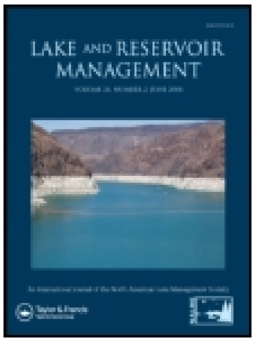

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The aquarium trade: A potential risk for nonnative plant introductions in Connecticut, USA

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NOTE

The aquarium trade: A potential risk for nonnative plant introductions in Connecticut, USA

Abstract

The aquarium trade has been shown to be a source for introductions of nonnative aquatic macrophyte species. Improvements are needed in identification, labeling, and retailer awareness of banned aquatic plants. In the state of Connecticut, United States, 20 nonnative macrophytes are banned from sale by state statute. At least 13 of these species are already established in the state's lakes, while the remainder are either present in near-shore wetlands or absent but thought to be capable of naturalizing. We documented the sale of banned plants in the Connecticut aquarium trade by visiting 23 retailers in 2008 and 47 retailers in 2010. Plants that resembled Connecticut's banned species were purchased and identified using standard morphological techniques. In 2010, we also employed DNA sequencing to aid in plant identification. We found that nearly 30% of stores sold banned aquatic plants including *Cabomba caroliniana*, *Egeria densa*, *Myriophyllum aquaticum*, and *Myriophyllum heterophyllum*. *Cabomba caroliniana* represented more than half of the banned species being sold; it was found in 17% of the stores in 2008 and 19% of the stores in 2010. *Egeria densa* was mislabeled 50% of the time as *Egeria najas* or *Anacharis najas*; it was sold in 11% of the stores in 2008 and 17% of the stores in 2010. In 2010, *Myriophyllum* specimens from 6 stores were unidentifiable using morphological characteristics. Using these techniques, one of the specimens was identified as *Myriophyllum heterophyllum*. Of the 29 chain stores surveyed, 7% sold banned species compared to 56% of the 27 independent stores.

Key words: aquarium trade, *Cabomba caroliniana*, *Egeria densa*, molecular identification, *Myriophyllum* spp., nonnative aquatic macrophyte, regulations, transport pathways

Introduction pathways are routes that result in the invasion of nonnative species. Due to advancements in human movement and trade, the rate of nonnative species introductions has increased markedly (Mack et al. 2000). Invasive nonnative aquatic macrophytes represent a severe threat to lakes and ponds because they have few natural enemies to limit their spread (Pimentel et al. 2001). These plants can clog water intakes, decrease recreational opportunities, and reduce local real estate values (Fishman et al. 1998, Connecticut Aquatic Nuisance Species Working Group 2006). In addition to the human impacts, severe ecological effects such as declines in species richness, reduction in species diversity, and alteration in ecosystem processes may occur (Vitousek et al. 1996). Once nonnative macrophytes are introduced into aquatic environments, their eradication is difficult; thus, preventing introduction through key pathways such as restrictions on sale and transport of these species is a particularly attractive alternative to postinvasion control (Kolar and Lodge 2001, Cohen et al. 2007). The United States government and most states have adopted regulations on the sale and transport of specific nonnative aquatic macrophytes (www.invasivespeciesinfo.gov).

The state of Connecticut, United States, lists 20 nonnative aquatic macrophytes as banned from sale, distribution, and import in section 22a-381d of the General Statutes (Table 1). One or more of the listed species occurs in nearly two-thirds of Connecticut lakes and ponds (Fig. 1; Bugbee and Balfour 2010). Several of the listed species are not normally encountered in lakes and ponds because they either typically occur in near-shore wetlands or have not been found but are listed because they are considered capable of naturalizing in Connecticut.

The aquarium trade has been identified as a major pathway for nonnative aquatic plant introductions (Strecker et al. 2011, Cohen et al. 2007). Of the nonnative aquatic macrophytes present in Connecticut lakes and ponds, *Cabomba caroliniana*, Egeria *densa*, *Hydrilla verticillata*, *Myriophyllum aquaticum*, *Myriophyllum heterophyllum*, *Myriophyllum spicatum*, and *Trapa natans* are reported as being sold by aquarium or water garden retailers (Kay and Holye 2001). Stopping the sale of invasive aquarium plants could eliminate this introduction pathway. Regulations that ban aquatic plants can only be effective if retailers are aware of the laws and can readily identify the plants. At least 124 taxa of aquatic macrophytes are commonly sold in the aquarium trade (Strecker et al. 2011, Kay and Holye 2001); most are from the tropics and not likely to overwinter in temperate climates. These plants are usually not banned but sometimes have similar morphological characteristics to regulated species and can be difficult to identify for even the most highly qualified taxonomists. Moreover, wholesalers of aquatic aquarium plants may not know the true identity of the plant they are marketing or give it an undocumented name out of convenience. Given these limitations, the only method to accurately identify certain species of aquatic plants may be to utilize molecular identification techniques.

The goals of this study were to (1) survey Connecticut aquarium retailers to determine the occurrence of banned aquatic plants; (2) evaluate the level of expertise needed to identify the plants; and (3) document mislabeling.

Methods

Pet store surveys

In 2008 and 2010, we sampled Connecticut aquarium retailers for invasive aquatic macrophytes banned from sale in

Table 1.-Aquatic macrophyte species banned under Connecticut State Statutes (2009; Sec.22a-381d). Invasive species found in Connecticut lakes and ponds in bold.

#	SCIENTIFIC NAME	COMMON NAME
1	*Butomus umbellatus* L.	Flowering rush
2	***Cabomba caroliniana* Gray**	**Fanwort**
3	***Callitriche stagnalis* Scop.**	**Pond water-starwort**
4	***Egeria densa* Planch.**	**Brazilian water-weed, Anacharis, Egeria**
5	***Hydrilla verticillata* (L. f.) Royle**	**Hydrilla**
6	*Iris pseudacorus* L.	Yellow iris, Yellow flag iris
7	*Lythrum salicaria* L.	Purple loosestrife
8	***Marsilea quadrifolia* L.**	**European waterclover, Water shamrock**
9	*Myosotis scorpioides* L.	Forget-me-not, Water scorpion-grass
10	***Myriophyllum aquaticum* (Vell.) Verdc.**	**Parrotfeather**
11	***Myriophyllum heterophyllum* Michx.**	**Variable-leaf watermilfoil**
12	***Myriophyllum spicatum* L.**	**Eurasian watermilfoil**
13	***Najas minor* All.**	**Brittle water-nymph, Minor naiad**
14	*Nelumbo lutea* (Willd.) Pers.	American water lotus
15	***Nymphoides peltata* (S.G. Gmel.) Kuntze**	**Yellow floating heart**
16	***Potamogeton crispus* L.**	**Curly leaf pondweed, Crispy-leaved pondweed**
17	*Rorippa microphylla* (Rchb.) H.Hyl.	Onerow yellowcress
18	*Rorippa nasturtium-aquaticum* L. Hayek	Watercress
19	*Salvinia molesta* D.S. Mitch.	Giant salvinia
20	***Trapa natans* L.**	**Water chestnut**

the state. Of the 106 total registered stores, 28 were visited in 2008, and 47 different stores were visited in 2010. Stores that did not sell live aquarium plants were not included in our results. At each store, an aquatic biologist purchased any plant that morphologically resembled a Connecticut banned species (Table 1). These specimens were brought back to the laboratory for identification. Each plant was identified morphologically using the taxonomy of Crow and Hellquist (2000a, 2000b) then pressed and stored in The Connecticut Agricultural Experiment Station herbarium (NHES). The aquatic macrophytes were also planted in greenhouse tanks to grow and develop structures, such as flowers, that could aid in identifying questionable specimens. In 2008, many plants could not be identified morphologically, so in 2010 we used genetic sequencing to determine the identity of questionable plants during our sampling of stores in that year.

Molecular identification/DNA sequencing

Fresh samples of each specimen were brought to the laboratory for DNA sequencing. DNA extraction was conducted using Qiagen DNeasy Plant and Mericon Food Kits. Once DNA was isolated from the individual plants, the following 3 regions were amplified using polymerase chain reaction (PCR) technology: (1) aptB-rbcL noncoding spacer region of the chloroplast genome (Chiang et al. 1998); (2) the ssrDNA- lsrDNA Internal Transcribed Spacer (ITS) region (White et al. 1990); and (3) the small subunit rDNA (ssrDNA; Lane et al. 1985). The amplification primers used were the following:

Chloroplast aptB-rbcL Non coding spacer region

ATPbF - ACATCKARTACKGGACCAATAA
RbclR2 - AACACCAGCTTTRAATCCAA

Internal Transcribed Spacer (ITS) region

16SF-FNG TGATATGCTTAAGTTCAGT
28SR-FNG ACAAGGTCTCCGTTGGTGAAC

Small subunit rDNA

25EF CTGGTTGATCCTGCCAG
1490ER TACGGAAACCTTGTTACGACTT

Thermocycling conditions were 94 C for 3 min, followed by 35 cycles of 94 C for 45 s, 45 C for 30 s, and 72 C for 1.5 min; followed by 72 C for 10 min. Sequencing was conducted by the DNA analysis facility at Yale University (New Haven, CT) in a 3730XL Genetic Analyzer. The derived sequences were compared to known sequences using the BLAST search engine at the NCBI GenBank facility.

DNA sequence information (bar coding) is helpful in distinguishing plants that cannot be identified with morphological techniques. Plant DNA bar coding is a developing science (Kress and Erickson 2007), and the guidelines to relate DNA

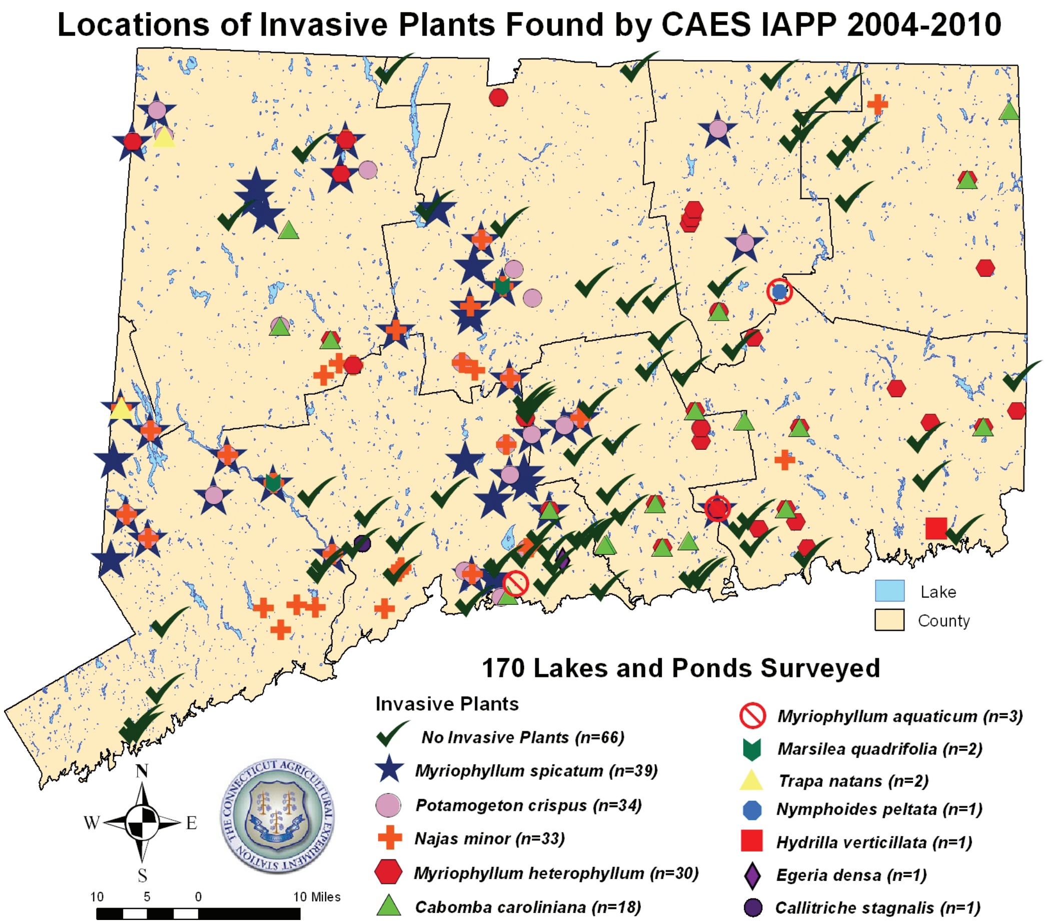

Figure 1.-Locations and frequencies of invasive aquatic macrophytes found in Connecticut lakes and ponds from 2004 to 2011 (color figure available online).

sequence similarity with species level identification has not been completely resolved. However, sequences that were identical (100% match) with the small subunit rDNA in GenBank were considered the same. Of equal importance, sequences that differed by 3 or more nucleotides in the ss-rDNA were considered different species. Additionally, sequences for which the small subunit rDNA was not present in GenBank were considered the same species if they differed by 9 or fewer nucleotides at either the chloroplast gene or ITS region. This threshold was chosen because we have observed similar sequence differences for these genes between isolates of the same species when all 3 genes were present in GenBank and the small subunit rDNA matched exactly.

Follow-up visits to surveyed stores

In 2011 we revisited all the surveyed stores and gave educational information to the store manager. The information included an identification guide for Connecticut's invasive aquatic and wetland plants (Bugbee and Balfour 2010), a poster with pictures of the banned invasive aquatic plants found in Connecticut, and a copy of the State Statutes (Sec 22a-381d) regarding the sale of banned plants.

Results and discussion

Morphological identification

We found that in 2008, 35% of the 23 surveyed stores (n = 8) sold banned aquatic plants compared to 30% of the 47 stores (n = 14) in 2010 (Table 2). *Cabomba caroliniana* was the most common banned plant for sale, available at 17% of the stores (n = 4) in 2008 and 19% of the stores (n = 11) in 2010. *Egeria densa* was the second most common banned plant found, available in 17% of the stores (n = 4) in 2008 and 11% of the stores (n = 5) in 2010. *Egeria densa* specimens were mislabeled as *Anacharis*, *Anacharis najas*, or *Egeria najas* 50% of the time. We found *Myriophyllum aquaticum* for sale at 9% of the stores (n = 2) in 2008 and 2% of the stores (n = 1) in 2010. In 2010 we found *Myriophyllum heterophyllum* for sale in 2% of the stores

Table 2.-Connecticut pet stores selling banned invasive aquatic macrophytes in 2008 and 2010.

Year	Stores Visited n	Stores Selling Banned Plants n (%)	Stores Selling *Cabomba caroliniana* n (%)	Stores Selling *Egeria densa* n (%)	Stores Selling *Myriophyllum aquaticum* n (%)	Stores Selling *Myriophyllum heterophyllum* n (%)	Stores Selling Unidentifiable *Myriophyllum* n (%)
2008	23	8 (35)	4 (17)	4 (17)	2 (9)	0	NA
2010	47	14 (30)	9 (19)*	5 (11)	1 (2)*	1 (2)*	5 (11)*

*Molecular techniques used to confirm identification

(n = 1). Positive identification of this plant required molecular techniques; 13% of the stores (n = 5) sold a *Myriophyllum* species that we could not identify using either morphological or molecular techniques.

Molecular identification

Specimens identified morphologically as *Egeria densa* were a 100% match to the species small subunit rDNA sequence in GenBank and differed by 5 nucleotides for the chloroplast gene and 1 nucleotide for the ITS region (Table 3). Our morphological identification of *Cabomba caroliniana* proved to be 2 different species based on DNA sequencing. Nine of the 11 individuals we obtained matched exactly the sequences for *C. caroliniana* in GenBank for the small subunit rDNA and chloroplast genes. There was a 7 base-pair (bp) difference in the ITS region, but this fell within our acceptable level (Table 3). Two individuals were found to differ from *C. caroliniana* in the small subunit (6 bp) and chloroplast gene (38 bp). We identified these 2 specimens as *Cabomba furcata*, which is not a banned species in Connecticut, based on a 9 nucleotide difference in the ITS region compared to the sequences of *Cabomba furcata* in GenBank (Table 3). These findings confirm that significant numbers of *C. caroliniana* are being sold in the market place and that morphological identifications are not entirely reliable for this species.

We obtained 7 *Myriophyllum* specimens but were only able to morphologically identify one specimen, *M. aquaticum*. Of the remaining 6 specimens, we were only able to identify one as *M. heterophyllum* based on DNA sequencing. This specimen exhibited an exact match in the small subunit and was different by 2 and 4 bp in the ITS region and the chloroplast gene respectively. We were unable to identify the remaining 5 *Myriophyllum* specimens using DNA sequencing because the DNA failed to amplify for one and there were no matching GenBank sequences for the others (Table 3). Overall, these results show that the morphological identification of *Myriophyllum* species sold in the aquarium trade is very difficult; therefore, the potential of aquarium plant retailers distributing banned *Myriophyllum* species is high. In addition, DNA sequences are not available online for all species in this genus. Database development for *Myriophyllum* species is necessary before molecular identification can be implemented.

Cultivated plants are responsible for the majority of introductions of nonnative aquatic plants in the Northeast United States (Les and Mehrhoff 1999). Moreover, the aquarium trade has been shown to be responsible for numerous nonnative species introductions (Rixon et al. 2005, Cohen et al. 2007). Aquatic ecosystems are species poor in regard to macrophytes compared to terrestrial systems (Capers et al. 2007, 2009) and thus are particularly susceptible to invasion by nonnative species (Shea and Chesson 2002). A focus on education and regulatory policy is therefore needed to limit import routes for nonnative aquatic macrophytes.

Connecticut's aquarium plant retailers are currently selling many freshwater plants regulated by state statute. Nearly 30% of stores in our study sold banned species. The most common banned plant was *Cabomba caroliniana*, and *Egeria densa* was also readily available. Though *E. densa* is not a widespread invasive in Connecticut, it has naturalized in a few lakes and is therefore capable of overwintering (Fig. 1). *Egeria densa* was often mislabeled as *Anacharis*, *Anacharis najas*, or *Egeria najas* (a nonbanned plant). The *Myriophyllum* species we purchased were unidentifiable 86% of the time (n = 6) with morphological techniques. We determined through DNA sequencing that one of the *Myriophyllum* specimens was the state-banned species *Myriophyllum heterophyllum*. We were not able to identify the remaining 5 *Myriophyllum* specimens even with molecular techniques. Misidentified and unidentified *Myriophyllum* species represent a risk for new invasions because it is unknown if they can inhabit Connecticut water bodies, and there are currently 3 naturalized species from this genus. Likewise, *C. furcata* and *E. najas* represent similar risks because other species from these genera are already invasive in Connecticut lakes.

Our revisits in 2011 to distribute educational information found most dealers to be cordial and interested in protecting the lakes and ponds from invasive species. Some felt the wholesale distributors should be prohibited from selling banned species and provide proper identification of their

Table 3.-Genetic identification table for sampled individuals. Species in bold are confirmed identifications based on comparisons with GenBank NCBI database and morphological characteristics.

Original Morphological Identification	Closes Sequence Match	Chloroplast Gene (nt difference)	ITS Region (nt difference)	Small Subunit (nt difference)	Accession Numbers	Number of Individuals (n)
Myriophyllum aquaticum	***Myriophyllum aquaticum***	5	0	0	EF529704.1, EF526367.1, EF526314.1	1
Myriophyllum spp.	***Myriophyllum heterophyllum***	4	2	0	EF529718.1, EF526365.1, EF526349.1	1
Myriophyllum spp.	*Gratiola aurea*	N/A	104	15	EF526401.1, EF526352.1	1
Myriophyllum spp.	*Myriophyllum aquaticum*	11	25	NA	EF529704.1, EF526367.1	1
Myriophyllum spp.	—	N/A	N/A	N/A	None	1
Myriophyllum spp.	*Myriophyllum heterophyllum*	72	30	10	EF529718.1, EF526398.1, EF526310.1	1
Myriophyllum spp.	*Myriophyllum aquaticum*	22	88	2	EF529704.1, EF526367.1, EF526314.1	1
Egeria najas	***Egeria najas***	NS	4	NS	JF805750, AY330708.1, JF805757	5
Egeria densa	***Egeria densa***	5	1	0	EF529712.1, AY330707.1, EF526327	5
Cabomba caroliniana	***Cabomba caroliniana***	0	7	0	EF529722.1, AY620424.1, AY165511.1	9
Cabomba caroliniana	***Cabomba furcata***	NS	9	NS	JF805755, AY620425.1, JF805759	2

nt difference – refers to the number of nucleotides that differ between sample and database sequences; N/A – indicates genes that failed to replicate; NS – indicates a new sequence that was uploaded to GenBank during this study due to the lack of comparable sequences for these species.

plants. Many wholesalers are from out of state, thus complicating the regulatory process.

Conclusions

The Connecticut aquarium trade poses a risk of introducing nonnative aquatic macrophytes because 30% of stores surveyed were selling banned species. *Cabomba caroliniana* and *Egeria densa* are the 2 most common banned plants offered for sale. *Myriophyllum aquaticum* and *Myriophyllum heterophyllum* are also available. The incidence of species misidentification by wholesalers and retailers is high with *Egeria densa.* Members of the genus *Myriophyllum*, sold by aquarium retailers, often look similar and cannot be readily identified morphologically. Molecular identification is necessary, but matching sequences in a GenBank are not currently available. *Cabomba furcata* may be confused with *C. caroliniana*; therefore, it may require that the genera *Myriophyllum* and *Cabomba* be banned entirely to limit further spread and species introductions. Greater education is also needed to curtail wholesale and retail sales of restricted species. Finally, enforcement and the regulatory procedures may need to incorporate molecular techniques to insure plants are correctly identified.

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