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Genetic and Morphological Differences of Water Chestnut (Myrtales: Lythraceae: *Trapa*) Populations in the Northeastern United States, Japan, and South Africa

Lynde L. Dodd, Nancy Rybicki, Ryan Thum, Yasuro Kadono, and Kadiera Ingram

March 2019

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Cover image: Dried fruits of water chestnut (species, population code). Top, left to right: *Trapa natans*, BR-MD-16; *T. natans*, CH-RI-16; *T.* sp 2, EM-KZN-16; *T. incisa*, NAK-16. Bottom: *Trapa* sp., WP-VA-16; *T. japonica*, ONO-J; *T. natans var. pumila*, ONO-J; *T. natans*, TEM-J. Photo Credit: Pablo Jimenez-Reyes.

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Abstract

Cryptic introductions are non-native species that have been introduced outside of native ranges; these introductions are undetected because the species have morphology similar to native or other non-native species naturalized within the same region. While non-native, invasive *Trapa natans* has been present in the Northeastern (NE) United States (U.S.) since the late 1800s, unpublished data suggests a new introduction of *Trapa* has occurred in the Commonwealth of Virginia. This population was distinct: it had 2-spined fruit as opposed to the typical 4-spined fruit associated with *T. natans*. It was therefore suspected as a cryptic introduction of *Trapa* species.

This work aims to elucidate genetic and morphological differences of naturalized *Trapa* taxa (water chestnut) in the NE U.S. Comparisons of morphological characteristics and genetics were made between *Trapa* populations from the native regions of Eurasia and Africa versus those of the NE U.S. Results of the morphological analysis supported genetic results that 2-spine *Trapa* sp. and 4-spine *T. natans* in the U.S. were different, with the number of spines and the presence of a crown (*Trapa* sp. lacks a crown) as morphological taxonomic indicators.

Given the problems associated with introduced water chestnut in the U.S., further investigation into the genetic and ecological characteristics of each distinct taxa are warranted.

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Preface

This study was conducted for the Aquatic Plant Control Research Program (APCRP). The APCRP is sponsored by Headquarters, U.S. Army Corps of Engineers (HQUSACE), and is assigned to the U.S. Army Engineer Research and Development Center (ERDC) under the purview of the Environmental Laboratory (EL), Vicksburg, Mississippi. The APCRP Program Manager is Dr. Linda Nelson.

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This report was prepared under the general supervision of Dr. Timothy E. Lewis, chief, Aquatic Ecology and Invasive Species Branch (CEERD-EEA); Mr. Mark D. Farr, chief, Ecosystem Evaluation and Engineering Division (CEERD-EE); and Dr. Ilker R. Adiguzel, Director, EL.

At the time of the publication of this report, COL Ivan P. Beckman was the Commander of ERDC, and Dr. David W. Pittman was the Director.

Acronyms and Abbreviations

Acronym	Meaning
AFLP	Amplified Fragment Length Polymorphisms
APCRP	Aquatic Plant Control Research Program
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
EE	Ecosystem Evaluation and Engineering Division
EEA	Aquatic Ecology and Invasive Species Branch
EL	Environmental Laboratory
ERDC	Engineer Research and Development Center
NE	Northeastern
PCoA	Principal Coordinates Analysis
TR	Technical Report
U.S.	United States
USGS	U.S. Geological Survey
HQUSACE	Headquarters, U.S. Army Corps of Engineers

1 Introduction

1.1 Background

Native to Eurasia and Africa, water chestnut (Myrtales: Lythraceae: *Trapa* L.) (Graham 2005; ITIS 2015; USDA 2016) is an annual, floating-leaved aquatic plant (Pemberton 2002; Crow and Hellquist 2000; Hummel and Kiviat 2004). Considered invasive in the United States (U.S.), its aggressive growth negatively influences aquatic ecosystem biodiversity and function and impedes hydroelectric power generation, irrigation, and recreation (Rawls 1964a, b; Carter and Rybicki 1994; Caraco and Cole 2002; Pemberton 2002; Naylor 2003; Hummel and Kiviat 2004; Ding and Blossey 2005; Hummel and Findlay 2006; LaManche 2007). Water chestnut has been reported in a number of states in the U.S., including Connecticut, Delaware, Massachusetts, Maryland, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, and the District of Columbia (EDDMaps 2017; Pfingsten et al. 2017). The plant has been particularly problematic in the Northeastern (NE) U.S. since shortly after its introduction during the latter half of the 19th century (Gwathmey 1945; Carter and Rybicki 1994; Les and Mehrhoff 1999; Naylor 2003). Although it has not been listed as a federal noxious weed, the following states have proactively listed water chestnut as noxious and/or prohibited: Alabama, Arizona, Connecticut, Delaware, Florida, Idaho, Illinois, Maine, Maryland, Massachusetts, Michigan, Minnesota, New Hampshire, New York, North Carolina, Oregon, Pennsylvania, South Carolina, Tennessee, Vermont, Virginia, Washington, and Wisconsin (EDDMaps 2017; Pfingsten et al. 2017).

While only one species of *Trapa* was known to be introduced to the U.S. (*Trapa natans*) (EDDMaps 2017; Pfingsten et al. 2017), a morphologically distinct population was discovered in Gunston Cove on the Potomac River within the Commonwealth of Virginia (Figure 1; Rybicki 2017 unpublished data). This population was distinct in that it had 2-spined fruit as opposed to the typical 4-spined fruit associated with *T. natans* and was therefore suspected as a cryptic introduction of *Trapa* species[1]. Further investigation into the distribution of this led to additional observations of

[1] Rybicki, N. 2014. Personal communication with Nathan Harms via email. 9 September 2014. USGS and USACE ERDC.

populations in Virginia of the 2-spined *Trapa* (hereafter referred to as *Trapa* sp.). The 2-spined *Trapa* sp. is not morphologically different from descriptions of *T. japonica* (Kadono 1987; Kadono 2018). However, the taxonomy and species identification of *Trapa* is confusing due to the wide variability in morphological traits (Kim et al. 2010; Li et. al 2017). Cook (1990) indicated there may be only one polymorphic species or up to 20 species worldwide within the genus. There also appears to be many synonyms in different geographic regions (Kadono 1987). Therefore, it is unclear whether the 2-spine *Trapa* sp. is a morphological variant of *T. natans*, or whether it is a genetically distinct and cryptic species of *Trapa*.

Figure 1. *Trapa* sp. 2-spine fruit (left) and pink flower (right) collected from the Potomac River September 201 Virginia.

Determining whether the 2-spined and 4-spined *Trapa* in the U.S. are distinct, cryptic taxa is important for effectively managing them. Currently, management strategies used to control *Trapa* in the U.S. (whether physical, chemical, or biological) are based upon the understanding that *Trapa natans* is the only *Trapa* taxon found in the U.S. Differences in phenology or ecology (i.e., anthesis, biomass, number of fruit produced, and/or competitive ability) of cryptic species can potentially affect the implementation and effectiveness of management strategies employed by water resource managers.

1.2 Objectives

The objectives of this work were the following: (1) determine the genetic and morphological differences of *Trapa* taxa in the NE U.S., (2) compare those to *Trapa* populations from native and introduced regions of Eurasia

and Africa, and (3) summarize variation in morphological characteristics of genetically distinct groups and populations of *Trapa* in this study.

1.3 Approach

***Trapa* collection.** Specimens of the genus *Trapa* were collected in the 2016 growing season (June through November) from a variety of populations by U.S. Geological Survey (USGS) and U.S. Army Corps of Engineers (USACE) researchers, and including other numerous collaborators (Table 1). Varying numbers of rosettes were collected in the field (3–30, depending on availability) at least 1 to 2 meters apart to avoid sampling the same plant, with at least one mature, fully ripe fruit (when available) for each population. For populations within the U.S., each rosette was rinsed and placed into a labeled plastic bag and shipped overnight in coolers to USGS (Reston, VA) for morphological evaluation. Specimens from outside the U.S. were dried prior to shipment. One to three leaves from each rosette were dried in silica gel and sent to Montana State University for molecular analysis. The putative species for Japanese specimens were assigned based on the width of the fruit and followed the nomenclature in Table 1 of Takono and Kadono (2005). The nomenclature for specimens collected in the U.S. from NY, RI, and VT follows Britton and Brown (1970), but was not specified for specimens collected in VA and will be referred to as *Trapa* sp. Specimens from S. Africa did not have mature fruits, were not identified to species, and will be referred to as *Trapa* sp. 2 in this technical report (TR).

1.4 Availability of specimens

Pressed specimens, dried leaves, and fruits are archived and available on request by contacting the author, Ms. Lynde Dodd, ERDC-EL. Pressed specimens of *Trapa* sp. (Fairfax County, VA, collected in 2015) and *T. natans* (Baltimore County, MD, collected in 2015) are also available at George Mason University Herbarium, Fairfax, VA (digital images on-line: http://sernecportal.org/portal/index.php#). Specimens and data related to DNA are archived and available upon request by contacting Dr. Ryan Thum, Montana State University.

Table 1. Populations of *Trapa* taxa collected in 2016 for this study (*indicates 11 to 12 corresponding quality fruits available for morphological analysis).

Population Code	Country	County, City, District	State, Prefecture	Putative Species
WP-VA-16*	U.S.	Fairfax	VA	*Trapa* sp.
VCB-VA-16*	U.S.	Fairfax	VA	*Trapa* sp.
ML-VA-16*	U.S.	Fairfax	VA	*Trapa* sp.
BR-MD-16	U.S.	Baltimore	MD	*Trapa natans*
CH-RI-16*	U.S.	Washington	RI	*Trapa natans*
CP-RI-16*	U.S.	Providence	RI	*Trapa natans*
IB-NY-16*	U.S.	Greene	NY	*Trapa natans*
SC-NY-16*	U.S.	Columbia	NY	*Trapa natans*
TP-NY-16*	U.S.	Albany	NY	*Trapa natans*
MM-NY-16*	U.S.	Cayuga	NY	*Trapa natans*
SSB-NY-16*	U.S.	Wayne	NY	*Trapa natans*
RC-NY-16*	U.S.	Wayne	NY	*Trapa natans*
CDL-VT-16*	U.S.	Rutland	VT	*Trapa natans*
CCD-NY-16*	U.S.	Washington	NY	*Trapa natans*
CMB-NY-16*	U.S.	Warren	NY	*Trapa natans*
TEM-J-16*	Japan	Kako	Hyogo	*Trapa natans*
KO-J-16*	Japan	Kobe City	Hyogo	*Trapa natans*
IWA-J-16*	Japan	Kobe City	Hyogo	*Trapa japonica*
ONO-J-16*	Japan	Ono City	Hyogo	*Trapa japonica* dominant & *Trapa natans* var. *pumila*
MIK-J-16	Japan	Mikata	Fukui	*Trapa japonica* & *Trapa natans* var. *pumila* mixed
NAK-J-16*	Japan	Tsuruga City	Fukui	*Trapa incisa*
EM-KZN-16	South Africa	Empangeni	KwaZulu-Natal	*Trapa* sp. 2

*indicates 11 to 12 corresponding quality fruits available for morphological analysis

2 Genetic and Morphological Differences of *Trapa* taxa

2.1 Genetic

Both introduced and native *Trapa* taxa specimens collected in 2016 for this project were processed for genetic analysis using amplified fragment length polymorphisms (AFLPs). For each of the 22 populations, one to six individuals and one duplicate was sampled for Deoxyribonucleic acid (DNA). DNA was extracted using Qiagen DNeasy Plant DNA extraction kits. Preparation of AFLPs followed Thum et al. (2011) using ~100 ng of total genomic DNA and two primer pairs (EcoR1-CGA/Mse1-AGG and EcoR1-CTG/Mse1-AGG). In order to estimate scoring error rates, duplicate AFLPs were performed on approximately 20% of all samples.

Amplified fragment length polymorphism data were scored with GeneMapper v4.0 (Applied Biosystems) and analysis of fragments was limited between 100 and 500 base pairs in length. SpAGedi version 1.5 (Hardy and Vekemans 2002) was used to remove loci, which were not repeatable by estimating heritability in the subset of samples for which there were duplicates using $F_{ST} \geq 0.8$. The final dataset for this analysis contained 475 AFLP markers. Principal Coordinates Analysis (PCoA), as implemented in GenAlEx 6.5 (Peakall and Smouse 2006, 2012) was used to analyze the AFLP dataset (no a priori groupings were determined for the PCoA, distances were standardized, and covariance method was used for this analysis).

2.2 Morphology

For each of the 22 populations, measurements of the morphological attributes of specimens of *Trapa* were made to quantify taxonomic characteristics to differentiate potentially genetically distinct groups of specimens or populations following the procedure of Kadono (1987) (Table 2 and Figure 2). In addition to the procedure used by Kadono (1987), for each rosette sampled, the following observations were recorded using the following fresh specimens: the width of stem below the rosette (within 10–20 cm from the base), flower petal color, and the color of the abaxial surface of leaves.

Figure 2. Mode of measuring the size of fruit. A: angle (°) between upper spines. W: width of the fruit across upper spines. H: height of the fruit. D: thickness of the fruit. L: width across lower projections (pseudohorns or spines). B. Types of lower projections. I: Pseudohorns shorter than 2 mm. II: Pseudohorns with the length between 2 and 4 mm. III: Pseudohorns longer than 4 mm. IV: Lower spines with acute apex. Illustration and descriptions from Kadono (1987).

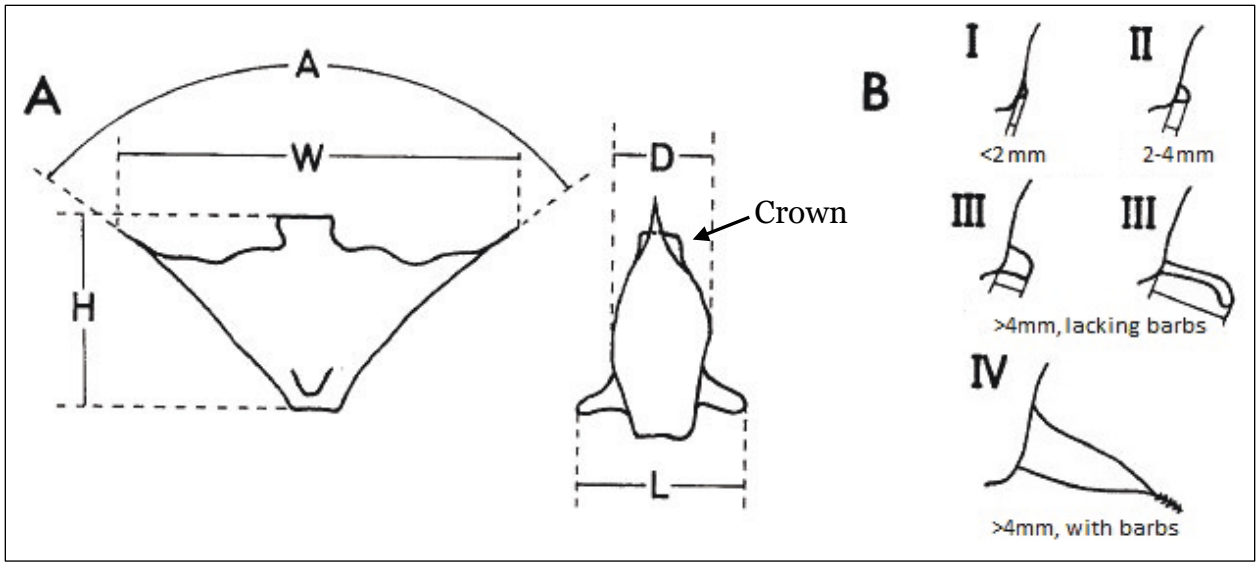


Table 2. Morphological characteristics observed and recorded for *Trapa* specimens collected in 2016.

Plant part	Morphological description
Leaf	Color (underside); teeth (count)
Flower	Color (petal)
Fruit *General*	Crown (presence); dry weight (g); height (mm); thickness (mm); number of spines
Upper Horns	Width (mm); orientation (ascending, descending, horizontal) Upper horn reflex (presence)
Lower Projections	Apex (acute, obtuse, both, none); base (narrow, wide, combination, none); orientation (ascending, descending, horizontal, none, undetermined); width (mm), length (mm)
Stem	Width (mm) below rosette

Additionally, one typical leaf was selected–representative in size and overall appearance of its rosette–and the number of teeth was counted. At least one mature fruit was selected for each rosette and dried. The following observations were recorded using dried fruits (see Figure 2): dry weight, presence or absence of a crown, the number of spines, width across upper spines, width across lower projections, height, thickness (across

main fruit body), angle and orientation of upper spines, lower projection length and type, shape of lower projection apex, shape of lower projection base (where projection meets main fruit body), and presence or absence of an oxbow-like or recurving shape in the upper spines. Furthermore, the presence and severity of any shriveling or damage to each fruit was noted, and those fruits that were not shriveled during drying or did not have sufficient damage to effect a measurement were considered quality fruit. For each population, the first 11 or 12 rosettes collected that had quality fruits were distinguished from the others and selected for morphological and genetic analysis and used to summarize correspondence between morphological and genetic characteristics. Fruit characteristics for three populations, BR-MD (n=5), MIK-J (n=8), and EM-KZN (n=1) were not included in the summary of fruit morphology because they did not have a sufficient number of quality fruits to represent the variability of a population.

3 Results and Discussion

3.1 Genetic and morphological differences of *Trapa* taxa

Specimens from 22 populations of six putative species from both the introduced and native ranges of *Trapa* taxa were included in the study (Figure 3, Table 1).

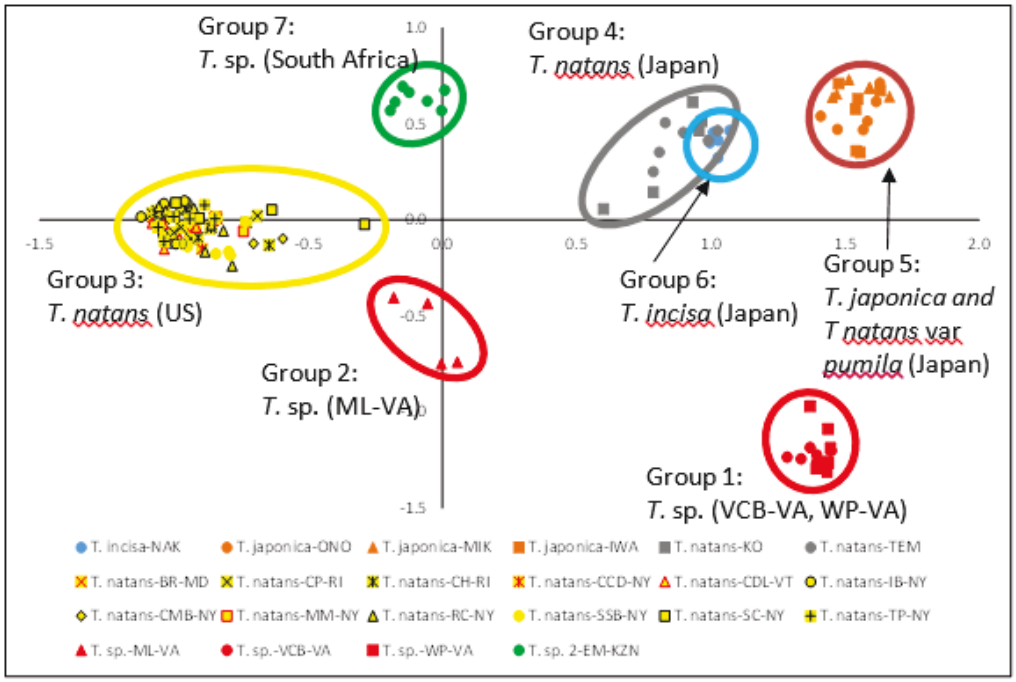
Figure 3. Twenty-two populations of *Trapa* taxa were collected in 2016 from the U.S. (n = 15), South Africa (n = 1), and Japan (n = 6).

3.1.1. Genetic

Amplified fragment length polymorphism data analysis showed a clear genetic separation between the newly discovered 2-spine *Trapa* sp. and 4-spine *T. natans* in the U.S., confirming them as genetically distinct (PCoA Axis 1 and 2 explained 33% and 8% of the variation, respectively, Figure 4, Appendix Table 1). U.S. *Trapa* taxa were also compared to four taxa collected from Japan (*T. incisa*, *T. natans*, *T. natans* var. *pumila*, and *T. japonica*), and one unidentified species collected from South Africa (*Trapa* sp. 2, EM-KZN-16). U.S. populations identified as *T. natans* did not show a genetic affinity to Japanese samples identified as *T. natans*, indicating a possible cryptic introduction within what is currently recognized as *T. natans*. The U.S. 2-spine *Trapa* sp. taxon did show a genetic affinity to Japanese samples identified as *T. japonica* and *T. natans* var. *pumila*, although they were separated along axis 2 of the PCoA. One *Trapa* sp. U.S. population, ML-VA, showed genetic separation from all other species, indicating the potential for yet another cryptic introduction, or possibly a hybrid between *Trapa natans* and *Trapa* sp. in

the U.S. The genetic analysis differentiated seven groups used for discussion of morphology (Table 3).

Figure 4. Two-dimensional representation of principal coordinates analysis of amplified fragment length polymorphism for *Trapa* populations collected in 2016. Circles represent distinct separation.



3.1.2. Morphology

A summary of morphology by genetic groups (groups are described in Table 3 and Figure 4). While the genetic analysis differentiated the population of *Trapa* sp. in ML-VA (group 2) as a separate group from *Trapa* sp. in WP-VA and ML-VA (group 1), the morphological characteristics were not different (Figure 5 A to F). The U.S. *Trapa* sp. groups from populations in Virginia had similar width of upper spines, dry weight of fruit, number of teeth on the leaf, width of stem, and percentage of 2-spine fruits. *Trapa* sp., however, was morphologically different from the group of *T. natans* in the U.S. (group 3) and from all other groups because *Trapa* sp. lacked a crown on the fruit (Figure 5 F), the underside of the leaf was dark red, and flowers were pink (Table 3). In contrast to *Trapa* sp. in the U.S., for all other genetic groups and populations in the U.S. or Japan, a crown was consistently present (except for NAK-J), the underside of the leaf was green (or green and brown), and flowers were described as white. Six of the individuals were lacking a crown in group 3

and those were in the three populations, RC-NY (n=1), MM-NY (n=4), and SC-NY (n=1).

Table 3. Summary of genetic groups, species, country, populations, and selected morphological parameters. ND = no data.

Genetic group	Putative species	Populations	Flower color	Color on underside of leaf
1	Trapa sp. (U.S.)	WP-VA, VCB-VA	Pink	Dark red
2	*Trapa* sp. (U.S.)	ML-VA	Pink	Dark red
3	*Trapa natans* (U.S.)	BR-MD[1], CH-RI, CP-RI, IB-NY, SC-NY, TP-NY, MM-NY, SSB-NY, RC-NY, CDL-VT, CCD-NY, CMB-NY	White	Green and brown
4	*Trapa natans* (Japan)	TEM-J, KO-J	White	Green with pink veins, Green and brown
5	*Trapa japonica, Trapa natans var pumila* (Japan)	MIK-J[1], IWA-J, ONO-J	White	Green and brown
6	*Trapa incisa* (Japan)	NAK-J	White	Green
7	*Trapa* sp. *2* (S. Africa)	EM-KZN[1]	ND	Green and brown

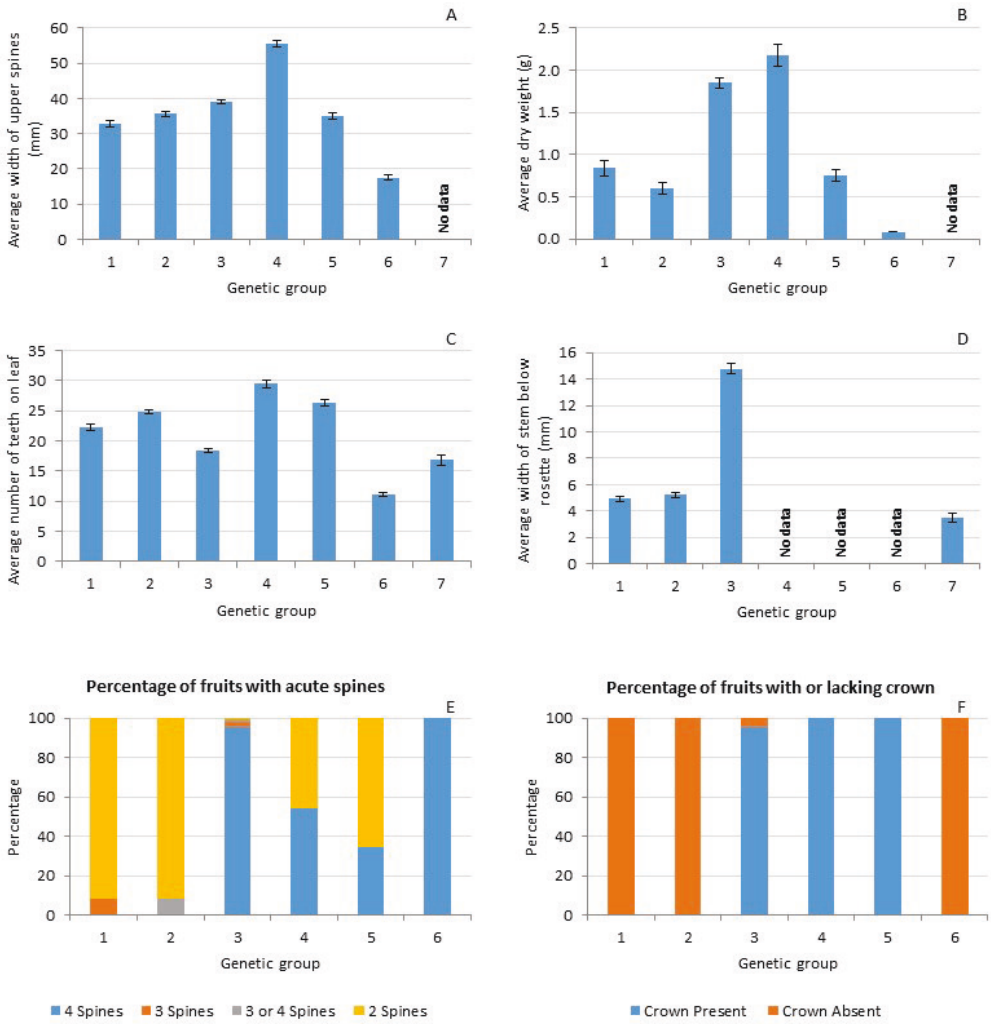
[1]Insufficient number of quality fruits to summarize fruit morphology in the population

The Japanese *T. incisa (*group 6) fruit was the smallest in width and dry weight (Figure 5 A and B). The S. African *Trapa* sp. 2 (group 7) had no flowers present and the only fruit present was lacking a crown and had two spines. This population had a leaf teeth count of < 20 (Figure 5 C) and the underside of the leaf was green and brown (Table 3). The stem width was 5 mm or less for both the S. African *Trapa* sp. 2 (group 7) and U.S. *Trapa* sp. (groups 1 and 2), while it was three times the width in *T. natans* in the U.S. (group 3) (Figure 5 D). Fruits with two spines, characteristic of *Trapa* sp., were also prevalent in two of the Japanese groups, the *T. natans* (group 4) and *T. natans* var *pumila* and *T. japonica* (group 5). However, the U.S. *T. natans* (group 3) and the Japanese *T. incisa* (group 6) groups had 4 spines (Figure 5 E).

The U.S. *T. natans* (group 3) had lower values than the Japanese *T. natans* (group 4) for fruit width and weight, and number of teeth on the leaf, and had a greater percentage of 4-spine fruit (Figure 5 A–C, E). The Japanese *T. natans* (group 4) had greater values than the Japanese *T. japonica* and *T. natans* var *pumila* (group 5) or *T. incisa* (group 6) for fruit width and

weight and the number of teeth on the leaf. The percentage of 4-spine fruit was 100% for *T. incisa* (group 6), and the number of spines varied between 2-spine and 4-spine for other Japanese groups (groups 4 and 5).

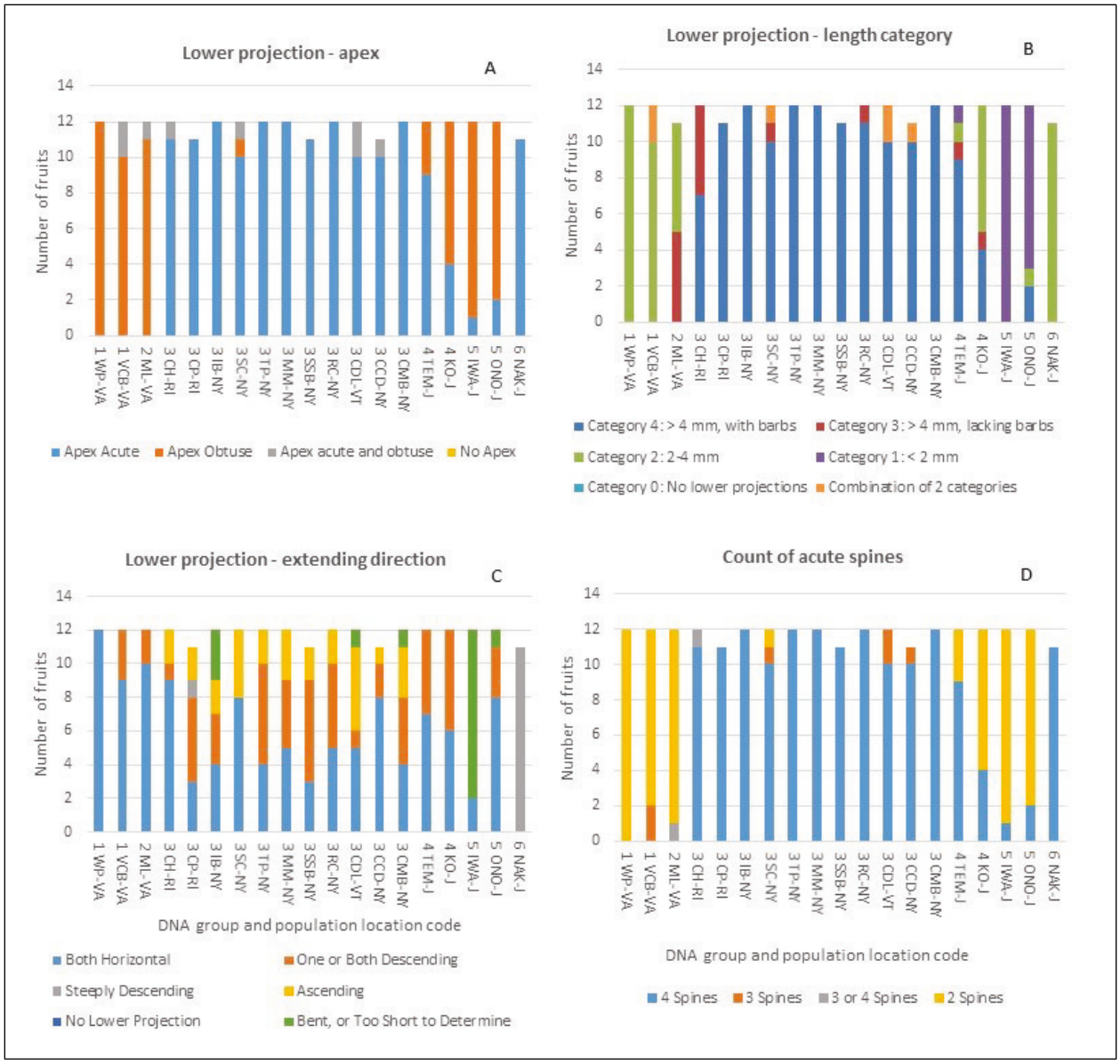
Figure 5. Morphological characteristics by the seven genetic groups shown in Figure 4 and Table 3. A, Width of upper spines of fruit; B, Dry weight of fruit; C, Number of teeth on leaf; D, Width of stem below rosette; E, Percentage of 2, 3, or 4 spine fruits; F, Presence of fruit crown for populations in each of the genetic groups numbered 1 to 7. The genetic group, putative species (location) and population for the groups are as follows: 1 is *Trapa* sp. (U.S.) WP-VA, VCB-VA; 2 is *Trapa* sp. (U.S.) ML-VA; 3 is *T. natans* (U.S.) BR-MD[1], CH-RI, CP-RI, IB-NY, SC-NY, TP-NY, MM-NY, SSB-NY, RC-NY, CDL-VT, CCD-NY, CMB-NY; 4 is *T. natans* (Japan) TEM-J, KO-J; 5 is *T. japonica, T. natans var pumila* (Japan) MIK-J[1], IWA-J, ONO-J; 6 is *T. incisa* (Japan) NAK-J; 7 is *Trapa* sp. *2* (S. Africa) EM-KZN[1]. Each bar represents the mean ± standard error for panels A–D.



Summary by population (populations are described in Table 1 and Appendix Table 2a, b, and c; genetic groups are described in Table 3 and Figure 4). For the nineteen populations with sufficient quality fruits, the qualitative (Figure 6) and quantitative (Figure 7) morphological parameters were summarized in plots. The populations shown in the plots (Figures 5–7) are arranged and labeled on the *x*-axis by the seven genetic groups identified in Figure 4, and groups are numbered in order of the latitude of each population in the group. By arranging them by latitude, the groups collected in the same country are adjacent and can be examined to determine whether proximity in latitude helps explain patterns of the variation in the morphological characteristics.

Upper and lower spine characteristics. The lower projection apex was generally obtuse in U.S. *Trapa* sp. (group 1 and 2), Japanese *T. japonica* and *T. natans* var. *pumila* (group 5) populations and was generally acute in U.S. *T. natans* (group 3) and Japanese *T. incisa* (group 6) populations (Figure 6 A). The upper spine orientation showed little variation and was ascending in all populations except where the upper spine was descending at KO-J in group 5. The lower projection length was highly variable among sites; however, the U.S. *Trapa* sp. (group 1) and Japanese *T. incisa* (groups 6) were similar in this trait (Figure 6 B). The lower projection base showed little variation and was wide at all sites, except the base was narrow on one of the fruits at site ML-VA and ONO-J. The lower projection's extending direction was horizontal for U.S. *Trapa* sp. (group 1 and 2), and the extending direction was less variable than at other sites (Figure 6 C). All fruits in Japan (with the exception of NAK-J) and the NE U.S. had a crown, while none of the *Trapa* sp. at any Virginia sites had a crown. Populations of *T. natans* in the U.S. had 4-spine fruits with few exceptions; there were rarely 3-spine fruits at two populations and a 2-spine fruit at one population (Figure 6D). Two-spine fruits were prevalent for U.S. *Trapa* sp. (group 1 and 2), except there were several 3-spine fruits at VCB-VA, and there was one 4-spine fruit at ML-VA. Two-spine fruits were also prevalent at Japanese populations ONO-J and IWA-J for *T. japonica* or *T. natans* var. *pumila* (group 5) and at KO-J, one of two Japanese populations with *T. natans* (group 4).

Figure 6. A, Lower projection - apex; B, Lower Projection - length category; C, Lower projection - extending direction; D, Number of acute spines for populations in each of the genetic groups numbered 1 to 6. The genetic groups are described in Figure 4 and Table 3. The genetic group, putative species (location) and population for the groups are as follows: 1 is *Trapa* sp. (U.S.) WP-VA, VCB-VA; 2 is *Trapa* sp. (U.S.) ML-VA; 3 is *T. natans* (U.S.) BR-MD[1], CH-RI, CP-RI, IB-NY, SC-NY, TP-NY, MM-NY, SSB-NY, RC-NY, CDL-VT, CCD-NY, CMB-NY; 4 is *T. natans* (Japan) TEM-J, KO-J; 5 is *T. japonica, T. natans var pumila* (Japan) MIK-J[1], IWA-J, ONO-J; 6 is *T. incisa* (Japan) NAK-J; 7 is *Trapa* sp. *2* (S. Africa) EM-KZN[1].

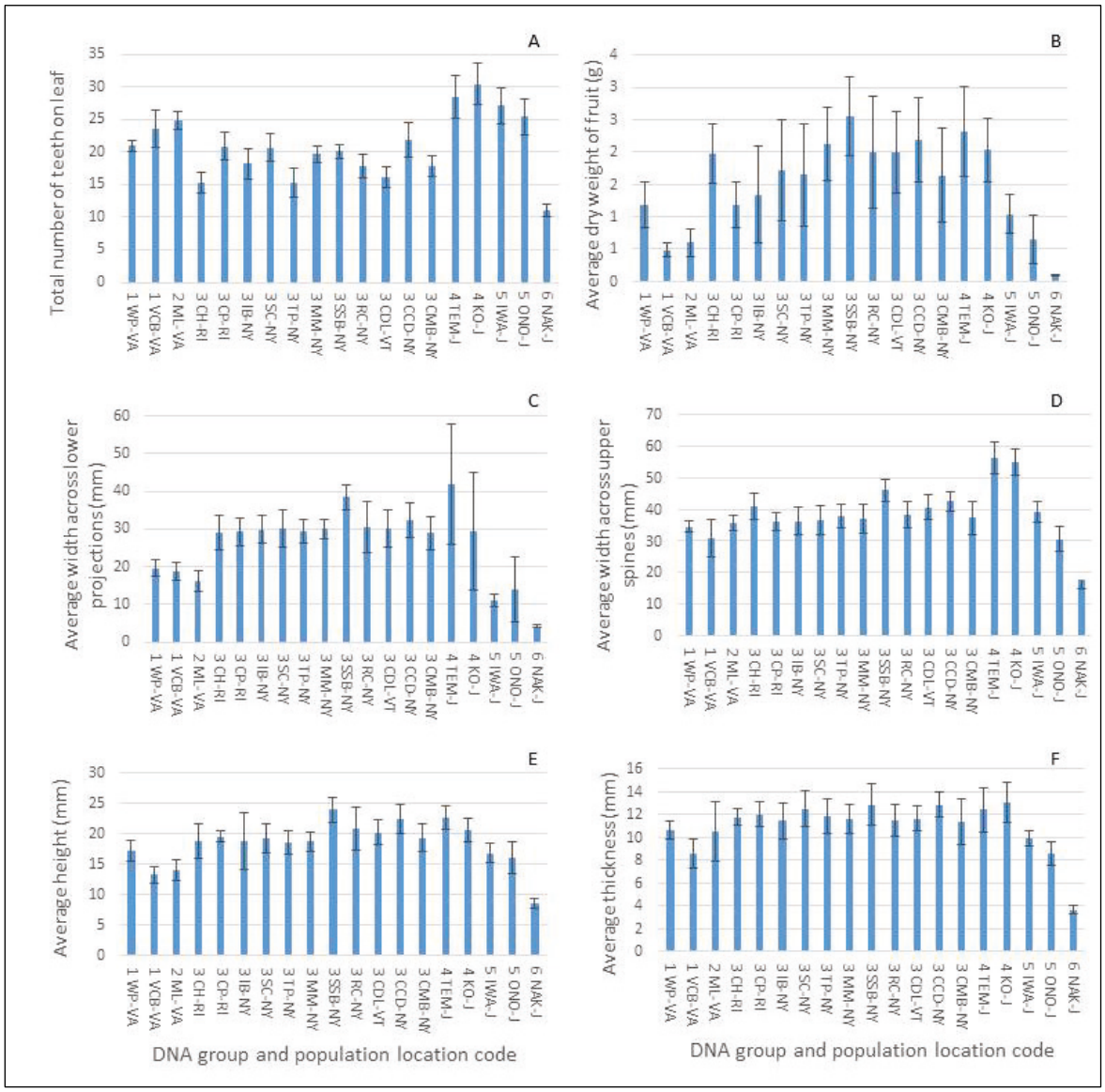


[1]Insufficient number of quality fruits to summarize fruit morphology in the population.

Leaf and fruit characteristics: The mean total number of leaf teeth was largest for the U.S. *T. natans* population, SC-NY, and smallest for the Japanese *T. incisa* population, NAK-J (Figure 7 A). The mean fruit dry weight ranged from 0.5 to 3.0 g, except in the NAK-J population, where it was < 0.25 g (Figure 7 B). The Japanese population from NAK-J had the

smallest (8 mm) and the *T. natans* TEM-J had the largest (45 mm) mean width across the lower spines (Figure 7 C). The mean width across the upper spines ranged from < 20 mm at NAK-J to about 55 mm for the Japanese, *T. natans* populations, KO-J and TEM-J (Figure 7 D). Mean fruit height was between 15 and 25 mm, except it was only 9 mm at the *T. incisa* population (Figure 7 E). While the fruit thickness of *T. incisa* was 4 mm, the range was 8 to 14 mm at all other populations (Figure 7F).

Figure 7. Mean ± standard deviation for leaf and fruit characteristics. A, total number of teeth on leaf; B, Average dry weight of the fruits; C, Average width across upper spines; D, Average width across lower projections; E, Average height; F, Average thickness for populations in each of the genetic groups numbered 1 to 6. The genetic groups are described in Figure 4 and Table 3. The genetic group, putative species (location) and population for the groups are as follows: 1 is *Trapa* sp. (U.S.) WP-VA, VCB-VA; 2 is *Trapa* sp. (U.S.) ML-VA; 3 is *T. natans* (U.S.) BR-MD[1], CH-RI, CP-RI, IB-NY, SC-NY, TP-NY, MM-NY, SSB-NY, RC-NY, CDL-VT, CCD-NY, CMB-NY; 4 is *T. natans* (Japan) TEM-J, KO-J; 5 is *T. japonica, T. natans var pumila* (Japan) MIK-J[1], IWA-J, ONO-J; 6 is *T. incisa* (Japan) NAK-J; 7 is *Trapa* sp. *2* (S. Africa) EM-KZN[1].



[1]Insufficient number of quality fruits to summarize fruit morphology in the population.

4 Conclusions

The preliminary results presented for both the morphological and genetic data after one year of surveys indicate there are cryptic introductions of *Trapa* naturalized in the NE U.S. While efforts have been undertaken to clarify the taxonomy of *Trapa* (by the authors and other researchers), at this time, it is possible that more than one taxa is currently distributed and has naturalized in the U.S.

4.1 Results

The results of this study are preliminary and how these results impact management of *Trapa* in the U.S. remains unclear. For example, the use of herbicides (chemical control) is a commonly used strategy for management of water chestnut in the U.S. and has been limited to the use of two herbicides, 2,4-D (2,4-dicholorophenoxy acetic acid) and triclopyr [93,5,6-trichloro-2-pyridinyl)oxy]acetic acid (Hummel and Kiviat 2004; Poovey and Getsinger 2007; GLMRIS 2012). Further investigation into the effectiveness of these products (and potentially new products approved for use in aquatic systems) on these newly discovered cryptic introductions is warranted.

Biocontrol (an alternative method to physical and chemical control of *Trapa*) includes the use of co-evolved herbivores to reduce the competitive influence of *Trapa*. This is especially relevant for the current biocontrol paradigm of matching specific herbivores to plant taxa in a geographically specific way. Currently, two species of the genus *Galerucella* (leaf-beetle) (*Coleoptera: Chrysomelidae*) have been shown to impact *Trapa natans* (Pemberton 1999; Pemberton 2002). A native water lily leaf beetle, *Galerucella nymphaeae*, widely distributed in Europe and North America, can contribute to a reduction in *Trapa* plant performance (Ding and Blossey 2005). Feeding by *G. nymphaeae* has been reported on the *Trapa* sp. population observed at Gunston Cove on the Potomac River[1], however, the long-term effects upon this population have not been evaluated. *Galerucella birmanica is* a promising potential biological control agent of *T. natans* (Ding et. al 2006), and may have the potential as a biocontrol agent for *Trapa* sp. Use of *G. birmanica* as a biocontrol for *Trapa* in the U.S. may reduce its photosynthetic ability and overall plant vigor leading

[1] Rybicki, N. 2014. Personal communication with Nathan Harms via email. September 9. USGS and USACE ERDC.

to decreased competitive ability, increasing the chances of native, more desirable submersed aquatic vegetation to compete for niche occupation (i.e., space, light, nutrients). It should be noted, however, that to date none of the insect agents found overseas and evaluated as biocontrol for water chestnut have been approved for release in the U.S. for the management of water chestnut.

4.2 Future work

The efforts of this work detail results from one year of morphological and genetics work for *Trapa* taxa in both its introduced and native ranges. Given the history and current problems associated with introduced water chestnut in the U.S., further investigation into the genetic and ecological characteristics of each distinct taxa are warranted, and will contribute to the knowledge base necessary for making decisions about managing this invasive plant.

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Appendix A: Raw Data Used in Morphology and Genetic Analyses

Table A-1. Principal Coordinates Analysis — X, Y data for Axis 1 and Axis 2 for Figure 4.

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0087	NE-Test -87	Trapa sp.	USA	VA	Waples Mill	Fairfax	1.437	-1.087
TRA-0087D	NE-Test -87	Trapa sp.	USA	VA	Waples Mill	Fairfax	1.370	-0.969
TRA-0109	SSB-NY1	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.733	-0.011
TRA-0114	SSB-NY6	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.789	-0.185
TRA-0120	SSB-NY12	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.967	-0.127
TRA-0126	SSB-NY18	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.949	-0.139
TRA-0132	SSB-NY24	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.796	-0.165
TRA-0132D	SSB-NY24	Trapa natans	USA	NY	South Sodus Bay	Wayne	-0.843	-0.176
TRA-0139	RC-NY1	Trapa natans	USA	NY	Red Creek	Wayne	-0.783	-0.239
TRA-0144	RC-NY6	Trapa natans	USA	NY	Red Creek	Wayne	-0.861	-0.025
TRA-0150	RC-NY12	Trapa natans	USA	NY	Red Creek	Wayne	-0.883	-0.166
TRA-0156	RC-NY18	Trapa natans	USA	NY	Red Creek	Wayne	-0.925	0.061
TRA-0162	RC-NY24	Trapa natans	USA	NY	Red Creek	Wayne	-0.815	-0.057
TRA-0168	RC-NY30	Trapa natans	USA	NY	Red Creek	Wayne	-1.030	0.046
TRA-0168D	RC-NY30	Trapa natans	USA	NY	Red Creek	Wayne	-1.055	0.069
TRA-0169	BR-MD1	Trapa natans	USA	MD	Bird River	Baltimore	-0.842	0.017
TRA-0171	BR-MD3	Trapa natans	USA	MD	Bird River	Baltimore	-0.918	-0.031
TRA-0173	BR-MD5	Trapa natans	USA	MD	Bird River	Baltimore	-0.939	-0.061
TRA-0175	BR-MD7	Trapa natans	USA	MD	Bird River	Baltimore	-0.993	-0.081
TRA-0177	BR-MD9	Trapa natans	USA	MD	Bird River	Baltimore	-0.838	0.006
TRA-0177D	BR-MD9	Trapa natans	USA	MD	Bird River	Baltimore	-1.079	0.014
TRA-0179	MM-NY1	Trapa natans	USA	NY	Montezuma Marsh	Cayuga	-0.740	-0.060
TRA-0209	CDL-VT1	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-1.080	-0.015
TRA-0214	CDL-VT6	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-0.978	-0.100

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0220	CDL-VT12	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-0.917	-0.038
TRA-0226	CDL-VT18	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-0.852	-0.003
TRA-0232	CDL-VT24	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-1.039	-0.023
TRA-0238	CDL-VT30	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-1.036	-0.152
TRA-0238D	CDL-VT30	Trapa natans	USA	VT	Drowned Lands, Lake Champlain	Rutland	-0.999	0.019
TRA-0239	CMB-NY1	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-0.943	0.015
TRA-0244	CMB-NY6	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-0.703	-0.123
TRA-0250	CMB-NY12	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-0.594	-0.098
TRA-0256	CMB-NY18	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-0.974	0.056
TRA-0262	CMB-NY24	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-1.053	0.024
TRA-0262D	CMB-NY24	Trapa natans	USA	NY	Mill Bay, Lake Champlain	Warren	-1.061	-0.010
TRA-0269	CCD-NY1	Trapa natans	USA	NY	Chubbs Dock, Lake Champlain	Washington	-0.895	-0.153
TRA-0274	CCD-NY6	Trapa natans	USA	NY	Chubbs Dock, Lake Champlain	Washington	-1.035	0.045
TRA-0292	CCD-NY24	Trapa natans	USA	NY	Chubbs Dock, Lake Champlain	Washington	-0.849	0.008
TRA-0299	TP-NY1	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-1.002	0.016
TRA-0304	TP-NY6	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-0.886	0.076
TRA-0310	TP-NY12	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-0.846	0.005
TRA-0316	TP-NY18	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-0.942	0.003

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0322	TP-NY24	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-1.036	-0.112
TRA-0322D	TP-NY24	Trapa natans	USA	NY	Tivoli Lake Park	Albany	-1.059	-0.038
TRA-0329	IB-NY1	Trapa natans	USA	NY	Inbocht Bay	Greene	-0.992	-0.124
TRA-0334	IB-NY6	Trapa natans	USA	NY	Inbocht Bay	Greene	-1.040	-0.078
TRA-0340	IB-NY12	Trapa natans	USA	NY	Inbocht Bay	Greene	-1.122	0.016
TRA-0346	IB-NY18	Trapa natans	USA	NY	Inbocht Bay	Greene	-1.037	0.084
TRA-0352	IB-NY24	Trapa natans	USA	NY	Inbocht Bay	Greene	-0.954	0.092
TRA-0352D	IB-NY24	Trapa natans	USA	NY	Inbocht Bay	Greene	-0.960	0.098
TRA-0359	SC-NY1	Trapa natans	USA	NY	Stockport Creek	Columbia	-0.635	0.051
TRA-0364	SC-NY6	Trapa natans	USA	NY	Stockport Creek	Columbia	-0.906	0.008
TRA-0370	SC-NY12	Trapa natans	USA	NY	Stockport Creek	Columbia	-0.289	-0.025
TRA-0376	SC-NY18	Trapa natans	USA	NY	Stockport Creek	Columbia	-0.997	0.015
TRA-0388D	SC-NY30	Trapa natans	USA	NY	Stockport Creek	Columbia	-0.992	0.088
TRA-0400	ML-VA13	Trapa sp.	USA	VA	Myrtle Leaf dr.	Fairfax	-0.054	-0.434
TRA-0406	ML-VA19	Trapa sp.	USA	VA	Myrtle Leaf dr.	Fairfax	-0.181	-0.405
TRA-0412	ML-VA25	Trapa sp.	USA	VA	Myrtle Leaf dr.	Fairfax	0.056	-0.740
TRA-0412D	ML-VA25	Trapa sp.	USA	VA	Myrtle Leaf dr.	Fairfax	-0.003	-0.749
TRA-0421	CP-RI3	Trapa natans	USA	RI	Central Pond	Providence	-0.730	-0.014
TRA-0426	CP-RI8	Trapa natans	USA	RI	Central Pond	Providence	-0.866	-0.043
TRA-0432	CP-RI14	Trapa natans	USA	RI	Central Pond	Providence	-0.973	-0.040
TRA-0438	CP-RI20	Trapa natans	USA	RI	Central Pond	Providence	-0.937	-0.097
TRA-0444	CP-RI26	Trapa natans	USA	RI	Central Pond	Providence	-1.082	0.033
TRA-0444D	CP-RI26	Trapa natans	USA	RI	Central Pond	Providence	-1.000	-0.067
TRA-0450	CP-RI32	Trapa natans	USA	RI	Central Pond	Providence	-0.688	0.021
TRA-0451	CH-RI1	Trapa natans	USA	RI	Chapman Pond	Washington	-0.952	0.050
TRA-0456	CH-RI6	Trapa natans	USA	RI	Chapman Pond	Washington	-1.063	0.029
TRA-0462	CH-RI12	Trapa natans	USA	RI	Chapman Pond	Washington	-1.063	0.048
TRA-0468	CH-RI18	Trapa natans	USA	RI	Chapman Pond	Washington	-1.035	0.005

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0474	CH-RI24	Trapa natans	USA	RI	Chapman Pond	Washington	-0.861	-0.041
TRA-0480	CH-RI30	Trapa natans	USA	RI	Chapman Pond	Washington	-0.909	-0.092
TRA-0480D	CH-RI30	Trapa natans	USA	RI	Chapman Pond	Washington	-0.644	-0.132
TRA-0483	EM-KZN1	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.003	0.568
TRA-0488	EM-KZN6	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.056	0.618
TRA-0494	EM-KZN12	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.123	0.664
TRA-0500	EM-KZN18	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	0.009	0.675
TRA-0500D	EM-KZN18	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.147	0.694
TRA-0502	EM-KZN20	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.176	0.613
TRA-0502D	EM-KZN20	Trapa sp.	South Africa	KZN	Empangeni	KwaZulu-Natal	-0.193	0.569
TRA-0503	WP-VA1	Trapa sp.	USA	VA	Waples	Fairfax	1.399	-1.271
TRA-0509	WP-VA7	Trapa sp.	USA	VA	Waples	Fairfax	1.426	-1.261
TRA-0515	WP-VA13	Trapa sp.	USA	VA	Waples	Fairfax	1.446	-1.187
TRA-0521	WP-VA19	Trapa sp.	USA	VA	Waples	Fairfax	1.439	-1.263
TRA-0527	WP-VA25	Trapa sp.	USA	VA	Waples	Fairfax	1.433	-1.309
TRA-0527D	WP-VA25	Trapa sp.	USA	VA	Waples	Fairfax	1.393	-1.294
TRA-0533	VCB-VA2	Trapa sp.	USA	VA	Nutley	Fairfax	1.449	-1.200
TRA-0539	VCB-VA8	Trapa sp.	USA	VA	Nutley	Fairfax	1.285	-1.234
TRA-0545	VCB-VA14	Trapa sp.	USA	VA	Nutley	Fairfax	1.396	-1.264
TRA-0550	VCB-VA19	Trapa sp.	USA	VA	Nutley	Fairfax	1.370	-1.186
TRA-0556	VCB-VA25	Trapa sp.	USA	VA	Nutley	Fairfax	1.337	-1.243
TRA-0556D	VCB-VA25	Trapa sp.	USA	VA	Nutley	Fairfax	1.395	-1.223
TRA-0563	ONO-J1	Trapa japonica	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.568	0.468
TRA-0569	ONO-J7	Trapa japonica	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.584	0.513
TRA-0575	ONO-J13	Trapa japonica	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.410	0.539

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0581	ONO-J19	Trapa natans var. pumila	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.472	0.471
TRA-0587	ONO-J25	Trapa japonica	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.630	0.712
TRA-0587D	ONO-J25	Trapa japonica	Japan	Hyogo Prefecture	Irrigation pond in Kashiyama-cho	Ono City	1.617	0.616
TRA-0593	IWA-J1	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.541	0.577
TRA-0599	IWA-J7	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.548	0.630
TRA-0605	IWA-J13	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.632	0.672
TRA-0611	IWA-J19	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.476	0.710
TRA-0617	IWA-J25	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.543	0.358
TRA-0617D	IWA-J25	Trapa japonica	Japan	Hyogo Prefecture	Oh-ike Pond in Iwaoka-cho	Kobe City	1.557	0.350
TRA-0623	MIK-J1	Trapa natans var. pumila	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.666	0.641
TRA-0629	MIK-J7	Trapa japonica	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.515	0.730
TRA-0635	MIK-J13	Trapa natans var. pumila	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.593	0.688
TRA-0641	MIK-J19	Trapa japonica	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.592	0.687
TRA-0647	MIK-J25	Trapa natans var. pumila	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.469	0.653
TRA-0647D	MIK-J25	Trapa natans var. pumila	Japan	Fukui Prefecture	Lake Mikata in Wakasa-cho	Mikata District	1.454	0.638
TRA-0653	KO-J1	Trapa natans	Japan	Hyogo Prefecture	Ohtoribami-ike Pond in Kande-cho	Kobe City	0.601	0.057
TRA-0659	KO-J7	Trapa natans	Japan	Hyogo Prefecture	Ohtoribami-ike Pond in Kande-cho	Kobe City	0.957	0.464
TRA-0671	KO-J19	Trapa natans	Japan	Hyogo Prefecture	Ohtoribami-ike Pond in Kande-cho	Kobe City	0.786	0.143
TRA-0677	KO-J25	Trapa natans	Japan	Hyogo Prefecture	Ohtoribami-ike Pond in Kande-cho	Kobe City	0.934	0.613

MSU Sample name	USGS sample name	Morphological Species ID	Country	State	Specific location	County	PCoA Axis 1 X	PCoA Axis 2 Y
TRA-0677D	KO-J25	Trapa natans	Japan	Hyogo Prefecture	Ohtoribami-ike Pond in Kande-cho	Kobe City	0.967	0.515
TRA-0683	TEM-J1	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	0.809	0.352
TRA-0689	TEM-J7	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	0.786	0.250
TRA-0695	TEM-J13	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	0.831	0.505
TRA-0701	TEM-J19	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	0.898	0.455
TRA-0707	TEM-J25	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	1.026	0.465
TRA-0707D	TEM-J25	Trapa natans	Japan	Hyogo Prefecture	Temma-Oh-ike in Inami-cho	Kako District	0.991	0.413
TRA-0713	NAK-J1	Trapa incisa	Japan	Fukui Prefecture	Nakaikemi Marsh	Tsuruga City	1.070	0.464
TRA-0719	NAK-J7	Trapa incisa	Japan	Fukui Prefecture	Nakaikemi Marsh	Tsuruga City	1.009	0.455
TRA-0725	NAK-J17	Trapa incisa	Japan	Fukui Prefecture	Nakaikemi Marsh	Tsuruga City	1.027	0.414
TRA-0731	NAK-J23	Trapa incisa	Japan	Fukui Prefecture	Nakaikemi Marsh	Tsuruga City	0.997	0.402
TRA-0731D	NAK-J23	Trapa incisa	Japan	Fukui Prefecture	Nakaikemi Marsh	Tsuruga City	1.027	0.323

Table A-2a. Summary of populations sampled and their respective genetic groups, species, latitude, and longitude.

Population code	Genetic group	Putative species	Latitude	Longitude
1 WP-VA	1	sp.	38.87362	-77.33995
1 VCB-VA	1	sp.	38.88058	-77.26836
2 ML-VA	2	sp.	38.84248	-77.39606
3 BR-MD	3	natans	39.37445	-76.38227
3 CH-RI	3	natans	41.38061	-71.79996
3 CP-RI	3	natans	41.86245	-71.33634
3 IB-NY	3	natans	42.16271	-73.89591
3 SC-NY	3	natans	42.30986	-73.77333
3 TP-NY	3	natans	42.67101	-73.76099

3 MM-NY	3	natans	43.08148	-76.64871
3 SSB-NY	3	natans	43.21568	-76.92658
3 RC-NY	3	natans	43.30000	-76.78114
3 CDL-VT	3	natans	43.61807	-72.41948
3 CCD-NY	3	natans	43.64984	-73.42343
3 CMB-NY	3	natans	43.74027	-73.37408
4 TEM-J	4	natans	34.73361	134.90861
4 KO-J	4	natans	34.74000	134.98389
5 IWA-J	5	japonica	34.68139	134.97111
5 ONO-J	5	japonica and natans var. pumila	34.81694	134.95694
5 MIK-J	5	japonica and natans var. pumila	35.55972	135.89250
6 NAK-J	6	incisa	35.65806	136.09000
7 EM-KZN	7	sp. 2	-28.80938	31.89342

Table A-2b. Summary of selected morphological parameters by population and genetic group (N = number of samples, nd = no data).

Population code Or Group	N_Flower Petal Color	Count_Petals White	Count_Petals Pink	Count_No Petals	N_Leaf Underside Color	Count_Underside Dark Red	Count_Underside Green	Count_Underside Green and Brown	Count_Underside Green with pink veins	N_Orientation upper spines	Count_Ascending	Count_Descending	Count_Horizontal
1 WP-VA	12	0	3	9	12	12	0	0	0	12	12	0	0
1 VCB-VA	10	0	5	5	12	12	0	0	0	12	12	0	0
Group 1	*22*	*0*	*8*	*14*	*24*	*24*	*0*	*0*	*0*	*24*	*24*	*0*	*0*
2 ML-VA	5	0	3	2	12	12	0	0	0	12	12	0	0
Group 2	*5*	*0*	*3*	*2*	*12*	*12*	*0*	*0*	*0*	*12*	*12*	*0*	*0*
3 BR-MD	8	4	0	4	10	0	10	0	0	nd	nd	nd	nd
3 CH-RI	0	0	0	0	12	0	12	0	0	12	12	0	0
3 CP-RI	0	nd	nd	nd	11	0	11	0	0	11	11	0	0
3 IB-NY	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
3 SC-NY	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
3 TP-NY	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
3 MM-NY	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
3 SSB-NY	11	9	0	2	11	0	11	0	0	11	11	0	0
3 RC-NY	12	8	0	4	12	0	1	11	0	12	12	0	0
3 CDL-VT	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
3 CCD-NY	2	2	0	0	11	0	11	0	0	11	11	0	0
3 CMB-NY	0	nd	nd	nd	12	0	12	0	0	12	12	0	0
Group 3	*33*	*23*	*0*	*10*	*139*	*0*	*128*	*11*	*0*	*129*	*129*	*0*	*0*
4 TEM-J	1	1	0	0	12	0	5	0	7	12	12	0	0
4 KO-J	1	1	0	0	12	0	11	1	0	12	11	1	0
Group 4	*2*	*2*	*0*	*0*	*24*	*0*	*16*	*1*	*7*	*24*	*23*	*1*	*0*
5 IWA-J	1	1	0	0	12	0	2	10	0	12	12	0	0
5 ONO-J	1	1	0	0	12	0	11	1	0	12	12	0	0
5 MIK-J	1	1	0	0	12	0	11	1	0	nd	nd	nd	nd
Group 5	*3*	*3*	*0*	*0*	*36*	*0*	*24*	*12*	*0*	*24*	*24*	*0*	*0*
6 NAK-J	0	nd	nd	nd	11	0	11	0	0	11	11	0	0
Group 6	*0*	*nd*	*nd*	*nd*	*11*	*0*	*11*	*0*	*0*	*11*	*11*	*0*	*0*
7 EM-KZN	0	nd	nd	nd	12	0	0	12	0	nd	nd	nd	nd
Group 7	*0*	*nd*	*nd*	*nd*	*12*	*0*	*0*	*12*	*0*	*nd*	*nd*	*nd*	*nd*

Population code Or Group	N_Upper Horn Reflexed	Upper Horn of Fruit Reflexed?	N_ Lower projections category	Count_Lower Projection = 4 (> 4 mm, barbed)	Count_Lower Projection = 3 (> 4 mm, not barbed)	Count_Lower Projection = 2 (2 – 4 mm)	Count_Lower Projection = 1 (< 2 mm)	Count_Lower Projection = 0 (no lower projection)	Count_Lower projection = mixture
1 WP-VA	12	0	12	0	0	12	0	0	0
1 VCB-VA	12	0	12	0	0	10	0	0	2
Group 1	*24*	*0*	*24*	*0*	*0*	*22*	*0*	*0*	*2*
2 ML-VA	12	0	12	0	5	6	0	0	0
Group 2	*12*	*0*	*12*	*0*	*5*	*6*	*0*	*0*	*0*
3 BR-MD	nd	nd	nd	nd	nd	nd	nd	nd	nd
3 CH-RI	12	0	12	7	5	0	0	0	0
3 CP-RI	11	0	11	11	0	0	0	0	0
3 IB-NY	12	0	12	12	0	0	0	0	0
3 SC-NY	12	0	12	10	1	0	0	0	1
3 TP-NY	12	0	12	12	0	0	0	0	0
3 MM-NY	12	0	12	12	0	0	0	0	0
3 SSB-NY	11	0	11	11	0	0	0	0	0
3 RC-NY	12	0	12	11	1	0	0	0	0
3 CDL-VT	12	0	12	10	0	0	0	0	2
3 CCD-NY	11	0	11	10	0	0	0	0	1
3 CMB-NY	12	0	12	12	0	0	0	0	0
Group 3	*129*	*0*	*129*	*118*	*7*	*0*	*0*	*0*	*4*
4 TEM-J	12	0	12	9	1	1	1	0	0
4 KO-J	12	0	12	4	1	7	0	0	0
Group 4	*24*	*0*	*24*	*13*	*2*	*8*	*1*	*0*	*0*
5 IWA-J	12	0	12	0	0	0	12	0	0
5 ONO-J	12	0	12	2	0	1	9	0	0
5 MIK-J	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 5	*24*	*0*	*24*	*2*	*0*	*1*	*21*	*0*	*0*
6 NAK-J	11	0	11	0	0	11	0	0	0
Group 6 Totals	*11*	*0*	*11*	*0*	*0*	*11*	*0*	*0*	*0*
7 EM-KZN	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 7	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Population code Or Group	N_Lower projection apex	Count_Apex Acute	Count_Apex Obtuse	Count_Apex mixture	Count_No Apex	N_Lower projection base	Count_Base Narrow	Count_Base Wide	Count_Base mixture
1 WP-VA	12	0	12	0	0	12	0	12	0
1 VCB-VA	12	0	10	2	0	12	0	12	0
Group 1	*24*	*0*	*22*	*2*	*0*	*24*	*0*	*24*	*0*
2 ML-VA	12	0	11	1	0	12	1	11	0
Group 2	*12*	*0*	*11*	*1*	*0*	*12*	*1*	*11*	*0*
3 BR-MD	nd	nd	nd	nd	nd	nd	nd	nd	nd
3 CH-RI	12	11	0	1	0	12	0	12	0
3 CP-RI	11	11	0	0	0	11	0	11	0
3 IB-NY	12	12	0	0	0	12	0	12	0
3 SC-NY	12	10	1	1	0	12	0	12	0
3 TP-NY	12	12	0	0	0	12	0	12	0
3 MM-NY	12	12	0	0	0	12	0	12	0
3 SSB-NY	11	11	0	0	0	11	0	11	0
3 RC-NY	12	12	0	0	0	12	0	12	0
3 CDL-VT	12	10	0	2	0	12	0	12	0
3 CCD-NY	11	10	0	1	0	11	0	11	0
3 CMB-NY	12	12	0	0	0	12	0	12	0
Group 3	*129*	*123*	*1*	*5*	*0*	*129*	*0*	*129*	*0*
4 TEM-J	12	9	3	0	0	12	0	12	0
4 KO-J	12	4	8	0	0	12	0	12	0
Group 4	*24*	*13*	*11*	*0*	*0*	*24*	*0*	*24*	*0*
5 IWA-J	12	1	11	0	0	12	0	12	0
5 ONO-J	12	2	10	0	0	12	1	11	0
5 MIK-J	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 5	*24*	*3*	*21*	*0*	*0*	*24*	*1*	*23*	*0*
6 NAK-J	11	11	0	0	0	11	0	11	0
Group 6	*11*	*11*	*0*	*0*	*0*	*11*	*0*	*11*	*0*
7 EM-KZN	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 7	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Population code Or Group	N_Lower projection extending direction	Count_Both Horizontal	Count_One or Both Descending	Count_Steeply Descending	Count_Ascending	Count_No Lower Projection	Count_Bent, or too short to determine
1 WP-VA	12	12	0	0	0	0	0
1 VCB-VA	12	9	3	0	0	0	0
Group 1	*24*	*21*	*3*	*0*	*0*	*0*	*0*
2 ML-VA	12	10	2	0	0	0	0
Group 2	*12*	*10*	*2*	*0*	*0*	*0*	*0*
3 BR-MD	nd	nd	nd	nd	nd	nd	nd
3 CH-RI	12	9	1	0	2	0	0
3 CP-RI	11	3	5	1	2	0	0
3 IB-NY	12	4	3	0	2	0	3
3 SC-NY	12	8	0	0	4	0	0
3 TP-NY	12	4	6	0	2	0	0
3 MM-NY	12	5	4	0	3	0	0
3 SSB-NY	11	3	6	0	2	0	0
3 RC-NY	12	5	5	0	2	0	0
3 CDL-VT	12	5	1	0	5	0	1
3 CCD-NY	11	8	2	0	1	0	0
3 CMB-NY	12	4	4	0	3	0	1
Group 3	*129*	*58*	*37*	*1*	*28*	*0*	*5*
4 TEM-J	12	7	5	0	0	0	0
4 KO-J	12	6	6	0	0	0	0
Group 4	*24*	*13*	*11*	*0*	*0*	*0*	*0*
5 IWA-J	12	2	0	0	0	0	10
5 ONO-J	12	8	3	0	0	0	1
5 MIK-J	nd	nd	nd	nd	nd	nd	nd
Group 5	*24*	*10*	*3*	*0*	*0*	*0*	*11*
6 NAK-J	11	0	0	11	0	0	0
Group 6	*11*	*0*	*0*	*11*	*0*	*0*	*0*
7 EM-KZN	nd	nd	nd	nd	nd	nd	nd
Group 7	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Population code Or Group	N_Number of barbed spines	Count_4 Spines	Count_3 Spines	Count_2 Spines	N_Number of acute spines	Count_4 Spines	Count_3 Spines	Count_3 or 4 Spines	Count_2 Spines	N_Crown	Count_Crown Present	N_Shriveled?	Sum_Shriveled
1 WP-VA	12	0	0	12	12	0	0	0	12	12	0	12	0
1 VCB-VA	12	0	2	10	12	0	2	0	10	12	0	12	3
Group 1	*24*	*0*	*2*	*22*	*24*	*0*	*2*	*0*	*22*	*24*	*0*	*24*	*3*
2 ML-VA	12	0	0	12	12	0	0	1	11	12	0	12	0
Group 2	*12*	*0*	*0*	*12*	*12*	*0*	*0*	*1*	*11*	*12*	*0*	*12*	*0*
3 BR-MD	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3 CH-RI	12	7	0	5	12	11	0	1	0	12	12	12	0
3 CP-RI	11	11	0	0	11	11	0	0	0	11	11	11	0
3 IB-NY	12	12	0	0	12	12	0	0	0	12	12	12	0
3 SC-NY	12	10	1	1	12	10	1	0	1	12	11	12	0
3 TP-NY	12	12	0	0	12	12	0	0	0	12	12	12	0
3 MM-NY	12	12	0	0	12	12	0	0	0	12	8	12	0
3 SSB-NY	11	11	0	0	11	11	0	0	0	11	11	11	0
3 RC-NY	12	11	0	1	12	12	0	0	0	12	11	12	0
3 CDL-VT	12	10	2	0	12	10	2	0	0	12	12	12	0
3 CCD-NY	11	10	0	1	11	10	1	0	0	11	11	11	0
3 CMB-NY	12	12	0	0	12	12	0	0	0	12	12	12	1
Group 3	*129*	*118*	*3*	*8*	*129*	*123*	*4*	*1*	*1*	*129*	*123*	*129*	*1*
4 TEM-J	12	9	0	3	12	9	0	0	3	12	12	12	0
4 KO-J	12	4	0	8	12	4	0	0	8	12	12	12	0
Group 4	*24*	*13*	*0*	*11*	*24*	*13*	*0*	*0*	*11*	*24*	*24*	*24*	*0*
5 IWA-J	12	0	0	12	12	1	0	0	11	12	12	12	0
5 ONO-J	12	2	0	10	12	2	0	0	10	12	12	12	1
5 MIK-J	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 5	*24*	*2*	*0*	*22*	*24*	*3*	*0*	*0*	*21*	*24*	*24*	*24*	*1*
6 NAK-J	11	11	0	0	11	11	0	0	0	11	0	11	0
Group 6	*11*	*11*	*0*	*0*	*11*	*11*	*0*	*0*	*0*	*11*	*0*	*11*	*0*
7 EM-KZN	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 7	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Appendix Table A-2c. Sample size (N), average, and standard error (SE) of selected morphological parameters by population and genetic group (nd = no data).

Appendix Table 2c. Sample size (N), average, and standard error (SE) of selected morphological parameters by population and genetic group (nd = no data). Population code Or Group	N_Width of Stem (mm)	Average_Width of stem (mm)	SE_Width of stem (mm)	N_Number of teeth on leaf	Average_Number of teeth on leaf	SE_Number of teeth on leaf	N_ Dry weight of fruit (g)	Average_ Dry weight of fruit (g)	SE_Dry weight of fruit (g)	N_Width across upper spines (mm)	Average_Width across upper spines (mm)	SE_Width across upper spines (mm)
1 WP-VA	12	5.00	0.30	12	21.00	0.25	12	1.19	0.10	12	34.49	0.49
1 VCB-VA	12	4.88	0.20	12	23.58	0.85	12	0.48	0.03	12	30.86	1.70
Group 1	*24*	*4.94*	*0.17*	*24*	*22.29*	*0.51*	*24*	*0.84*	*0.09*	*24*	*32.68*	*0.94*
2 ML-VA	12	5.21	0.20	12	24.83	0.41	12	0.60	0.42	12	35.64	0.72
Group 2	*12*	*5.21*	*0.20*	*12*	*24.83*	*0.41*	*12*	*0.60*	*0.06*	*12*	*35.64*	*0.72*
3 CH-RI	0	nd	nd	12	15.33	0.47	12	1.97	0.13	12	40.90	1.16
3 CP-RI	0	nd	nd	10	20.90	0.67	11	1.19	0.11	11	36.02	0.89
3 IB-NY	12	20.21	0.33	11	18.18	0.69	12	1.34	0.22	12	36.30	1.28
3 SC-NY	12	15.50	0.74	7	40.43	1.56	12	1.71	0.22	12	36.64	1.32
3 TP-NY	12	15.75	0.83	12	15.33	0.66	12	1.65	0.23	12	37.83	1.09
3 MM-NY	12	13.00	0.85	12	19.67	0.38	12	2.12	0.16	12	37.13	1.34
3 SSB-NY	11	10.27	0.73	11	20.09	0.31	11	2.56	0.18	11	46.14	1.07
3 RC-NY	12	11.83	1.34	12	17.83	0.52	12	2.00	0.25	12	38.42	1.18
3 CDL-VT	12	18.29	0.63	12	16.08	0.47	12	2.00	0.18	12	40.68	1.09
3 CCD-NY	11	12.50	1.33	11	21.91	0.81	11	2.19	0.20	11	42.59	0.95
3 CMB-NY	12	15.13	1.11	12	17.92	0.47	12	1.64	0.21	12	37.34	1.49
Group 3	*106*	*14.78*	*0.42*	*122*	*18.38*	*0.26*	*129*	*1.85*	*0.07*	*129*	*39.03*	*0.43*
4 TEM-J	0	nd	nd	12	28.50	0.95	12	2.32	0.20	12	56.25	1.47
4 KO-J	0	nd	nd	12	30.42	0.92	12	2.03	0.14	12	54.87	1.20
Group 4	*0*	*nd*	*nd*	*24*	*29.46*	*0.68*	*24*	*2.17*	*0.12*	*24*	*55.56*	*0.94*
5 IWA-J	0	nd	nd	12	27.17	0.80	12	1.04	0.09	12	39.06	0.95
5 ONO-J	0	nd	nd	12	25.42	0.81	12	0.65	0.11	12	30.56	1.16

Appendix Table 2c. Sample size (N), average, and standard error (SE) of selected morphological parameters by population and genetic group (nd = no data). Population code Or Group	N_Width of Stem (mm)	Average_Width of stem (mm)	SE_Width of stem (mm)	N_Number of teeth on leaf	Average_Number of teeth on leaf	SE_Number of teeth on leaf	N_ Dry weight of fruit (g)	Average_ Dry weight of fruit (g)	SE_Dry weight of fruit (g)	N_Width across upper spines (mm)	Average_Width across upper spines (mm)	SE_Width across upper spines (mm)
Group 5	0	nd	nd	24	26.29	0.59	24	0.84	0.08	24	34.81	1.15
6 NAK-J	*0*	*nd*	*nd*	*11*	*11.09*	*0.59*	*11*	*0.08*	*0.00*	*11*	*17.51*	*0.80*
Group 6	0	nd	nd	11	11.09	0.28	11	0.08	0.00	11	17.51	0.80
7 EM-KZN	*12*	*3.50*	*0.31*	*12*	*16.83*	*0.83*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Group 7	12	3.50	0.31	12	16.83	0.83	nd	nd	nd	nd	nd	nd

Appendix Table A-2c - continued. Sample size (N), average, and standard error (SE) of selected morphological parameters by population and genetic group (nd = no data).

Population code Or Group	N_Width across lower projections (mm)	Average_Width across lower projections (mm)	SE_Width across lower projections (mm)	N_Fruit Height (mm)	Average_Fruit Height (mm)	SE_Fruit Height (mm)	N_Fruit Thickness (mm)	Average_Fruit Thickness (mm)	SE_Fruit Thickness (mm)
1 WP-VA	12	19.64	0.63	12	17.20	0.51	12	10.64	0.23
1 VCB-VA	12	18.69	0.70	12	13.25	0.38	12	8.58	0.36
Group 1	*24*	*19.16*	*0.47*	*24*	*15.22*	*0.52*	*24*	*9.61*	*0.30*
2 ML-VA	12	16.20	0.81	12	14.11	0.49	12	10.48	0.75
Group 2	*12*	*16.20*	*0.81*	*12*	*14.11*	*0.49*	*12*	*10.48*	*0.75*
3 CH-RI	12	29.00	1.31	12	18.78	0.80	12	11.78	0.22
3 CP-RI	11	29.24	1.10	11	19.58	0.30	11	12.01	0.33
3 IB-NY	12	29.95	1.10	12	18.79	1.37	12	11.42	0.45
3 SC-NY	12	30.09	1.41	12	19.19	0.70	12	12.47	0.45
3 TP-NY	12	29.38	0.94	12	18.59	0.56	12	11.84	0.43
3 MM-NY	12	30.09	0.75	12	18.68	0.49	12	11.59	0.35
3 SSB-NY	11	38.50	1.00	11	23.91	0.62	11	12.86	0.54

Population code Or Group	N_Width across lower projections (mm)	Average_Width across lower projections (mm)	SE_Width across lower projections (mm)	N_Fruit Height (mm)	Average_Fruit Height (mm)	SE_Fruit Height (mm)	N_Fruit Thickness (mm)	Average_Fruit Thickness (mm)	SE_Fruit Thickness (mm)
3 RC-NY	12	30.51	1.96	12	20.77	1.00	12	11.52	0.40
3 CDL-VT	12	30.13	1.45	12	20.25	0.62	12	11.63	0.32
3 CCD-NY	11	32.48	1.39	11	22.46	0.70	11	12.83	0.32
3 CMB-NY	12	28.99	1.28	12	19.28	0.66	12	11.33	0.58
Group 3	*129*	*30.70*	*0.44*	*129*	*19.98*	*0.26*	*129*	*11.92*	*0.13*
4 TEM-J	12	41.91	4.59	12	22.66	0.58	12	12.42	0.56
4 KO-J	12	29.50	4.48	12	20.63	0.56	12	13.07	0.52
Group 4	*24*	*35.71*	*3.39*	*24*	*21.64*	*0.45*	*24*	*12.70*	*0.38*
5 IWA-J	12	11.08	0.49	12	16.82	0.47	12	9.90	0.19
5 ONO-J	12	14.08	2.48	12	16.09	0.75	12	8.55	0.29
Group 5	24	12.58	1.28	24	16.45	0.44	24	9.23	0.22
6 NAK-J	*11*	*4.16*	*0.11*	*11*	*8.64*	*0.24*	*11*	*3.65*	*0.12*
Group 6	11	4.16	0.11	11	8.64	0.24	11	3.65	0.12
7 EM-KZN	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Group 7	nd	nd	nd	nd	nd	nd	nd	nd	nd

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14. ABSTRACT

Cryptic introductions are non-native species that have been introduced outside of native ranges; these introductions are undetected because the species have morphology similar to native or other non-native species naturalized within the same region. While non-native, invasive *Trapa natans* has been present in the Northeastern (NE) United States (U.S.) since the late 1800s, unpublished data suggests a new introduction of *Trapa* has occurred in the Commonwealth of Virginia. This population was distinct: it had 2-spined fruit as opposed to the typical 4-spined fruit associated with *T. natans.* It was therefore suspected as a cryptic introduction of *Trapa* species.

This work aims to elucidate genetic and morphological differences of naturalized *Trapa* taxa (water chestnut) in the NE U.S. Comparisons of morphological characteristics and genetics were made between *Trapa* populations from the native regions of Eurasia and Africa versus those of the NE U.S. Results of the morphological analysis supported genetic results that 2-spine *Trapa* sp. and 4-spine *T. natans* in the U.S. were different, with the number of spines and the presence of a crown (*Trapa* sp. lacks a crown) as morphological taxonomic indicators.

15. SUBJECT TERMS
Aquatic plants, Cryptic introductions, Invasive vegetative species, Morphological analysis, *Trapa natans*, Water chestnut

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