resistant genotype, or more than one resistant genotype evolved within the same waterbody. Lake Toho hydrilla accessions collected in 2000 and 2010, representing both the CAT and AGT genotypes, differ from other US dioecious hydrilla by possession of only two detectable haplotypes rather than three. Therefore, the presence of both of these resistant genotypes in Lake Toho hydrilla was likely the result of two independent, in situ somatic mutations. Unfortunately, the initial *pds* genotype frequencies within Lake Toho, or any lake with resistant hydrilla, are unknown. Understanding the changes in frequencies of resistance mutations within a lake following cessation of fluridone treatments could provide valuable information on the relative fitness of the different genotypes.

Prolonged use of an effective herbicide with a single mode of action often can lead to the evolution of resistance (Beckie 2006, Koschnick et al. 2006), as in the case of fluridone and resistant dioecious hydrilla. However, there is a lack of published data on treatment histories for lakes with either the dioecious or monoecious biotypes. A comparison of hydrilla management histories for a sample of infested lakes revealed different treatment patterns between lakes with dioecious versus monoecious hydrilla (Table 2). Fluridone use at sites with dioecious hydrilla was characterized by long-term (8 to10 years) continuous annual treatments and the lack of alternative treatments (Table 2). Alternative treatments included nonPDS enzyme inhibiting herbicides or physical control methods such as mechanical removal, or stocking sterile triploid grass carp (*Ctenopharyngodon idella*) that eat aquatic vegetation, including hydrilla. Fluridone use at sites with US monoecious hydrilla, in contrast, ranged from 2 to 8 years, and three of the five sites had at least one alternative treatment method in use. Only one water body containing monoecious hydrilla, Long Pond in Massachusetts, had a fluridone use history (i.e., eight continuous years) comparable to those of the dioecious sites. None of the monoecious hydrilla sites possessed herbicide-resistant genotypes, but dioecious hydrilla at three of five sites developed resistance to fluridone.

Individual treatment regimens at the scale of a single lake or, more broadly, at the scale of water bodies within a state, generate geographically discontinuous selection pressures (Délye et al. 2010). Such variability in selective pressure may give rise to geographic variability in plant population adaptive events. In Florida, where selection was high because of frequent use of fluridone, multiple independent mutations were selected in various lakes. Fluridone use, in general, was much lower in other states, such as South Carolina, Delaware and Connecticut, where alternate herbicides and mechanical methods were often used. Subsequently, no herbicide-resistant mutations have been detected in these states. No mutations were detected in any location with monoecious hydrilla, and decreased effectiveness of fluridone treatments of monoecious hydrilla has never been reported. Monoecious hydrilla may have been subjected to less frequent fluridone treatments, with the exception of one locality in Massachusetts. However, monoecious hydrilla may be less susceptible to mutation at codon 304 and, therefore, less likely to evolve resistance to fluridone, as it does not possess the unique dioecious haplotype that is prone to mutation at codon 304. Managers still should proceed with caution and use alternate herbicides and physical removal methods. The mutation rate for codon 304 in monoecious hydrilla may be lower than rates for dioecious hydrilla, for as yet undetermined reasons, but it is likely not zero. Given enough time, a mutation in codon 304 that confers herbicide resistance may arise in monoecious hydrilla.

Resistance to the herbicide fluridone has evolved quite rapidly in hydrilla since fluridone was first approved in 1986 by the USEPA. Previously developed molecular tools and methodologies used to identify herbicide-resistant mutations, or to test for resistance in vitro, require significant time, effort and expense. The development, described in this study, of a PCR and sequencing-based molecular method to amplify the hydrilla *pds* gene region containing codon 304 sequences, streamlines the process of identifying codon 304 point mutations, and makes cloning unnecessary in most cases. Treating herbicide-resistant hydrilla with fluridone is not only a waste of resources, but may exacerbate the problem by

killing any susceptible plants and increasing the frequency of resistant biotypes. Using this new method, lake managers may have hydrilla tested for codon 304 mutations prior to selecting a control treatment. A similar protocol may be in use already as the SePRO Corporation recently began to offer a service, called GenTEST, to identify specific genetic sequences in hydrilla.

Large scale screening of US accessions and accessions from other countries revealed that all codon 304 mutations were found only in US dioecious hydrilla, and all were located on the same homologous allele. No codon 304 mutations were detected in any of the US monoecious hydrilla populations that have been exposed to fluridone, or in accessions outside the US that never have been exposed to this herbicide. Additional research should be done to elucidate the etiology of resistance in only one hydrilla haplotype.

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Appendix 1. Hydrilla sample collection sites.[a]

Samples no.	Collection site	Country	Collector	Date collected/received	Comments	Biotype
US						
AR 838	Millwood Lake, Little River County, Arkansas	US	D. Les	July 10, 2010		D
CA D2.1	Spruce Lateral 4, Brawley, Imperial County, California	US	M.R. Mizumoto	1995	USDA[b], gDNA	D
CT 07	Mystic Seaport pond, Mystic, New London County, Connecticut	US	L. Benoit	July 13, 2005		M
CT 32B	Silvermine River, Norwalk, Fairfield County, Connecticut	US	L. Benoit	July 27, 2006		M
CT 55	Mystic Seaport pond, Mystic, New London County, Connecticut	US	L. Benoit	August 8, 2007		M
CT 56	Silvermine River, Norwalk, Fairfield County, Connecticut	US	L. Benoit	September 24, 2007		M
NM 09_005	Crystal Lake, Weston, Fairfield County, Connecticut	US	N. Murray	September 15, 2009		M
DE 50	Concord Pond, Sussex County, Delaware	US	L. Benoit	July 29, 2007	Close to 100% coverage	M
DE 52	Wagamons Pond, Milton, Sussex County, Delaware	US	L. Benoit	July 29, 2007		M
DE 53	Haven Lake, Milford, Sussex County, Delaware	US	L. Benoit	July 29, 2007		M
DE 54	Griffith Lake, Milford, Sussex County, Delaware	US	L. Benoit	July 29, 2007		M
DC 48	Kenilworth Aquatic Gardens, District of Columbia	US	L. Benoit	July 28, 2007		M
FL 08	Lake, Disney Caribbean Beach Resort, Orlando, Orange County, Florida	US	L. Benoit	March 6, 2006	No flowers or turions.	D
FL 09	Canal at Jaycee Park, Okeechobee, Okeechobee County, Florida	US	L. Benoit	May 7, 2006		D
FL 13	Creek under Hoover Road overpass, Winter Haven, Polk County, Florida	US	L. Benoit	May 7, 2006		D
FL 14	Lake Howard, Winter Haven, Polk County, Florida	US	L. Benoit	May 7, 2006		D
FL 15	Lake Rochelle, Winter Haven, Polk County, Florida	US	L. Benoit	May 7, 2006		D
FL 16	Lake Fannie, Winter Haven, Polk County, Florida	US	L. Benoit	May 7, 2006		D
FL 21	Lake Seminole, Sneads, Jackson County, Florida	US	L. Benoit	May 9, 2006	Sneads Landing	D
FL 64	Lake Okeechobee, Okeechobee, Okeechobee County, Florida	US	L. Benoit	December 28, 2009		D
FL 65	Lake Tohopekaliga, Osceola County, Florida	US	L. Benoit	December 29, 2009		D
FL 66	Lake Okahumpka, Wildwood, Sumter County, Florida	US	L. Benoit	December 30, 2009		D
GA 22	Lake Seminole, Faceville, Decatur County, Georgia	US	L. Benoit	May 9, 2006		D
GA 23	Gray's Creek, J. Strom Thurman Lake, Lincolnton, Lincoln County, Georgia	US	L. Benoit	May 10, 2006		M
TWsn	Idaho	US	T. Woolf			D
LA 1.1	Louisiana	US			USDA[b], gDNA	D
LA 840	Caddo Lake, Crouch Dam Park, Caddo Parish, Louisiana	US	D. Les	July 11, 2010		D
MA 06	Long Pond, Barnstable, Barnstable County, Massachusetts	US	K. Gazaille	July 13, 2005		M
ME 04	Pickerel Pond, Limerick, York County, Maine	US	J. McPhedran	June 23, 2005		M
MD 43A	Otter Point Creek, Smith State Park, Leight Park, Hartford County, Maryland	US	L. Benoit	July 27, 2007		M
MD 49	Patuxent River Park, Jug Bay, Upper Marlboro, Prince Georges County, Maryland	US	L. Benoit	July 28, 2007		M
NY SKsn	New York	US	S. Kishbaugh			M
NC 61	Lake Gaston, Summit, Halifax County, North Carolina	US	L. Benoit	September 19, 2008	Two pieces have single open female flower. Others have unopened male flower buds.	M
NC 62	Lake Gaston, Littleton, Warren County, North Carolina	US	L. Benoit	September 19, 2008	Two pieces have female flowers.	M
NC 63	Lake Gaston, Henrico, Northampton County, North Carolina	US	L. Benoit	September 19, 2008		M
SC 25	Durham Creek, Moncks Corner, Berkeley County, South Carolina	US	L. Benoit	May 11, 2006		D
SC 27	Lake Moultrie, Chicora, Berkeley County, South Carolina	US	L. Benoit	May 11, 2006		D
TX 1	Lake Palestine, Harrison County, Texas	US	N. Harms	June 19, 2009		D
TX 842	Brandy Branch Reservoir, Texas	US	D. Les	July 11, 2010		D
VA 30	Lake Gaston NW, Town of Bracey, Mecklenburg County, Virginia	US	L. Benoit	May 12, 2006		M
VA 46	Potomac River, Alexandria, Fairfax County, Virginia	US	L. Benoit	July 27, 2007	One piece w/ four female flowers.	M
VA 47	Lake Anna State Park pond, Spotsylvania County, Virginia	US	L. Benoit	July 28, 2007	No aquatic plants in lake proper.	M
WI MNsn	Wisconsin	US	M. Netherland			M
Outside US						
Aus 9839	Alstonville, Bullwinkle Park, Maguires Creek, Australia	Australia	S. Jacobs	February 9, 2007	SJ: native to Australia, "Australia 1"	U
Aus 9841	Rocky Creek Dam, N. of Lismore, Australia	Australia	S. Jacobs	February 10, 2007	SJ: native to Australia, "Australia 2"	U

Appendix 1. Cont.

Samples no.	Collection site	Country	Collector	Date collected/received	Comments	Biotype
Aus 9877	Ord. River Dam, WA, Australia	Australia	S. Jacobs N. Tippery	June 30, 1905	SJ: native to Australia	U
Aus 9895	Howard Springs, NT, Australia	Australia	S. Jacobs N. Tippery	June 30, 1905	Cultivated. Ex Singapore	U
Aus 9903	Wildman River, 4 Mile Hole, NT	Australia	S. Jacobs N. Tippery	June 30, 1905	"Australia 4"	U
Aus 9936	Kelso (Townsend)	Australia	S. Jacobs N. Tippery	June 30, 1905	Cultivated	U
Aus 9937	Kelso (Townsend)	Australia	S. Jacobs N. Tippery	June 30, 1905	Cultivated	U
Australia 6	Alice River, Queensland, Australia	Australia	J. Balciunas	June 9, 1905	USDA[b], gDNA	U
Burundi 58	Lake Tanganyika, Burundi	Burundi	W. van Vierssen	Jul-1989	USDA[b], gDNA	U
Bj 4	Beijang River, Qingyuan, Guangdong Province	China	L. Chen	October 22, 2009	silica dried	U
CH 01	Changdu Lake, Shilin, Yunan Province	China	L. Chen	June 11, 2010	silica dried "China 1"	U
DA 1	Donganjiang River, Fengkai, Guangdong Province	China	L. Chen	October 26, 2009	silica dried	U
EH 01	Erhai Lake, Dali, Yunan Province	China	L. Chen	June 9, 2010	silica dried	U
Hj 1	Heijang River, Fengkai, Guangdong Province	China	L. Chen	October 26, 2009	silica dried	U
Jz 1	Jinzhuang River, Fengkai, Guangdong Province	China	L. Chen	October 25, 2009	silica dried	U
Sj 1	Suijiang River, Huaiji, Guangdong Province	China	L. Chen	October 25, 2009	silica dried, "China 2"	U
China 14	Beijing, China	China	J. Tsai	Nov-1984	USDA[b], gDNA	U
India 21	Bangalore, India	India	J. Tsai	Jan-1986	USDA[b], gDNA	U
India 61	Rajasthan, India	India	W. van Vierssen	Jul-1989	USDA[b], gDNA, "India 1"	U
India 64	Kashmir, India	India	W. van Vierssen	Jul-1989	USDA[b], gDNA, "India 3"	U
Indonesia 66	Rawa Penig, Java, Indonesia	Indonesia	W. van Vierssen	Jul-1989	USDA[b], gDNA	U
Java	Pangalengam, Java	Indonesia	W. van Vierssen	Jul-1989	USDA[b], gDNA	U
UK 500-501	Ballinakill Lough, Connemara, County Galway, Ireland	Ireland	U. King	June 10, 2010		U
UK 531	Rusheenduff Lough, Connemara, County Galway, Ireland	Ireland	U. King	July 25, 2010		U
Japan 49	Kobe, Japan	Japan	G. Buckingham	Dec-1992	USDA[b], gDNA	U
UK 505	Lake Citss, Aglona, Latvia	Latvia	U. King	July 10, 2010		U
UK 509-510	Lake Sila (Sila Ezers), Skrudaliena, Latvia	Latvia	U. King	July 12, 2010		U
UK 514	Lake Skujines, South of Dārzini, Ilūske, Latvia	Latvia	U. King	July 13, 2010		U
Malaysia 7	Panang Island, Malaysia	Malaysia	J. Balciunas	Before 1989	USDA[b], gDNA	U
T35	Bich Dong caves, North Vietnam	North Vietnam			USDA[b], gDNA	U
Pakistan 72	Rawalpundi, Pakistan	Pakistan	W. van Vierssen	Jul-1989	USDA[b], gDNA	U
SA1	South Africa	South Africa			USDA[b], gDNA	U
KR 277	Korea, Gyeongsangbuk-do, Uiseong-gun, Bian-myeon, Bangchoji; reservoir	South Korea	H. R. Na	August 7, 2008	Only female flower	likely D
KR 278	Korea, Gyeongsangbuk-do, Gunwi-gun, Sobo-myeon, stream	South Korea	H. R. Na	August 7, 2008	After cultivation in the pond in AJOU, female flower occurred.	likely D
KR 434	Korea, Gyeongsangbuk-do, Yeongcheon-si, Bonchon-dong, ditch	South Korea	H. R. Na	August 8, 2008	Only female flower	likely D
KR S 1-4	Korea, Gyeongsangbuk-do, Seongju-gun, Gyejeong-ri, Hoecheon	South Korea	H. R. Na	July 23, 2009	gDNA samples, "Korea S1"	U
KR U 1	Korea, Gyeongsangbuk-do, Uiseong-gun, Bian-myeon, Bangchoji	South Korea	H. R. Na	July 24, 2009	gDNA samples	U
KR Y 1	Korea, Gyeongi-do, Yeoju-gun, Gangcheon-ri, Bawineupgubi	South Korea	H. R. Na	July 25, 2009	gDNA samples	U
Korea 51	Palang Reservoir, Han R., Seoul, South Korea	South Korea	B. Pemberton	Oct-1989	USDA[b], gDNA	M
Taiwan 37	Wildman River, 4 Mile Hole, NT	Taiwan	J. Tsai	Jan-1986	USDA[b], gDNA	U
T4	Bang Phra Reservoir, Thailand	Thailand			USDA[b], gDNA	U
T8	Tha Thung Na Dam, Thailand	Thailand			USDA[b], gDNA	U

[a] Abbreviations: M, monoecious; D, dioecious; U, unknown; gDNA, genomic DNA; USDA, United States Department of Agriculture.
[b] Collection information from Madiera et al. (1997)