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Multiple introductions of invasive Eurasian watermilfoil and recurrent hybridization with northern watermilfoil in North America

MATTHEW P. ZUELLIG AND RYAN A. THUM*

ABSTRACT

Aquatic plant managers and scientists have become increasingly interested in the potential for genetic variation to explain differences in the ecology or management response of invasive Eurasian watermilfoil. To date, genetic studies of invasive milfoil populations have focused on genetic identification techniques to distinguish Eurasian from hybrid watermilfoils. However, genetic variation within and among Eurasian and hybrid milfoil populations has been neglected, despite the potential for genetically distinct populations to exhibit unique ecological characteristics (including response to management efforts). Here we fill a gap in genetic studies of Eurasian and hybrid milfoils by employing amplified fragment length polymorphism markers (AFLPs) to assess patterns of genetic variation within and among Eurasian and hybrid watermilfoil. We demonstrate that Eurasian watermilfoil in North America consists of at least two distinct genetic lineages that were introduced to different parts of the continent on separate occasions. We also show that "hybrid watermilfoils" constitute a genetically diverse group that likely reflects a pattern of recurrent hybridization between native northern watermilfoil and both Eurasian lineages. The uncovering of genetic variability in Eurasian and hybrid watermilfoils demonstrates a need for further study of these groups' ecology and management response. Finally, given the increasing interest in molecular identification methods, we compare identifications based on AFLPs to those of internal transcribed spacer (ITS) sequences. We found ITS identifications were commonly congruent with ITS, but a small fraction of ITS identifications incorrectly identified hybrid watermilfoils as Eurasian or northern milfoil.

Key Words: aquatic plant, biological invasions, cryptic genetic diversity, DNA fingerprinting, management, invasive species, *Myripohyllum sibiricum, Myriophyllum spicatum.*

INTRODUCTION

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is one of North America's most problematic invasive aquatic weeds and is frequently and selectively managed, especially in the northern tier of the United States (Netherland and Getsinger 1992, Newman et al. 1996, Unmuth et al. 1998, Parsons et al. 2001, Getsinger et al. 2002, Madsen et al. 2002). Populations of Eurasian watermilfoil can exhibit considerable variation in the extent of nuisance growth and/or response to efforts to reduce growth by various management methods (e.g., herbicides and biocontrol), and the factors that determine this variability are unknown. In general, variation among populations to the extent that they exhibit nuisance growth and/or different responses to management may be determined by environmental factors (e.g., lake conditions, management regime, human precision in applying treatments), genetic factors (e.g., genetic differences among populations), or both.

The initial recognition that genetic factors might influence milfoil invasiveness and/or control efficacy came from the identification of several invasive hybrid populations formed between Eurasian and northern watermilfoil (*Myriophyllum sibiricum* Komarov), a close relative of Eurasian watermilfoil that is native to North America (Moody and Les 2002). Hybrids were identified using DNA sequences from a biparentally inherited nuclear gene (nuclear ribosomal internal transcribed spacers; ITS), and it was noted these hybrid populations were "noticeably aggressive" with respect to parental species. They also noted that one milfoil population that showed reduced susceptibility to damage by the milfoil weevil (*Euhrychiopsis lecontei*) in an earlier study was composed of hybrids (Jester et al. 2000). Subsequent genetic studies have documented the occurrence of hybrid watermilfoils across the northern tier of the United States (Moody and Les 2007, Sturtevant et al. 2009), and the potential for hybrid watermilfoils to exhibit unique challenges to management is increasingly recognized by members of the aquatic plant management community.

To date, genetic analyses have focused on distinguishing hybrid from Eurasian watermilfoil, whereas little is known about the potential for genetic differentiation among populations. Genetic diversity within and among Eurasian and hybrid watermilfoil populations might be limited and therefore plays a limited role in determining the variation in growth and/or control efficacy among populations. For example, Eurasian watermilfoil is thought to reproduce primarily through vegetative reproduction and therefore spreads among waterbodies through human movements such as boats, boat trailers, and fishing equipment (Johnstone et al. 1985, Madsen et al. 1988, Madsen and Smith 1997). If so, genetic diversity within or among Eurasian watermilfoil populations may be limited, especially if the founding populations of introduced Eurasian watermilfoil consisted of a very small number of genetically distinct individuals. Similarly, genetic diversity within

*First author: Graduate Student, Department of Genetics, University of Georgia, Athens, GA 30602; second author: Assistant Professor, Annis Water Resources Institute, Grand Valley State University, Muskegon, MI 49441. Corresponding author's E-mail: zuelligm@uga.edu. Received for publication June 20, 2011 and revised in form November 14, 2011.

or among hybrid watermilfoil populations may be limited if only one or a very small number of hybrid clones exist and spread primarily through vegetative propagation. However, studies of other invasive taxa increasingly demonstrate that genetic diversity within or among populations is considerably higher than originally anticipated and suggests that genetic diversity may be a critical factor influencing the "success" of invaders (Lavergne and Molofsky 2007 Roman and Darling 2007, Crawford and Whitney 2010), although no such study has been conducted for Eurasian watermilfoil.

Two observations suggest that genetic diversity within or among Eurasian or hybrid watermilfoil populations may be higher than previously recognized. First, distinct genetic lineages may have been introduced to different locations and/or on separate occasions in North America. Couch and Nelson (1985) hypothesized that Eurasian watermilfoil may have been introduced to North America on as many as four different occasions based on its concurrent identification in geographically distant portions of the country (Arizona, California, Ohio, and Chesapeake Bay) in the 1940s. Second, in addition to asexual reproduction through vegetative fragments, Eurasian watermilfoil is monoecious and frequently produces flowers in natural populations, providing ample opportunities for genetic recombination through sexual reproduction. Eurasian watermilfoil populations have been estimated to produce as many as four million viable seeds per hectare in North American lakes (Aiken 1979 and citations within). Presumably, the prolific flowering that occurs in Eurasian watermilfoil provides numerous opportunities for hybridization with northern watermilfoil, yet the extent to which hybrids are formed is not currently known. As such, an analysis of genetic diversity among Eurasian and hybrid populations is warranted.

While the potential for genetic diversity among Eurasian or hybrid watermilfoil populations exists, the molecular marker employed in previous studies (ITS DNA sequences) is unlikely to identify potentially meaningful patterns of genetic variation within or among populations. ITS is well-suited for distinguishing species and detecting early generation interspecific hybrids (the focus of previous studies) because it exhibits strong genetic differentiation between species. However, ITS rarely exhibits large amounts of variation within species, making it difficult to detect genetic variation among individuals or distinct genetic groups (biotypes; Hodkinson et al. 2000, Xu and Sun 2001). In addition, ITS may also be limited in its ability to identify later-generation hybrids (e.g., F2 and later) because of genetic homogenization resulting from subsequent sexual reproduction of early generation hybrids. In contrast, a variety of highly polymorphic markers are routinely employed for the express purposes of examining fine-scale intraspecific genetic diversity and hybridization within and among populations (Freeland 2005).

In this study, we sampled Eurasian and hybrid watermilfoils from across North America (with intensive sampling in Michigan) to identify genetic differentiation among populations. We developed amplified fragment length polymorphism (AFLP) molecular markers to detect genetic variation within and among populations of Eurasian and hybrid watermilfoil. Specifically, we used AFLPs to test the following hypotheses: (1) Eurasian watermilfoil consists of multiple genetically distinct lineages stemming from multiple introductions in North America; and (2) hybridization between Eurasian and northern watermilfoil has resulted in the formation of many hybrid lineages that harbor large amounts of genetic variation. Additionally, given the increasing reliance on molecular methods for milfoil species identifications for management decisions (e.g., Thum et al. 2006, Moody et al. 2008), we compared taxonomic identifications based on AFLPs and ITS.

MATERIALS AND METHODS

Sample collection and DNA extraction

To investigate broad patterns of genetic variation in Eurasian and hybrid watermilfoils, we examined plants primarily from lakes in the northwestern, northeastern, and Great Lakes regions of the United States as well as some lakes in Canada. We intensively sampled in Michigan as a result of the numerous collaborations with lake managers and regulators in that state. While our primary purpose was to examine Eurasian and hybrid watermilfoils, we also sampled northern watermilfoil. We sampled 1016 plants from 106 lakes during the summers of 2008 to 2010 (Appendix 1). Many of these samples were single individuals sent to us by lake managers for the purpose of genetically identifying the specimen as Eurasian, northern, or hybrid, and many of our sampling locations are therefore represented by a single individual.

We washed all plants thoroughly in distilled water to limit contaminant DNA from symbionts and epibionts (e.g., periphyton, insects, snails) and extracted total genomic DNA from fresh, submerged vegetative meristem tissue using DNeasy Plant Mini Kits (Qiagen).

AFLP molecular biology and genotyping

AFLPs were prepared as described in Thum et al. (2011) using ~100 ng of total genomic DNA. We present results from a single selective primer pair (EcoR1-ACA and Mse1-CAT) because a pilot study on a subset of our samples showed that the qualitative results presented here did not change when adding additional primer pairs. We replicated the entire AFLP process on 29 individuals to calculate an error rate using the Jaccard Index. We genotyped 1016 individuals, resulting in 192 unique AFLP chromatograms.

Genetic identification and diversity: AFLPs

We used Structure v2.3.2 (Pritchard et al. 2000, Falush et al. 2007) to (1) identify individuals as Eurasian, northern, or hybrid watermilfoil based on their AFLP profiles, (2) determine whether any additional substructure (i.e., genetically distinct groups) exist within any of these taxa, and (3) identify evidence for gene flow between any distinct genetic groups within these taxa. We assumed that individuals with identical chromatograms most likely represented different ramets of the same genet (i.e., identical "clones") that formed via vegetative propagation. Thus, to reduce bias in allele frequency estimates in our Structure analyses due to

vegetative propagation of single clones, we included only the unique AFLP profiles from each population sampled in our analyses (i.e., only a single clone; see Appendix 1 for number of unique clones in each lake). For example, if 10 individuals were sampled in a lake and nine had identical AFLP chromatograms, we included in our Structure analysis one individual of the nine with identical chromatograms, as well as the chromatogram from the single individual that differed from the other nine. We used an admixture model with no priors, correlated allele frequencies, and a single α. However, we evaluated models employing all possible combinations of the above parameters, and our results were robust to different combinations. We initially ran Structure for values of K from 1 to 10 and evaluated the number of distinct genetic clusters (i.e., true value of K) by examining the plot of the likelihood of the data against each value of K, and using the ΔK statistic from Evanno et al. (2005; Figure 1). Although the ΔK method indicated K = 2, the likelihood scores continued to rise across values of K. Moreover, ΔK is indicative only of the highest level of structure in the dataset (e.g., minimum value of K for which structure could be explained). Therefore, we used the hierarchical approach of Coulon et al. (2008) to identify further genetic structuring; we repeated the analysis of ΔK for each group of the K groups identified in the previous step until no further substructuring was evident. For each analysis, we ran Structure for 250,000 generations, preceded by a burn-in period of 50,000 generations. We utilized the ANCESTDIST function in Structure v2.3.2 to calculate a 95% confidence interval around the assignment of each individual in our analysis, which we utilized to assign individuals to different genetic groups. Individuals that did not contain 1 in their confidence intervals were considered admixed between the two groups that made up the majority of their genome.

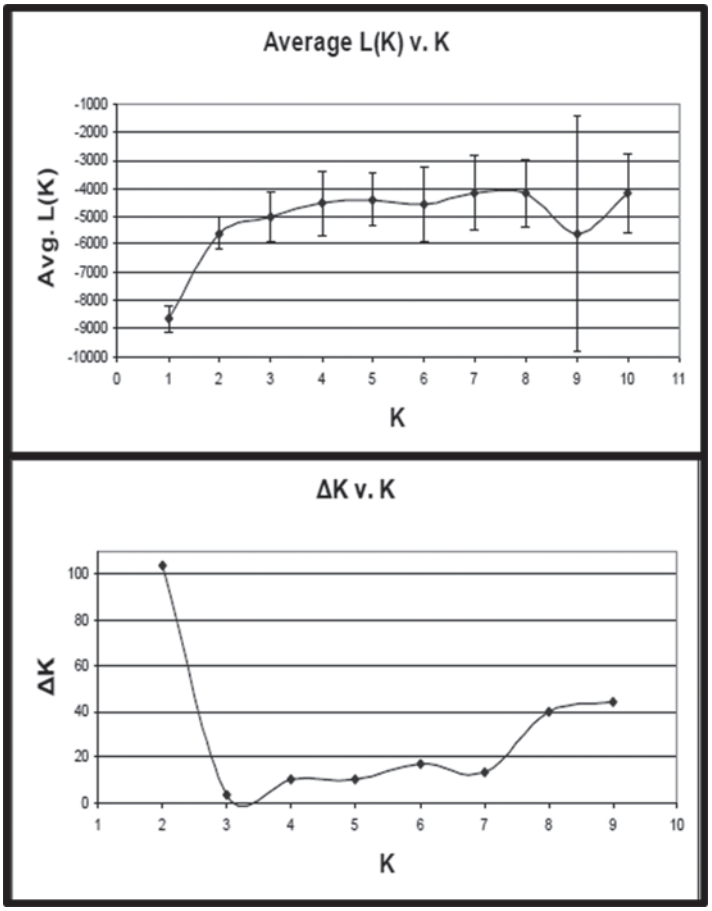

Figure 1. Top: -Ln Likelihood of Data (L(K)) vs. K. –Ln likelihood of data estimated at different values of K using Structure v.2.3.2 (Pritchard et al. 2000, Falush et al. 2007). Each value of K is averaged over three runs, and error bars represent standard deviation. Bottom: ΔK vs. K for our full AFLP dataset. ΔK was calculated from method in Evanno et al. (2005) using three replicate runs of Structure v.2.3.2 (Pritchard et al. 2000, Falush et al. 2007

We also estimated several parameters of genetic diversity for all taxa (i.e., Eurasian, northern, and hybrid watermilfoil), including (1) Clonal diversity, or the number of distinct AFLP profiles within each group, which is assumed to reflect the number of distinct milfoil clones, (2) Genotype diversity (Nei 1987), which describes the probability of sampling two distinct genotypes within a group, where groups with more genetic diversity have higher values, and (3) Gene diversity (or expected heterozygosity; Nei 1973), which reflects the probability that two randomly sampled individuals differ at a given AFLP marker. For the above measures, we used the full dataset of 1016 individuals in our analysis, as opposed to the reduced dataset we used for our Structure analysis, which included only unique AFLP chromatograms from each population. The above measures of genetic diversity were all calculated using the "Clones" option in the program AFLPdat (Ehrich 2006). Because genotyping errors can lead to different AFLP chromatograms for genetically identical individuals (e.g., vegetative clones), we calculated the above measures of genetic diversity for different assumed levels of scoring error: 0 band differences (i.e., no error), 1 band difference, 2 band differences, and 4 band differences. Finally, we determined whether any clones were shared between populations by identifying identical genotypes (i.e., no band differences) across different lakes, which gives the minimum estimate of the number of clones shared among lakes in our dataset.

Genetic identification and diversity: ITS

We sequenced ITS on a subset of our plants (481) to compare molecular identifications and genetic diversity with ITS sequences to AFLPs. We utilized established protocols for amplifying ITS DNA sequences (e.g., Moody and Les 2002, 2007, Sturtevant et al. 2009; for exact details see Thum et al. 2011). We aligned our sequences with previously published Eurasian and northern watermilfoil accessions (FJ426346-FJ426357 from Sturtevant et al. 2009; Supplementary Appendix 2) using Sequencher v4.8. We identified individuals as hybrids on the basis of sequence polymorphisms at sites that have previously been shown to represent fixed differences between Eurasian and northern watermilfoil (Moody and Les 2002, 2007, Sturtevant et al. 2009). Due to the presence of small indels and sequence polymorphisms, we were unable to obtain clean reads throughout the entire ITS gene using direct sequencing of Eurasian, northern, and hybrid watermilfoils; however, this did not affect our ability to make direct comparisons between ITS and AFLPs (Table 1).

Table 1. ITS variants identified in this study in relation to those reported by Sturtevant et al. (2009). Site refers to the base position relative to GenBank accession FJ426347 reported by Sturtevant et al. (2009). Site specific polymorphisms in hybrid variants described using the IUPAC ambiguity codes: Y = C and T, R = A and G, W = A and T, M = C and A; ** indicates species-specific sites used to identify hybrids with direct sequences; * indicates sites that can distinguish Eurasian from northern watermilfoil, but cannot be reliably determined by direct sequencing in hybrids because of insertion or deletion of bases at upstream locations that separate Eurasian and northern watermilfoil (e.g., sites 161, 181, 689, and 690); ?s represent portions of the ITS gene not analyzed in our study, or sites that were not resolved with direct sequencing due to indels (see text for details). All other sites correspond to intraspecific variants. Note that site 282 in NWM2 represents a fixed difference between Eurasian and northern in Sturtevant et al. (2009), but not in our study.

	Northern ITS Variants									Eurasian ITS Variants									Hybrid Variants	
Site	MI1	MI2	MI3	MI4	MI5	MI6	NWM1	NWM2	NWM3	MI1	MI2	MI3	MI4	MI6	MI7	EWM1	EWM2	EWM3	HYB1	HYB2
80*		T	T	T	T	T	?	?	?	C	C	C	C	C	C	?	?	?	?	?
102	G	G	G	A	G	G	?	?	?	G	G	G	G	C	C	?	?	?	?	?
105	C	C	C	C	C	C	?	?	?	A	C	C	C	C	C	?	?	?	?	?
131	A	A	A	A	A	A	?	?	?	G	A	G	G	G	G	?	?	?	?	?
140*	T	T	T	T	T	T	?	?	?	C	C	C	C	C	C	?	?	?	?	?
142	C	C	C	C	—	C	?	?	?	C	C	C	C	C	C	?	?	?	?	?
161	—	—	—	—	G	—	—	—	—	G	G	G	G	G	G	G	G	G	?	?
170	C	C	C	C	C	C	C	C	C	C	C	C	T	T	T	Y	C	Y	?	?
181*	—	—	—	—	G	—	—	—	—	T	T	T	T	T	T	T	T	T	?	?
209**	G	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	R	R
263**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	A	T	W
273*	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	R	R
282	C	C	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	C
413	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C
515**	T	T	T	T	T	T	T	T	T	A	A	A	A	A	A	A	A	A	T	T
518**	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	Y	Y
552**	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	Y	Y
559**	A	A	A	A	A	A	A	A	A	C	C	C	C	C	C	C	C	C	M	M
582	G	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	?	?
633*	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	?	?
676*	T	T	T	T	T	T	?	?	?	C	C	C	C	C	C	?	?	?	?	?
679	C	T	T	C	C	C	?	?	?	C	C	C	C	C	C	?	?	?	?	?
684	C	T	C	C	C	C	?	?	?	C	C	C	C	C	C	?	?	?	?	?
689	—	—	—	—	T	—	?	?	?	—	—	—	—	—	—	?	?	?	?	?
690	—	—	—	—	T	—	?	?	?	—	—	—	—	—	—	?	?	?	?	?

RESULTS AND DISCUSSION

Genetic identifications and diversity: AFLPs

The ΔK statistic for the complete AFLP dataset was highest at K = 2 (Figure 1), and as expected, these two genetic groups clearly corresponded to Eurasian and northern watermilfoil, as identified with ITS sequences (see ITS section below). All hybrids identified with ITS sequences were also clearly identified as hybrids at K = 2. Most of the individuals identified as hybrids in our analysis contained at least 40% genetic contribution from both Eurasian and northern watermilfoil, suggestive of F_1 hybrids. However, four individuals (MI180-clone1, MI121-clone2, MI121-clone4, and MI019-clone1) had admixture proportions (18 to 27% contribution from one parental taxon) that were more consistent with subsequent introgression (e.g., F_2, backcrosses). In addition, three individuals identified as Eurasian or northern watermilfoil with ITS sequences were unambiguously identified as hybrids with AFLPs (at least 40% of genetic makeup from both parents and did not contain 1 in their 95% confidence intervals).

To determine whether any distinct genetic groups, or biotypes, exist within Eurasian, northern, or hybrid watermilfoils, we performed a subsequent structure analysis on each of these groups separately (i.e., "hierarchical" analysis; Coulon et al. 2008). Indeed, hierarchical analysis identified two genetic groups in both Eurasian (Eurasian Group 1 and Eurasian Group 2) and hybrid watermilfoil (Hybrid Group 1 and Hybrid Group 2; Figure 2A). These genetic groups were further supported by associations with ITS sequence variants, where Eurasian Group 1 primarily contained the ITS variant EWM1 (all but one individual), and Eurasian Group 2 contained only ITS variants EWM2 and EWM3 (see ITS section below for descriptions of ITS variants). We did not detect any additional groups in northern watermilfoil; however, this likely reflects the relatively low sampling effort for northern watermilfoil, and future analyses examining a larger number of accessions may well reveal distinct northern watermilfoil biotypes. In addition to the hierarchical analysis, we show results for K = 3 of the full dataset (Figure 2B) because it simultaneously illustrates the two Eurasian watermilfoil groups (Eurasian Group 1 and Eurasian Group 2) and hybrids between both Eurasian groups and northern watermilfoil.

We found different levels of genetic diversity in Eurasian, northern, and hybrid watermilfoil groups. Generally speaking, hybrids are the most genetically diverse, followed by northern, and then Eurasian watermilfoil (Table 2). We note that genetic diversity in AFLPs can represent either true genetic diversity or errors associated with genotyping or scoring. However, two observations demonstrate that the genetic diversity we observe in our AFLPs is not the result of genotyping errors, but rather reflects true genetic diversity. First, our error rate was very low (average Jaccard Index 0.004%), corresponding to an average of less than one band difference between replicate samples. Second, pairwise comparisons of the number of AFLP band differences among individuals within

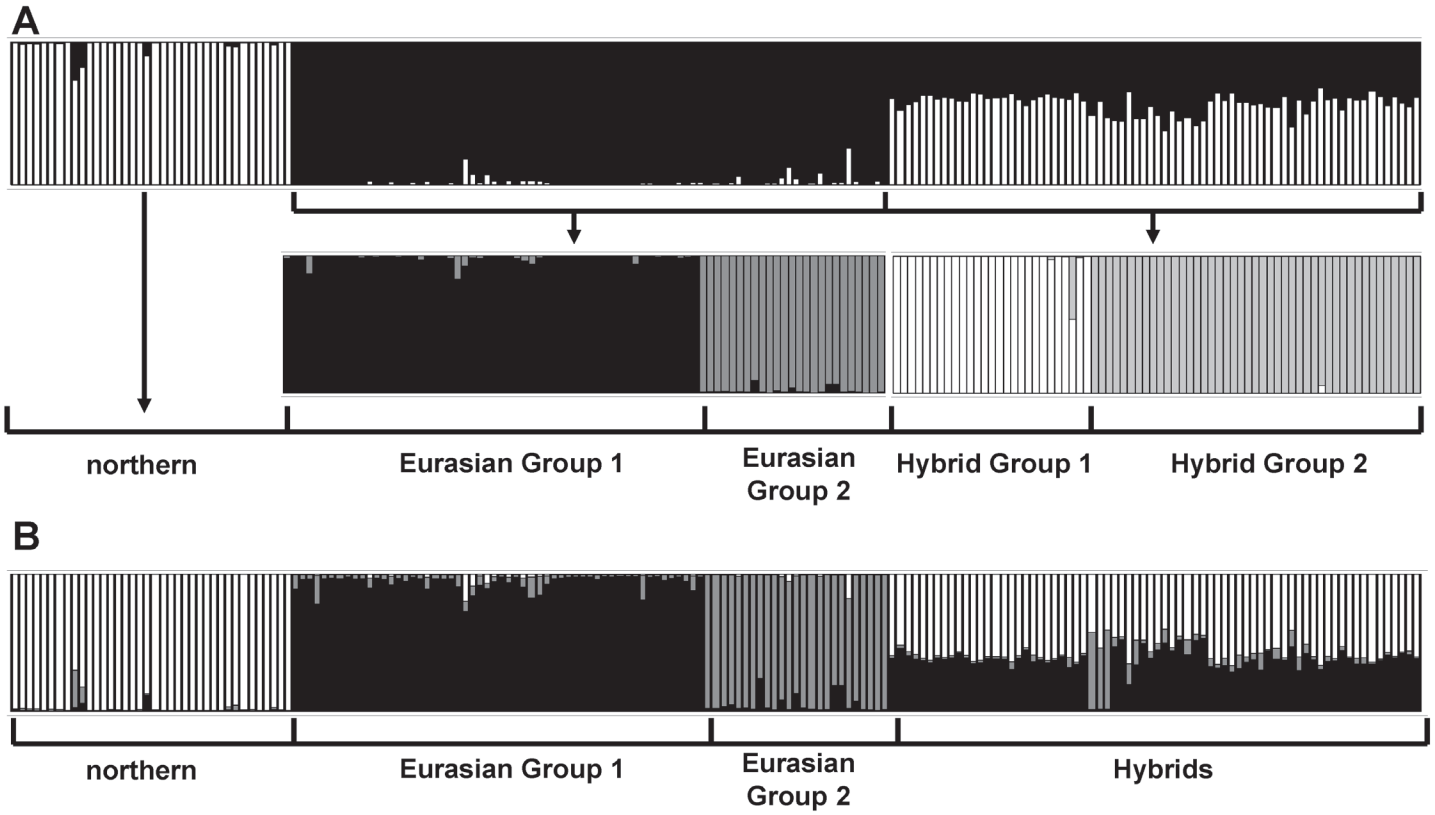

Figure 2. Structure results for our AFLP dataset. Vertical bars represent unique clones from each population identified with a visual inspection of AFLP chromatograms performed during scoring of AFLP chromatograms. A. (top): Structure results for the full dataset at K = 2; (bottom): Hierarchical Structure results for Eurasian and hybrid watermilfoils. Note that both contain two additional groups. B. Entire AFLP dataset at K = 3, shows both Eurasian groups hybridizing with northern watermilfoil.

Table 2. Measures of genetic diversity at different assumed genotyping error rates. Results for error rates with 0 band differences, 1 band difference, 4 band differences, and the maximum number of band differences necessary to consider each individual in a given group a single clone. Clonal diversity (i.e., number of clones), estimated genotype diversity, and estimated gene diversity at each value with the given error rate.

Species (number of individuals sampled)	Error Rate (# Bands)	Clonal Diversity	Genotype Diversity	Gene Diversity
Eurasian (301)	0	58	0.9	0.1
	1	41	0.82	0.16
	4	11	0.38	0.16
	Max. = 11	1	0	0
Northern (171)	0	35	0.93	0.09
	1	33	0.93	0.09
	4	16	0.76	0.11
	Max. = 11	1	0	0
Hybrid (544)	0	99	0.96	0.14
	1	80	0.92	0.16
	4	42	0.84	0.19
	Max. = 27	1	0	0

each taxon show a large amount of clonal variation that cannot be explained by even our most conservative estimates of error (4.3% or 4 band differences; Figure 3). Nevertheless, we calculated genetic diversity measures for a range of assumed error rates and found that even when assuming high scoring error rates (accepting 4 band differences among individuals as reflecting genotyping error, which corresponds to an error rate one order of magnitude higher than our actual estimated error rate, or 4.3%), genotype diversity was 0.38 in Eurasian watermilfoil as a group, whereas genotype diversity in northern and hybrid watermilfoils were 0.76 and 0.84, respectively (Table 2).

Genetic identifications and diversity: ITS

We identified 218 Eurasian, 69 northern, and 194 hybrid watermilfoils on the basis of ITS DNA sequences. We found intraspecific polymorphisms for both Eurasian and northern watermilfoil (three ITS sequence variants each; Table 1), and each sequence variant was readily identified to species when compared to sequences previously identified sequences by Moody and Les (2002, 2007) and Sturtevant et al. (2009; Table 1, Appendix 2). As with Eurasian and northern watermilfoil, we identified two unique hybrid ITS sequence variants (Table 1). Of the ITS variants identified here, two northern watermilfoil variants (NWM2 and NWM3, found in one population each), one Eurasian watermilfoil variant (EWM3, found in seven populations), and one hybrid watermilfoil variant (HYB2, found in two populations) have not been previously reported in the literature.

Multiple introductions of distinct Eurasian watermilfoil biotypes

Here we have identified clear genetic structuring in Eurasian watermilfoil that was not detected in earlier genetic studies that primarily focused on distinguishing hybrid and parental milfoils (e.g., Moody and Les 2002, 2007, Sturtevant et al. 2009). Our AFLP analysis clearly delineated two distinct

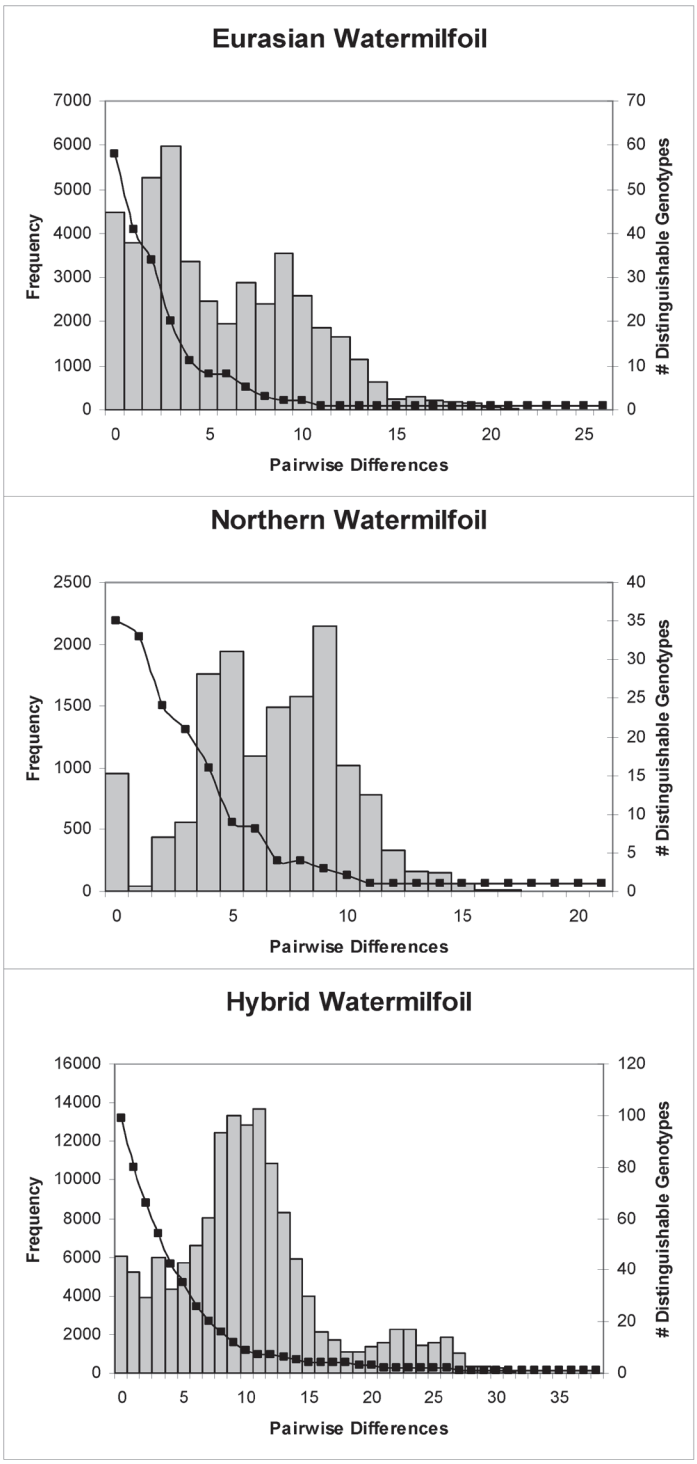

Figure 3. Frequency distribution of pairwise genetic distances (estimated as simple mismatch distances between individuals) based on AFLP markers among genetic lineages for Eurasian, northern, and hybrid watermilfoil (represented by gray bars, where the x-axis is the number of pairwise differences and the left y-axis is the frequency). Also shown is the number of distinguishable clones under a given error rate, where "pairwise differences" reflects the maximum genetic distance between two samples assigned to the same clone (represented by black squares, where the x-axis is the number of pairwise differences (i.e., estimated error rate) and the right y-axis is the number of distinguishable clones at that error rate).

genetic groups of Eurasian watermilfoil; furthermore, the two groups exhibited consistent differences in their ITS sequences. ITS variants EWM2 and EWM3 were only found in Eurasian Group 2 (Figure 2). Similarly, all but one individual with the ITS variant EWM1 were part of Eurasian Group 1. It is not currently known whether any ecological differences are coincident with this genetic structuring, but future studies should compare ecological characteristics to determine whether the two lineages exhibit different growth habits or responses to management efforts (e.g., herbicides, biological control).

The identification of two distinct genetic lineages of Eurasian watermilfoil provides new insight into its introduction history. Previous hypotheses regarding the origin of Eurasian watermilfoil in North America were limited to examination of herbarium specimens. For example, Couch and Nelson (1985) observed that the earliest herbarium specimens of Eurasian watermilfoil in North America were collected in the 1940s from several geographically widespread areas (Washington DC, Ohio, Arizona, and California). They hypothesized that Eurasian watermilfoil may have been introduced to these separate locations independently and then subsequently spread rapidly from each of these foci throughout North America. The two genetic lineages of Eurasian watermilfoil identified in our study support the hypothesis of multiple, independent introductions because the lineages likely represent at least two different introductions from two distinct sources from the native range (Figure 4). One lineage (Eurasian Group 1, Figure 2A) appears to be restricted to the Great Lakes region (Michigan, Ontario, Wisconsin, and one sample from Oregon), and it was likely introduced there. The second lineage (Eurasian Group 2, Figure 2A) was much more widely distributed throughout North America (Kansas, Michigan, New Hampshire, Ohio, Oregon, Texas, Washington, and Wisconsin), and it is not clear where this lineage was originally introduced. It is also possible that Eurasian Group 2 was introduced independently to several different geographic locations throughout North America. This group had higher genetic diversity than Eurasian Group 1 (higher gene diversity and genotype diversity with AFLPs, and two unique ITS sequence variants), which might reflect the independent introduction of this group from genetically similar source populations.

Note that the two Eurasian lineages identified here do not seem to have admixed extensively within the invaded range. The two lineages clearly overlap within the Great Lakes region and have both hybridized with northern watermilfoil in this area. However, we did not find any strong evidence for intraspecific hybridization between the two groups, as Structure placed all Eurasian watermilfoil individuals into either Eurasian Group 1 or 2 with 95% confidence. The lack of intraspecific hybrids may reflect the highly clonal nature of Eurasian watermilfoil or our limited sampling in other potential ranges of overlap. Alternatively, a lack of hybridization between the two lineages could reflect isolating mechanism(s) that limit gene flow between the two lineages. Further studies that investigate intraspecific hybridization within Eurasian watermilfoil are warranted (e.g., experimental crosses, more molecular markers, more intensive sampling) because intraspecific hybridization has been implicated as playing an important role in the evolution of invasiveness in other systems (e.g., Gaskin and Schall 2002, Kolbe et al. 2004) and could potentially lead to increased invasiveness in this already destructive species.

Recurrent hybridization and distinct hybrid milfoil biotypes

Our hierarchical Structure analysis with AFLP data revealed two major hybrid groups (Hybrid Group 1 and Hybrid Group 2) composed primarily from crosses between Eurasian Group 1

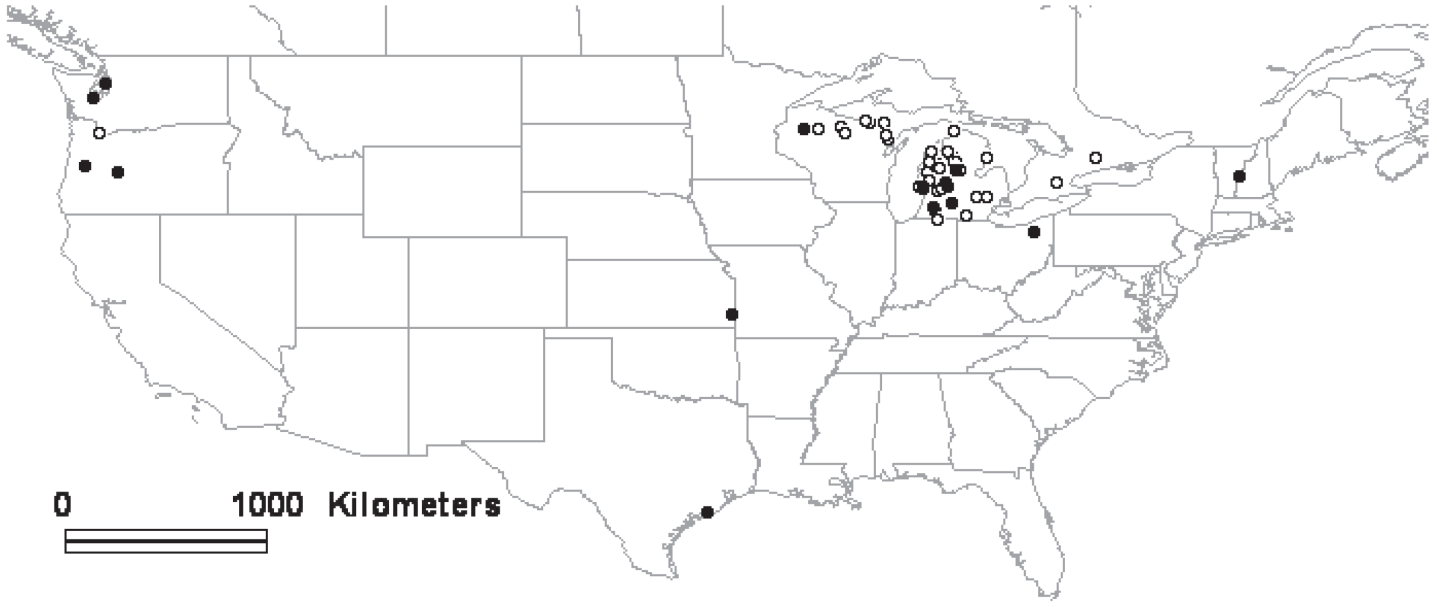

Figure 4. Distribution of Eurasian Group 1 and Eurasian Group 2 watermilfoil lineages in North America. Open circles represent populations where Eurasian Group 1 was found and black circles represent populations where Eurasian Group 2 was found.

and northern watermilfoil. Structure analysis at K = 3 revealed a third distinct group of hybrids that represent crosses between Eurasian Group 2 and northern watermilfoil. In addition, all of the individuals identified as "Eurasian Group 2 X northern watermilfoil hybrids" could also be distinguished by a unique ITS variant (HYB2), which only occurs when an individual with ITS variant EWM3 crosses with northern watermilfoil (based on our current knowledge of variation at ITS). This finding may suggest that Eurasian Group 2 generally does not produce successful hybrid offspring with northern watermilfoil, and perhaps only certain lineages within the group (i.e., individuals with ITS variant EWM3) are capable of successful reproduction; however, it may also reflect limited sampling range and/or sample size. Future studies that sample more extensively across a wider range or perform experimental crosses of these lineages are needed to resolve this issue. As with the cryptic diversity that we uncovered in Eurasian watermilfoil, it is not currently known whether any morphological or ecological differences are coincident with this genetic structuring of hybrid milfoils. Future studies should compare morphological and ecological characteristics to determine whether the genetically distinct lineages exhibit different growth habits or responses to management efforts (e.g., herbicides), and if so, whether morphological traits are sufficient to distinguish them in the field.

In addition to the discovery of Hybrid Groups 1 and 2 identified with our AFLP and ITS analyses, the extensive amount of clonal diversity in hybrids as a group relative to Eurasian watermilfoil suggests that hybrid watermilfoils have been formed repeatedly within North America. We found between 42 and 99 distinct clones (depending on the assumed error rate) in North America from the 50 populations that contained hybrids (544 total individuals). Furthermore, estimates of genotype diversity were highest in hybrids relative to both Eurasian and northern watermilfoil. While it is possible that hybrid watermilfoils were introduced to North America from Eurasia, where Eurasian and northern watermilfoil are reported to naturally overlap (Aiken and McNeill 1980), we find it much more likely that hybrids have repeatedly formed in North America as evidenced by our observations that (1) both groups of Eurasian watermilfoil introduced to North America have hybridized with northern watermilfoil, (2) multiple distinct hybrid clones occur within many lakes, and (3) a relatively large amount of genetic diversity occurs in hybrid watermilfoils as a group in comparison to Eurasian watermilfoil.

The repeated formation of hybrids and resultant genetic diversity may play an important role in the extent to which hybrid populations "behave" differently from Eurasian watermilfoil, a phenomenon that has been noted by some lake managers. Hybridization may stimulate the evolution of invasiveness in some taxa through several mechanisms including heterosis (hybrid vigor), increased genetic variation available to respond to selection, genetic novelty (i.e., novel traits in hybrids relative to parental taxa), and reducing genetic load (i.e., reducing the effects of deleterious alleles resulting from inbreeding or mutation accumulation during vegetative propagation; Ellstrand and Schierenbeck 2000). Any given hybrid lineage will possibly exhibit heterosis and nuisance growth patterns and/or require more stringent management efforts relative to Eurasian watermilfoil populations that exhibit "wild-type" desired levels of control under existing protocols. However, the repeated formation of hybrids from genetically distinct parental genotypes provides many opportunities to generate unique hybrid genotypes, and therefore different hybrid genotypes will likely exhibit different characteristics related to their growth patterns and response to management (Anderson and Stebbins 1954). Whether or not any or all of these mechanisms are important in determining the growth characteristics or response to control efforts in hybrid milfoils is presently unknown but warrants considerable attention in future research efforts.

Sexual versus asexual spread in Eurasian and hybrid watermilfoil

The genetic analyses performed here suggest that both asexual and sexual reproduction are important modes of reproduction and spread of Eurasian and hybrid watermilfoil. We found that asexual reproduction was very common within this system, with several clones shared among lakes and vegetative propagation common within all lakes. These findings are consistent with previous hypotheses that asexual reproduction is an important mode of establishment and spread of milfoils (Madsen et al. 1988, Madsen and Smith 1997). However, our analysis also identified a large number of genetically distinct clones within Eurasian, hybrid, and northern watermilfoils, suggesting that sexual reproduction is common in this system. Previous studies have suggested that sexual reproduction may be more common in northern watermilfoil than in Eurasian watermilfoil (Patten 1956, Furnier et al. 1995). Our estimates of genetic diversity support this notion because northern watermilfoil exhibits higher rates of genetic diversity within populations than Eurasian watermilfoil. Many of the lakes we surveyed were dominated entirely by unique clones that have likely formed through sexual reproduction and subsequently spread via vegetative fragments or seed (see Appendix 1). These findings suggest that while vegetative spread is important in the formation of large invasive populations within lakes, sexual reproduction may play a larger role in the colonization of new lakes (i.e., spread) or regeneration of populations following management efforts, and further consideration of the ecology and management of seed production is warranted.

AFLP versus ITS identifications

Given the increasing attention paid to hybrid watermilfoils by aquatic plant managers in combination with the reliance on molecular methods for their identification (Moody and Les 2007), we felt that a comparison of ITS identifications with our AFLP data was appropriate. Identifications based on ITS were generally supported by our AFLP analysis. Every individual identified as hybrid with ITS was also identified as a hybrid with AFLPs; however, AFLPs identified seven individuals as hybrids (including putative later generation hybrids; 1.7% misidentified) when the corresponding ITS sequences identified them as either Eurasian or northern watermilfoil. These misidentifications may be explained by gene conversion of ITS in some hybrids, extensive backcrossing with a single parental species, or possible PCR amplification bias of a particular ITS gene during DNA amplification. Our finding of three individuals that were clearly hybrids in our AFLP

analysis supports the possibility that gene conversion or PCR bias has occurred and can contribute to identification errors with ITS. Further supporting this claim, a second individual from each of these populations, and with the same AFLP genotype, were all identified as hybrid with ITS. The discovery of four individuals that seem to be introgressed beyond the first generation lends support to the hypothesis that introgression lowers the copy number of certain parental ITS genes, making highly introgressed hybrids more difficult to detect with ITS (see Moody and Les 2002, Moody et al. 2008). Furthermore, direct sequencing of ITS cannot always be used to accurately detect the major genetic groups identified in our Structure analysis with AFLPs (i.e., Eurasian Group 1 and Hybrid Groups 1 and 2) and cannot be used to differentiate between unique clones. Given these findings, we recommend genotyping multiple individuals with ITS and/or using AFLP genotypes when accurate identification of an individual is vital to the management or experimental plan (e.g., when different management protocols are used for different genotypes or when assaying differences between distinct genotypes).

CONCLUSIONS

Here we used AFLP molecular markers to study genetic variation in Eurasian, northern, and hybrid watermilfoil. We have shown that Eurasian watermilfoil in North America is composed of two genetically distinct lineages that probably represent independent introductions of this species. Both of these introduced lineages of Eurasian watermilfoil have hybridized with northern watermilfoil. Furthermore, hybrids are widespread and harbor a relatively large amount of genetic variation, suggesting that hybridization between Eurasian and northern watermilfoil occurs frequently in North America. Future studies should investigate whether distinct Eurasian and hybrid watermilfoil lineages differ ecologically or in their management susceptibility. Finally, our comparison of taxonomic identifications with ITS and AFLP markers shows that ITS generally provides reliable identifications. Nevertheless, we observed a low error rate (1.7%), illustrating that ITS does not always accurately distinguish some of the genetically distinct groups of Eurasian and hybrid milfoils. Thus, in situations where Eurasian, northern, and hybrid watermilfoil must be distinguished, we recommend obtaining ITS genetic identifications from multiple stems in a population.

SOURCES OF MATERIALS

[1]Qiagen Corp., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

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Appendix 1. Sampling locations and summary of genetic identifications for all samples using ITS sequences and AFLPs. States labeled according to U.S. postal codes (NA = Not Available). Additional information on study lakes may be available upon request from the corresponding author. Clones Per Lake = the number of times a distinct clone was sampled in a lake. Lake Sample Size = the number of individuals sampled at each location (individual lakes are separated by shading). ITS, the identification given to individuals with the ITS gene (see text for details on different ITS types). AFLP K = 2, the taxonomic identification given to individuals at K = 2 in Structure. AFLP K = 3, the taxonomic identification given to individuals at K = 3 in Structure. AFLP Sub, the taxonomic identification given to individuals in the first hierarchical level of Structure analysis. NWM = northern watermilfoil. EWM = Eurasian watermilfoil., number indicates AFLP group assignment (e.g., Eurasian watermilfoil Group 1 = EWM1). HYB = hybrid watermilfoil, number indicates AFLP group assignment. A "*" indicates a Eurasian watermilfoil Group 2 × northern watermilfoil hybrid. HYB × EWM1, HYB × EWM2, and HYB × NWM all represent individuals that are putatively introgressed beyond the first generation where the second species accounts for the higher proportion of the genome. HYB/NWM or HYB/EWM indicates two ITS identifications given to the same AFLP clone.

Clone	State	Clones Per Lake	Lake Sample Size	ITS	AFLP K=2	AFLP K=3	AFLP Sub
CA101-clone1	CA	1	1	NWM3	NWM	NWM	NWM
DL242-clone1	WA	1	1	NWM1	NWM	NWM	NWM
ID103-clone1	ID	1	2	NWM1	NWM	NWM	NWM
ID103-clone2	ID	1		NWM1	NWM	NWM	NWM
IN003-clone1	IN	2	2	HYB2	HYB	HYB*	HYB2
IN006-clone1	IN	1	1	HYB11	HYB	HYB	HYB2
KS003-clone1	KS	2	2	EWM2	EWM	EWM	EWM2
ME102-clone1	ME	1	2	NWM2	NWM	NWM	NWM
ME102-clone2	ME	1		NWM2	NWM	NWM	NWM
MI008-clone1	MI	7	7	EWM2	EWM	EWM	EWM1
MI015-clone1	MI	8	9	EWM3	EWM	EWM	EWM2
MI015-clone2	MI	1		NWM1	NWM	NWM	NWM
MI019-clone1	MI	1	1	EWM1	HYB	HYBxEWM1	HYBxEWM1
MI026-clone1	MI	23	25	HYB1	HYB	HYB	HYB2
MI026-clone2	MI	1		HYB1	HYB	HYB	HYB2
MI026-clone3	MI	1		HYB1	HYB	HYB	HYB2
MI101-clone1	MI	7	7	EWM3	EWM	EWM	EWM2
MI102-cloneA10	MI	2	4	HYB1/NWM1	HYB	HYB	HYB2
MI102-cloneA20	MI	1		HYB1	HYB	HYB	HYB2
MI102-cloneA30	MI	1		HYB1	HYB	HYB	HYB2
MI102-cloneA4	MI	1		HYB1	HYB	HYB	HYB2
MI104-clone1	MI	10	10	HYB1	HYB	HYB	HYB2
MI105-clone1	MI	2	2	EWM1	EWM	EWM	EWM1
MI106-clone1	MI	1	1	EWM2	EWM	EWM	EWM2

Appendix 1. (Continued)

MI107-clone1	MI	1	1	EWM2	EWM	EWM	EWM2
MI109-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI114-clone1	MI	4	6	HYB1	HYB	HYB	HYB2
MI114-clone2	MI	2		HYB1	HYB	HYB	HYB2
MI115-clone1	MI	10	12	EWM3	EWM	EWM	EWM2
MI115-clone2	MI	1		EWM3	EWM	EWM	EWM2
MI115-clone3	MI	1		EWM3	EWM	EWM	EWM2
MI116-clone1	MI	9	10	EWM1	EWM	EWM	EWM1
MI116-clone2	MI	1		EWM1	EWM	EWM	EWM1
MI118-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI119-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI120-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI121-clone1	MI	9	16	NWM1	NWM	NWM	NWM
MI121-clone2	MI	1		NWM1	HYB	HYBxNWM	HYBxNWM
MI121-clone4	MI	1		NWM1	HYB	HYBxNWM	HYBxNWM
MI121-clone5	MI	5			HYB	HYB	HYB1
MI124-clone1	MI	2	2	HYB1/NWM1	HYB	HYB	HYB1
MI127-clone1	MI	1	2	EWM1	EWM	EWM	EWM1
MI127-clone2	MI	1		EWM1	EWM	EWM	EWM1
MI128-clone1	MI	1	1	HYB1	HYB	HYB	HYB2
MI130-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI134-clone1	MI	5	18	EWM1	EWM	EWM	EWM2
MI134-clone2	MI	12		EWM1	EWM	EWM	EWM2
MI134-clone3	MI	1		EWM1	EWM	EWM	EWM2
MI136-clone1	MI	1	2	HYB1	HYB	HYB	HYB2
MI136-clone2	MI	1		HYB1	HYB	HYB	HYB2
MI137-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI139-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI140-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI141-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI142-clone1	MI	6	6	EWM1	EWM	EWM	EWM1
MI143-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI144-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI145-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI147-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI151-clone1	MI	9	9	HYB1	HYB	HYB	HYB2
MI152-clone1	MI	17	18	EWM1	EWM	EWM	EWM1
MI152-clone2	MI	1		EWM1	EWM	EWM	EWM1
MI153-clone1	MI	2	2	HYB2	HYB	HYB*	HYB2
MI154-clone1	MI	7	15	EWM1	EWM	EWM	EWM1
MI154-clone10	MI	1		EWM1	EWM	EWM	EWM1
MI154-clone7	MI	5		NWM1	NWM	NWM	NWM
MI154-clone8	MI	2		NWM1	NWM	NWM	NWM
MI157-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI160-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI161-clone1	MI	2	2	EWM1	EWM	EWM	EWM1
MI162-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI164-clone1	MI	4	4	EWM1	EWM	EWM	EWM1
MI165-clone1	MI	1	16	EWM1	EWM	EWM	EWM1
MI165-clone2	MI	1		EWM1	EWM	EWM	EWM1
MI165-clone3	MI	14		EWM1	EWM	EWM	EWM1
MI166-clone1	MI	2	2	HYB1/EWM1	HYB	HYB	HYB2
MI168-clone1	MI	3	3	EWM1	EWM	EWM	EWM1
MI169-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI170-clone1	MI	1	1	EWM1	EWM	EWM	EWM1
MI171-clone1	MI	1	1	HYB1	HYB	HYB	HYB2

Appendix 1. (Continued)

MI173-clone1	MI	8	8	EWM1	EWM	EWM	EWM1
MI174-clone1	MI	6	6	HYB1	HYB	HYB	HYB2
MI175-clone1	MI	4	16	EWM1	EWM	EWM	EWM1
MI175-clone2	MI	7		EWM1	EWM	EWM	EWM1
MI175-clone3	MI	5		EWM1	EWM	EWM	EWM1
MI176-clone1	MI	1	8	HYB1	HYB	HYB	HYB2
MI176-clone2	MI	7		HYB1	HYB	HYB	HYB2
MI177-clone1	MI	24	24	HYB1	HYB	HYB	HYB2
MI178-clone1	MI	4	4	HYB1	HYB	HYB	HYB2
MI179-clone4	MI	2	7	HYB1	HYB	HYB	HYB1
MI179-clone5	MI	2		HYB1	HYB	HYB	HYB1
MI179-clone8	MI	2		HYB1	HYB	HYB	HYB1
MI179-clone9	MI	1		HYB1	HYB	HYB	HYB1
MI180-clone1	MI	1	12	EWM2	HYB	HYBxEWM2	HYBxEWM2
MI180-clone2	MI	1		EWM2	EWM	EWM	EWM2
MI180-clone3	MI	3		EWM2	EWM	EWM	EWM2
MI180-clone4	MI	7		EWM2	EWM	EWM	EWM2
MI181-clone1	MI	15	15	HYB1	HYB	HYB	HYB2
MI182-clone1	MI	4	14	HYB1	HYB	HYB	HYB1
MI182-clone2	MI	1		NWM1	NWM	NWM	NWM
MI182-clone3	MI	9		NWM1	NWM	NWM	NWM
MI183-clone1	MI	2	7	NWM1	NWM	NWM	NWM
MI183-clone2	MI	1		NWM1	NWM	NWM	NWM
MI183-clone3	MI	1		NWM1	NWM	NWM	NWM
MI183-clone4	MI	3		HYB1	HYB	HYB	HYB1
MI184-clone1	MI	22	22		HYB	HYB	HYB1
MI185-clone1	MI	3	3	HYB1	HYB	HYB	HYB2
MI186-clone1	MI	4	5	NWM1	NWM	NWM	NWM
MI186-clone2	MI	1		EWM1	EWM	EWM	EWM1
MI187-cloneA1	MI	1	4	NWM1	NWM	NWM	NWM
MI187-cloneA10	MI	1		EWM1	EWM	EWM	EWM1
MI187-cloneA2	MI	1		NWM1	NWM	NWM	NWM
MI187-cloneA3	MI	1		EWM1	EWM	EWM	EWM1
MI188-clone1	MI	6	9		NWM	NWM	NWM
MI188-clone2	MI	1			HYB	HYB	HYB2
MI188-clone3	MI	1			HYB	HYB	HYB1
MI188-clone4	MI	1			NWM	NWM	NWM
MI189-clone1	MI	1	22		HYB	HYB	HYB1
MI189-clone2	MI	1			EWM	EWM	EWM1
MI189-clone3	MI	2			EWM	EWM	EWM1
MI189-clone4	MI	18			EWM	EWM	EWM1
MI190-clone1	MI	11	18		HYB	HYB	HYB2
MI190-clone2	MI	4			HYB	HYB	HYB2
MI190-clone3	MI	1			HYB	HYB	HYB2
MI190-clone4	MI	1			HYB	HYB	HYB2
MI190-clone5	MI	1			HYB	HYB	HYB1
MI191-clone6	MI	1	6		HYB	HYB	HYB2
MI191-clone7	MI	1			HYB	HYB	HYB2
MI191-clone8	MI	4			NWM	NWM	NWM
MI192-clone1	MI	16	21		HYB	HYB	HYB1
MI192-clone2	MI	5			HYB	HYB	HYB2
MI193-clone1	MI	12	21		HYB	HYB	HYB1
MI193-clone2	MI	9			HYB	HYB	HYB2
MI194-clone2	MI	4	9		NWM	NWM	NWM
MI194-clone3	MI	5			NWM	NWM	NWM
MI195-clone1	MI	28	28		NWM	NWM	NWM

Appendix 1. (Continued)

MI196-clone1	MI	28	28		EWM	EWM	EWM1
MI197-clone1	MI	38	38		HYB	HYB	HYB2
MI198-clone1	MI	29	30		HYB	HYB	HYB2
MI198-clone2	MI	1			NWM	NWM	NWM
MI199-clone1	MI	2	8	EWM1	EWM	EWM	EWM1
MI199-clone2	MI	3		HYB1	HYB	HYB	HYB2
MI199-clone3	MI	3		EWM1	EWM	EWM	EWM1
MI200-clone1	MI	22	22		NWM	NWM	NWM
MI201-clone1	MI	28	28		EWM	EWM	EWM1
MI202-clone1	MI	26	26		HYB	HYB	HYB1
MI203-clone1	MI	23	24		HYB	HYB	HYB1
MI203-clone2	MI	1			HYB	HYB	HYB1
MI204-clone1	MI	5	5	HYB1	HYB	HYB	HYB2
MI206-clone2	MI	16	22		EWM	EWM	EWM1
MI206-clone3	MI	6			HYB	HYB	HYB1
MI207-clone1	MI	5	5		NWM	NWM	NWM
MI208-clone2	MI	6	6		HYB	HYB	HYB2
MT102-clone1	MT	8	15	NWM1	NWM	NWM	NWM
MT102-clone2	MT	7		NWM1	NWM	NWM	NWM
NH402-clone1	NH	2	2	EWM3	EWM	EWM	EWM2
OH101-clone1	OH	1	1	EWM2	EWM	EWM	EWM2
ONT102-clone1	ONT	3	5	EWM1	EWM	EWM	EWM1
ONT102-clone2	ONT	2		NWM1	NWM	NWM	NWM
ONT103-clone1	ONT	2	2	EWM1	EWM	EWM	EWM1
ONT104-clone1	ONT	3	3	EWM1	EWM	EWM	EWM1
OR101-clone1	OR	2	2	EWM1	EWM	EWM	EWM1
OR102-clone1	OR	2	2	EWM2	EWM	EWM	EWM2
OR103-clone1	OR	2	2	EWM1	EWM	EWM	EWM2
OR104-clone1	OR	2	2	NWM1	NWM	NWM	NWM
SClone-clone1	NA	1	4	EWM3	EWM	EWM	EWM2
SClone-clone2	NA	3		EWM3	EWM	EWM	EWM2
TX002-clone1	TX	2	2		EWM	EWM	EWM2
WA107-clone1	WA	1	1	EWM3	EWM	EWM	EWM2
WA108-clone1	WA	1	1	HYB1	HYB	HYB*	HYB2
WA110-clone1	WA	1	1	NWM1	NWM	NWM	NWM
WA111-clone1	WA	2	2	EWM3	EWM	EWM	EWM2
WI102-clone1	WI	1	1	HYB1	HYB	HYB	HYB2
WI103-clone1	WI	1	1	HYB1	HYB	HYB	HYB1
WI105-clone1	WI	1	1	HYB1	HYB	HYB	HYB1
WI110-clone1	WI	1	1	HYB1	HYB	HYB	HYB2
WI111-clone1	WI	1	1	EWM3	EWM	EWM	EWM2
WI115-clone1	WI	2	2	EWM1	EWM	EWM	EWM1
WI116-clone1	WI	1	1	NWM1	NWM	NWM	NWM
WI117-clone1	WI	1	1	NWM1	NWM	NWM	NWM
WI125-clone1	WI	24	24	HYB1	HYB	HYB	HYB1
WI126-clone1	WI	18	18	HYB1	HYB	HYB	HYB2
WI134-clone1	WI	3	11		HYB	HYB	HYB2
WI134-clone2	WI	2			HYB	HYB	HYB1
WI134-clone3	WI	5			HYB	HYB	HYB1
WI134-clone4	WI	1			HYB	HYB	HYB1
WI135-clone1	WI	5	21		NWM	NWM	NWM
WI135-clone2	WI	16			NWM	NWM	NWM
WI136-clone1	WI	1	13		HYB	HYB	HYB1
WI136-clone2	WI	2			HYB	HYB	HYB1
WI136-clone3	WI	10			HYB	HYB	HYB1

Appendix 2. Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

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                 1                                                  50
FJ426346_SpiMI1  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426347_SpiMI2  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426348_SpiMI3  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426349_SpiMI4  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426350_SpiMI6  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426351_SpiMI7  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
EWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
FJ426352_SibMI1  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426353_SibMI2  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426354_SibMI3  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426355_SibMI4  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426356_SibMI5  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
FJ426357_SibMI6  G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G T G A A C C T G C G G A A G G A T C
NWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?

                 51                                                 100
FJ426346_SpiMI1  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
FJ426347_SpiMI2  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
FJ426348_SpiMI3  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
FJ426349_SpiMI4  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
FJ426350_SpiMI6  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
FJ426351_SpiMI7  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C C G T G A A C T A A T A A A C A C C C G G
EWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
FJ426352_SibMI1  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
FJ426353_SibMI2  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
FJ426354_SibMI3  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
FJ426355_SibMI4  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
FJ426356_SibMI5  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
FJ426357_SibMI6  A T T G T C G A A A C C T G C A C A G C A G A A C G A C C T G T G A A C T A A T A A A C A C C C G G
NWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?

                 101                                                150
FJ426346_SpiMI1  G G G G A G A G G A G G G A G C T G C A C T T G T G C G G C G C C A C C C C T C G C C C C C C A G T
FJ426347_SpiMI2  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T C G C C C C C C A G T
FJ426348_SpiMI3  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C G C C A C C C C T C G C C C C C C A G T
FJ426349_SpiMI4  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C G C C A C C C C T C G C C C C C C A G T
FJ426350_SpiMI6  G C G G C G A G G A G G G A G C T G C A C T T G T G C G G C G C C A C C C C T C G C C C C C C A G T
FJ426351_SpiMI7  G C G G C G A G G A G G G A G C T G C A C T T G T G C G G C G C C A C C C C T C G C C C C C C A G T
EWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
FJ426352_SibMI1  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C C A G T
FJ426353_SibMI2  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C C A G T
FJ426354_SibMI3  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C C A G T
FJ426355_SibMI4  G A G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C C A G T
FJ426356_SibMI5  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C —A G T
FJ426357_SibMI6  G G G G C G A G G A G G G A G C T G C A C T T G T G C G G C A C C A C C C C T T G C C C C C C A G T
NWM1             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM2             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM3             ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
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Appendix 2. (Continued) Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

	151	200
FJ426346_SpiMI1	**G C C T A G A C G C G C C C C C T G C C A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G**	
FJ426347_SpiMI2	G C C T A G A C G C G C C C C C T G C C A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
FJ426348_SpiMI3	G C C T A G A C G C G C C C C C T G C C A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
FJ426349_SpiMI4	G C C T A G A C G C G C C C C C T G C T A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
FJ426350_SpiMI6	G C C T A G A C G C G C C C C C T G C T A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
FJ426351_SpiMI7	G C C T A G A C G C G C C C C C T G C T A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
EWM1	? ? ? ? ? ? ? ? ? ? C C C C C T G C Y A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
EWM2	? ? ? ? ? ? ? ? ? ? C C C C C T G C C A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
EWM3	? ? ? ? ? ? ? ? ? ? C C C C C T G C Y A C A C C G G A C T T T G T T C G G C G T C G G C A G G A G	
FJ426352_SibMI1	G C C T A G A C G C —C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
FJ426353_SibMI2	G C C T A G A C G C —C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
FJ426354_SibMI3	G C C T A G A C G C —C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
FJ426355_SibMI4	G C C T A G A C G C —C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
FJ426356_SibMI5	G C C T A G A C G C G C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
FJ426357_SibMI6	G C C T A G A C G C —C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
NWM1	? ? ? ? ? ? ? ? ? ? C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
NWM2	? ? ? ? ? ? ? ? ? ? C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	
NWM3	? ? ? ? ? ? ? ? ? ? C C C C C T G C C A C A C C G G A C T T —G T T C G G C G T C G G C A G G A G	

	201	250
FJ426346_SpiMI1	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426347_SpiMI2	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426348_SpiMI3	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426349_SpiMI4	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426350_SpiMI6	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426351_SpiMI7	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
EWM1	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
EWM2	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
EWM3	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426352_SibMI1	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426353_SibMI2	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426354_SibMI3	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426355_SibMI4	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426356_SibMI5	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
FJ426357_SibMI6	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
NWM1	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
NWM2	G T C G T C C A T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	
NWM3	G T C G T C C G T G G C G A C A A T A A C A A A C C C C G G C G C G G A A A G C G C C A A G G A A A	

	251	300
FJ426346_SpiMI1	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426347_SpiMI2	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426348_SpiMI3	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426349_SpiMI4	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426350_SpiMI6	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426351_SpiMI7	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
EWM1	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
EWM2	T C A T G A C G A A C T T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
EWM3	T C A T G A C G A A C W T A G C A C A C C A C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426352_SibMI1	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426353_SibMI2	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426354_SibMI3	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426355_SibMI4	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426356_SibMI5	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
FJ426357_SibMI6	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
NWM1	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
NWM2	T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A C T T G T G C G G C A G C G G C G T T G	
NWM3	**T C A T G A C G A A C T T A G C A C A C C G C T A G C C G A Y T T G T G C G G C A G C G G C G T T G**	

Appendix 2. (Continued) Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

	301	350
FJ426346_SpiMI1	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426347_SpiMI2	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426348_SpiMI3	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426349_SpiMI4	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426350_SpiMI6	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426351_SpiMI7	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
EWM1	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
EWM2	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
EWM3	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426352_SibMI1	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426353_SibMI2	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426354_SibMI3	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426355_SibMI4	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426356_SibMI5	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
FJ426357_SibMI6	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
NWM1	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
NWM2	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	
NWM3	C A A A C T T C G A T A C C T A A A C G A C T C T C G G C A A C G G A T A T C T C G G C T C T C G C	

	351	400
FJ426346_SpiMI1	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426347_SpiMI2	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426348_SpiMI3	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426349_SpiMI4	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426350_SpiMI6	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426351_SpiMI7	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
EWM1	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
EWM2	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
EWM3	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426352_SibMI1	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426353_SibMI2	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426354_SibMI3	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426355_SibMI4	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426356_SibMI5	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
FJ426357_SibMI6	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
NWM1	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
NWM2	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	
NWM3	A T C G A T G A A G A A C G T A G C G A A A T G C G A T A C T T G G T G T G A A T T G C A G A A T C	

	401	450
FJ426346_SpiMI1	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426347_SpiMI2	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426348_SpiMI3	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426349_SpiMI4	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426350_SpiMI6	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426351_SpiMI7	C C G T G A A C C A T T G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
EWM1	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
EWM2	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
EWM3	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426352_SibMI1	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426353_SibMI2	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426354_SibMI3	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426355_SibMI4	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426356_SibMI5	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
FJ426357_SibMI6	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
NWM1	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
NWM2	C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C	
NWM3	**C C G T G A A C C A T C G A G T T T T G A A C G C A A G T T G C G C C C G A A G C C A T T C G G C**	

Appendix 2. (Continued) Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

	451	500
FJ426346_SpiMI1	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426347_SpiMI2	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426348_SpiMI3	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426349_SpiMI4	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426350_SpiMI6	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426351_SpiMI7	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
EWM1	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
EWM2	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
EWM3	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426352_SibMI1	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426353_SibMI2	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426354_SibMI3	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426355_SibMI4	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426356_SibMI5	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
FJ426357_SibMI6	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
NWM1	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
NWM2	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	
NWM3	C G A G G G C A C G T C T G C C T G G G C G T C A C G T A T C G C G T T G C T C C C A A A G C C C A	

	501	550
FJ426346_SpiMI1	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426347_SpiMI2	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426348_SpiMI3	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426349_SpiMI4	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426350_SpiMI6	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426351_SpiMI7	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
EWM1	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
EWM2	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
EWM3	C C C T T C A A G G A T A A G G C G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426352_SibMI1	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426353_SibMI2	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426354_SibMI3	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426355_SibMI4	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426356_SibMI5	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
FJ426357_SibMI6	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
NWM1	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
NWM2	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	
NWM3	C C C T T C A A G G A T A T G G T G C T G C G G A A G C A G A T A T T G G C C T C C C G T G C C T G	

	551	600
FJ426346_SpiMI1	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426347_SpiMI2	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426348_SpiMI3	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426349_SpiMI4	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426350_SpiMI6	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426351_SpiMI7	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
EWM1	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
EWM2	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
EWM3	C G C A C G G C T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426352_SibMI1	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426353_SibMI2	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426354_SibMI3	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426355_SibMI4	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426356_SibMI5	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
FJ426357_SibMI6	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G C T G A C G A A A G G G T C A C G A C A	
NWM1	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
NWM2	T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A	
NWM3	**T G C A C G G A T G G C C T A A A T G C A A G C C T G G G G G T G A C G A A A G G G T C A C G A C A**	

Appendix 2. (Continued) Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

	601 … **650**
FJ426346_SpiMI1	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
FJ426347_SpiMI2	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
FJ426348_SpiMI3	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
FJ426349_SpiMI4	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
FJ426350_SpiMI6	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
FJ426351_SpiMI7	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C C G T G C C C G C C G T G C C C C T
EWM1	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM2	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
EWM3	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G C ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
FJ426352_SibMI1	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
FJ426353_SibMI2	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
FJ426354_SibMI3	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
FJ426355_SibMI4	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
FJ426356_SibMI5	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
FJ426357_SibMI6	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T C G T G C C C G C C G T G C C C C T
NWM1	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM2	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
NWM3	A G C G G T G G T T G A T A A C T C A G C C T T T G T T G C G T ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?

	651 … **700**
FJ426346_SpiMI1	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426347_SpiMI2	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426348_SpiMI3	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426349_SpiMI4	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426350_SpiMI6	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426351_SpiMI7	T G G A G C T C A G C A T C C C C G A C G C G C C G T C T C G A C G G C G T T T — — G C A T C G C G
EWM1	? ?
EWM2	? ?
EWM3	? ?
FJ426352_SibMI1	T G G A G C T C A G C A T C C C C G A C G C G C T G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426353_SibMI2	T G G A G C T C A G C A T C C C C G A C G C G C T G T T T C G A T G G C G T T T — — G C A T C G C G
FJ426354_SibMI3	T G G A G C T C A G C A T C C C C G A C G C G C T G T T T C G A C G G C G T T T — — G C A T C G C G
FJ426355_SibMI4	T G G A G C T C A G C A T C C C C G A C G C G C T G T C T C G A C G G C G T T T — — G C A T C G C G
FJ426356_SibMI5	T G G A G C T C A G C A T C C C C G A C G C G C T G T C T C G A C G G C G T T T T T G C A T C G C G
FJ426357_SibMI6	T G G A G C T C A G C A T C C C C G A C G C G C T G T C T C G A C G G C G T T T — — G C A T C G C G
NWM1	? ?
NWM2	? ?
NWM3	? ?

	701 … **750**
FJ426346_SpiMI1	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426347_SpiMI2	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426348_SpiMI3	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426349_SpiMI4	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426350_SpiMI6	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426351_SpiMI7	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
EWM1	? ?
EWM2	? ?
EWM3	? ?
FJ426352_SibMI1	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426353_SibMI2	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426354_SibMI3	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426355_SibMI4	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426356_SibMI5	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
FJ426357_SibMI6	A C C C C A G G T C A G G C G G G A C T A C C C G C T G A G T T T A A G C A T A T C A A T A A G C G
NWM1	? ?
NWM2	? ?
NWM3	**? ?**

Appendix 2. (Continued) Alignment of our ITS sequence variants to Genbank accessions (FJ426346-FJ426357) identified in Sturtevant et al. 2009. "?'s" represent portions of the ITS gene not analyzed in our study.

	751
FJ426346_SpiMI1	G A G G A
FJ426347_SpiMI2	G A G G A
FJ426348_SpiMI3	G A G G A
FJ426349_SpiMI4	G A G G A
FJ426350_SpiMI6	G A G G A
FJ426351_SpiMI7	G A G G A
EWM1	? ? ? ? ?
EWM2	? ? ? ? ?
EWM3	? ? ? ? ?
FJ426352_SibMI1	G A G G A
FJ426353_SibMI2	G A G G A
FJ426354_SibMI3	G A G G A
FJ426355_SibMI4	G A G G A
FJ426356_SibMI5	G A G G A
FJ426357_SibMI6	G A G G A
NWM1	? ? ? ? ?
NWM2	**? ? ? ? ?**
NWM3	**? ? ? ? ?**

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Invasions and Impacts of Alligatorweed in the Upper Xiaoqing River Basin of Northern China

LIU DASHENG[1], J. F. HU[2], D. P. HORVATH[3], X. J. ZHANG[4], X. Y. BIAN[1], G. L. CHANG[5], X. H. SUN[6], J. TIAN[7]

ABSTRACT

Alligatorweed (*Alternanthera philoxeroides* (Mart.) Griseb), is a problematic and difficult to manage invasive weed. The recent invasion in the upper Xiaoqing River, northern China extends its range northwards through almost five degrees latitude and 500 km from the northern limit and main invasion area of the weed in China. The length of main branches of the weed in Jinan ranges from 198 cm to 382 cm, with an average value of 266.67 ± 24.01 cm. The average number of nodes and adventitious roots on the main branches are 27.01 ± 2.25 and 17.11 ± 0.84, respectively. The number of main branches per linear meter transect is 376-511, with an average of 436.52 ± 55.33. The main impact of alligatorweed is that it chokes the flood flow of the local river in rainy seasons, but was not found to cause obvious damage to agricultural production in the area covered by this study. However, the presence of this weed in northern China highlights its potential future risk, and questions the previous models used to predict the spread and distribution of this weed.

Key Words: *Alternanthera philoxeroides*, extend, northern limit, five degrees latitude, 500 km.

INTRODUCTION

Alligatorweed (*Alternanthera philoxeroides* (Mart.) Griseb), is a very difficult to manage invasive weed. It originated in the Parana River region of South America (Maddox 1968, Vogt et al. 1979) and was spread to the other areas of South America, North America, Asia, Australia and some adjacent island countries (Julien et al. 1995). It grows in both aquatic and terrestrial habitats, and in some areas it blankets water surfaces,

[1]Shandong Institute of Environmental Science, Jinan 250013, China; Corresponding author's E-mail: liu_sdiep@126.com
[2]College of Life Science, South China Normal University, Guangzhou 510631, China
[3]Biosciences Research Laboratory, Agricultural Research Service, US Department of Agriculture, Fargo, ND, 58102-2765, USA
[4]College of Life Science, Shandong Normal University, Jinan 250014, China
[5]Shandong Center of Environmental Monitoring, Jinan 250013, China
[6]College of Population, Resource and Environment, Shandong Normal University , Jinan 250014, China
[7]The management office of Xiaoqing River of Jinan, Jinan 250014, China
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and chokes waterways and ponds, and can also invade farm land. Alligatorweed was introduced to China's mainland in the late 1930s as horse feed by Japanese in Shanghai suburbs (Diao 1990), and was cultivated widely as a forage in southern China in the 1950s. Unfortunately it escaped cultivation and now is a significant ecological problem. It is currently listed on a shortlist of 16 invasive species requiring special control in China (State Environmental Protection Administration of China 2003).

Julien et al. (1995) used a climate matching program, CLIMEX, and the known distribution of alligatorweed in South and North America to infer areas suitable for its growth in the world, and the results showed that the northern limit in the region of China for the weed is about 32 degrees north latitude (Figure 1). Its main distribution in China is the southern areas along the middle and lower reaches of the Yangtze River (Ma and Wang 2005). The Yangtze River is the longest river in China, and the latitude of the main cities along its middle and lower reaches is from about 28 to 32 degrees north. Latitude and longitude of Jinan and main cities along the middle-lower reaches of the Yangtze River and main meteorological parameters are shown in Table 1 (data from China Meteorological Data Sharing Service System, cdc. cma. gov. cn). In contrast to predictions by Julien et al. (1995) and earlier documents in China (Ma and Wang 2005), we found alligatorweed to have significantly invaded the upper Xiaqing River in Jinan of Shandong province, located at 36.7 degrees north latitude (Liu et al. 2006). These later surveys suggest that the main invasion area of alligatorweed in China had moved north by almost 5 degrees latitude and nearly 500 km indicting a greater range than was previously predicted.

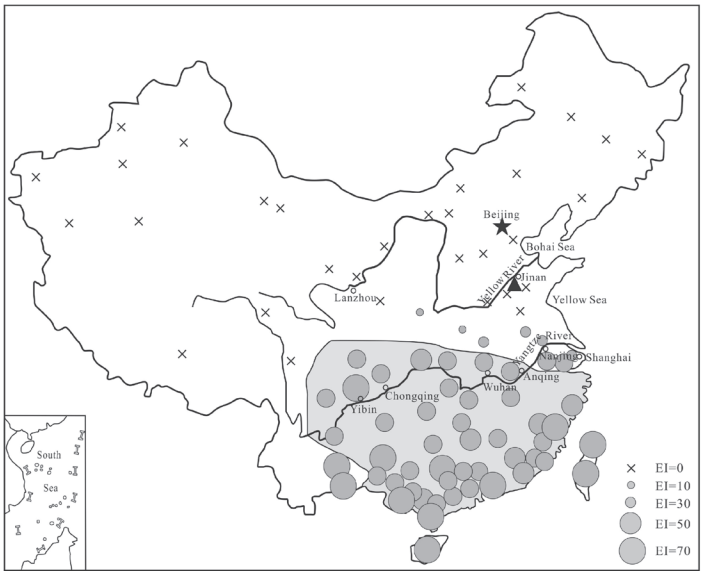

Figure 1. Location map showing the predicted and known historical distribution of alligatorweed in China (Julien et al. 1995) and our present field survey in Jinan, northern China (predicted distribution indicated by circle and cross, and known historical distribution by shadow, our present work by triangle). The map is based on the result and figure of Julien et al. (1995) with some modification. The annual ecoclimatic index (EI) describes the potential growth of the weed and is scaled between 0 and 100. The values of EI above 10 for locations favorable to the growth of alligatorweed are indicated by circles, and the areas of circles are proportional to the predicted suitability of the location. The values of EI between 0 and 10 for locations unfavorable for the weed are indicated by crosses. For a full description refer to Julien et al. (1995).

The primary invasion sites of alligatorweed were found near the source of the Xiaoqing River, however none of the general stand characteristics, for example stand density or distribution to the other parts of the upper Xiaoqing river, were known. The impact of these introductions on local rivers and agricultural production were also unknown. To answer these questions, we conducted a further field investigation during 2006 to 2007. In addition, the upper Xiaoqing River is very different in water quality depending on the distance from the source, with clear unpolluted waters in the region near the source to polluted waters through most parts of the upper Xiaoqing River. Thus, we also wanted to test the hypothesis that the distribution of alligatorweed is not limited by water quality in the upper Xiaoqing River.

MATERIALS AND METHODS

Study Sites and Scope of Investigation

Jinan, the provincial capital of Shandong, is located in North China on the south bank of the lower reaches of the Yellow River, the second longest river in China. The average annual temperature in Jinan is 14.7 C with an average of -0.4 C in January and 27.5 C in July. The average annual rainfall is 672.7 mm.

The Xiaoqing River is an important river in Shandong province, with a total length of 237 km, of which 70.3 km traverses in Jinan. The Xiaoqing River serves regionally for flood discharge, irrigation, and sewage discharge etc. The Xiaoqing River stems from Muli gate, which is located in the western suburbs of Jinan, then flows eastward through Jinan, Binzhou, Zibo, Dongying and Weifang, and finally joins into the Bohai Sea in Yangjiaokou, Weifang (Figure 2).

The 40 km-long Yufu River is a small seasonal river that mainly discharges water from hills of southern Jinan and then flows northward through Jinan suburban Zhonggong, Dangjia, Duandian, Pingandian, Wujiapu and finally joins into the Yellow River in Beidianzi. It can also flow into the Xiaoqing River through Muli gate.

Field Survey

The investigation was carried out over the Jinan section of the Xiaoqing River, from the source at the Muli gate to the end at Dashaliu gate (70 km), and over 10 km of the Yufu River. The field work was conducted in 2006 and 2007. We recorded the distribution and invasion of alligatorweed at the upper reaches of the Xiaoqing River and the Yufu River. We also interviewed local river management officers, local farmers, and local aquaculture peasants to investigate the impacts of alligatorweed on local rivers and agricultural production. Additionally, we randomly selected five quadrants of rice field (100 m^2), five quadrants of vegetable production areas (≥50 m^2) and five fishponds (≥200 m^2) to survey for the presence of the weed within the cultivated areas. The rice fields were located outside of the river bank and the vegetable production areas were located inside of the bank. The position of

Table 1. Latitude and longitude of Jinan and main cities along the middle-lower reaches of the Yangtze River and their main meteorological parameters

Cities/Parameters	Jinan	Shanghai	Nanjing	Anqing	Wuhan	Chongqing	Yibing
Latitude (N)	36.6	31.4	32.0	30.5	30.6	29.6	28.8
Longitude (E)	117.1	121.5	118.8	117.1	114.1	106.5	104.6
Lowest temperature (°C)	-14.9	-7.7	-13.1	-9.0	-18.1	-1.7	-1.7
Highest temperature (°C)	40.5	37.8	39.7	39.5	39.3	41.9	39.5
Mean temperature (°C)	14.7	16.6	15.4	16.7	16.6	18.2	17.8
Mean temperature of January (°C)	-0.4	4.7	2.4	4.0	3.7	7.8	7.8
annual precipitation (mm)	672.7	1184.4	1062.4	1474.9	1269.0	1104.5	1063.1

survey points was determined by GPS, and digital photos were taken. For the location of field survey points see Figure 2.

Data Collection and Measurements

At the field sites near Peng Zhuang in the upper Xiaoqing River, alligatorweed was the dominant community. We randomly selected three quadrants in the filed sites, and chose six or seven main stem branches that were as long and complete as possible in each quadrant for our data collection. The length, number of branches, number of nodes, number of adventitious roots, and the number of flowers on representative main branches were measured. Small branches with lengths below 5 cm were treated as one class and not measured individually. Because of the creeping, long and interlaced branching of alligatorweed which made counting difficult and counting confusion, we used the transects instead of 1 m^2 quadrants in the present study. We selected six 1m wide transects at random, cut all of the weeds within each transect, and counted the number of main branches in each unit.

Water Quality Data Collection

The recorded water quality data from 2005 to 2007 for the following four monitoring sites in the upper Xiaoqing River were used: Muli gate, Huanxiangdian, Damatou and Xinfeng Zhuang (Jinan Environmental Protection Bureau 2006, 2007, 2008). Muli gate is located at the source of the Xiaoqing River, while Xinfeng Zhuang is close to the Jinan eastern boundary. For the four water quality monitoring sites see Figure 2.

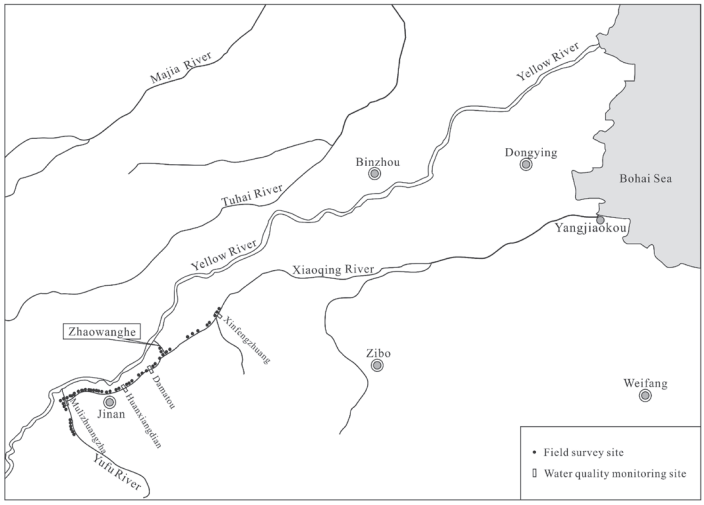

Figure 2. Location map of the Xiaoqing River, field survey points and water quality monitoring sites.

RESULTS AND DISCUSSION

Local Invasions

Alligatorweed was found along the entire study area (Figure 2). In the sections with greater infestation, the weed extended 5-6 m from both sides of the river bank, and occasionally covered the whole river surface where the river was narrow. It formed the dominant community at Peng Zhuang, West Second Circle Road Bridge, Ban Bridge and Huangtai. The infestation in the Yufu River and the Zhaowanghe was particularly serious. The Yufu River is the upstream branch of the Xiaoqing River in western Jinan, and the latter is a small waterway for flood discharge and irrigation between the Xiaoqing River and the Yellow River in eastern Jinan. The 3 km-long part of the Yufu River and a 1 km-long portion of the Zhaowanghe were covered or almost covered by mats of alligatorweed and the flow speed was slow or non-existent in these areas.

In the sections with low infestation, such as Shahe Bridge, Xiaoxu Jia, Hanguan Zhuang Bridge, Zong Jia Bridge and Fu Jia Bridge, the weed was only distributed in small patches. Between the two extremes mentioned above, a sheet-like distribution generally extended 3-5m from the river bank, and sometimes continued 100-200 m along the river.

Compared with the previously predicted northern distribution limits (Julien et al. 1995) and known main invasion areas in southern China (Ma and Wang 2005), the distribution of alligatorweed in the upper Xiaoqing River in Jinan has moved north almost five degrees latitude and nearly 500 km. The average temperature in the coldest month (January) is -0.4 C in Jinan, and 2.4-7.8 C in Shanghai, Nanjing, Anqing, Wuhan, Chongqing, and Yibing along the middle-lower reaches of the Yangzi River (data from China Meteorological Data Sharing Service System , cdc. cma. gov. cn).

Our survey of the upper Xiaoqing River showed that the natural condition of northern China were suitable for the alligatorweed, in contrast to the previous CLIMEX prediction (Julien et al. 1995). CLIMEX is a modeling package employed to predict the potential species distribution based on its current distribution and a huge database of meteorological information (Sutherst et al. 1999, Kriticos et al. 2003, Peterson 2003), and it features a climate-matching function with species-specific responses to key environmental param-

eters (Pattison and Mack 2008). However, CLIMEX has its limitations. As one of climate models, CLIMEX greatly relies on the number and distribution of meteorological stations (Poutsma et al. 2008). Areas with a small number of stations may not give a representative view of the climate in that region because the location of the meteorological stations is frequently unrepresentative for the surrounding area (Bennett et al. 1998).

The reason for the failed distribution predictions of alligatorweed may have been the lack of available meteorological data from northern China. Recently, interest in species distribution models of plant and animals has grown dramatically (Guisan and Thuiller 2005), and the current work of this kind of model could use climatic data of temperature and rainfall from 45000 locations and nearly 25000 locations separately, from www.worldclim.org. The predictions by Julien et al. (1995) used meteorological data from only 2500 locations. In addition, McFadyen (1991) pointed out that predictions of the likely exotic distribution of an organism based solely on knowledge of its native range may be quite erroneous. Julien et al. (1995) just used the distribution data of alligatorweed from South and North America to infer areas suitable for the growth of this weed in the whole world, but they did not use data from Asian, African and European infestations to build his model. This may be another reason that led to the inaccurate predictions in China.

Water Quality in Investigated Section

The distribution of alligatorweed was found in different water qualities from un-polluted to polluted (Figure 3 and 4). A large amount of wastewater is poured into the Xiaoqing River when it flows through Jinan. Although the majority of the wastewater is treated in wastewater plants, excluding the region near the source, most of the surveyed regions of the upper Xiaoqing River were still polluted. According to the 2005-2007 Jinan Environmental Status Bulletin (Jinan Environmental Protection Bureau, 2006, 2007, 2008), the average values of chemical oxygen demand (CODcr), biochemical oxygen demand (BOD_5), ammonia nitrogen, volatile phenols, cyanide, arsenic, Hg, Cr^{6+} and Pb in Muli gate comply with the limit value of Grade III of the "Environmental Quality Standard for Surface water (GB3838-2002) "(State Environmental Protection Administration of China 2002), and shows that the water quality at the source of the Xiaoqing River is clear. Grade III water corresponds to conservation district of drinking water, areas for fish over wintering and migration as well as swimming areas. Huanxiangdian, Damatou and Xinfeng Zhuang are located downstream of Muli gate about 25 km, 37 km and 65 km away, respectively. The annual average values of CODcr, BOD_5 and ammonia nitrogen of the three monitoring sites do not meet the demands of Grade V of the "Environmental Quality Standard for Surface water (GB3838-2002)"(State Environmental Protection Administration of China 2002), of which the values of ammonia nitrogen exceeded the limit greatly, and shows that water in the three sites was heavily polluted. Grade V water corresponds to the water for agriculture and general landscape, without exposure to the human body. The quality values of water in excess of limit values of Standard V means that water is polluted.

The four sites, Muli gate, Huanxiangdian, Damatou and Xinfeng Zhuang are very different in water quality, from unpolluted to polluted, but alligatorweed was found in all sections of the upper Xiaoqing River, which indicates that the differences in water quality did not affect its distribution.

Parameters of Alligatorweed

The length of main branches of alligatorweed measured in the Peng Zhuang's population in the upper Xiaoqing River ranged from 198 cm to 382 cm, with an average value of 266.67 ± 24.01 cm. The number of nodes on the main

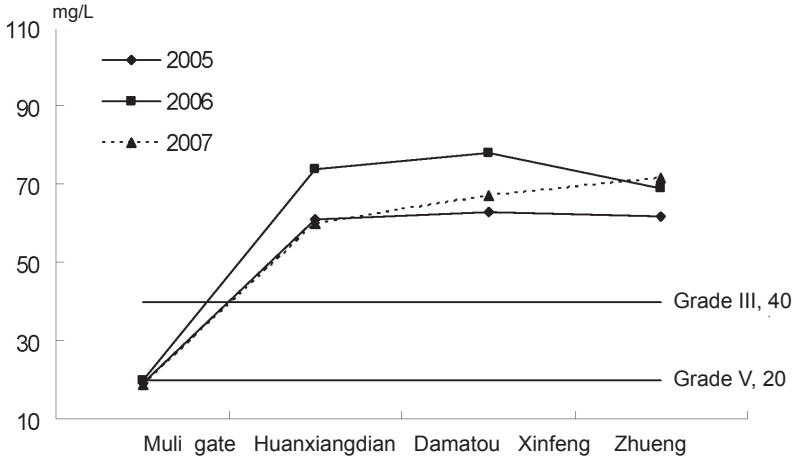

Figure 3. A: The values of chemical oxygen demand (CODcr) of the four monitoring sites of Muli gate, Huanxiangdian, Damatou and Xinfeng Zhuang in the upper Xiaoqing River. The limit values of Grade III and Grade V of "Environmental Quality Standard for Surface Water (GB3838-2002)" (State Environmental Protection Administration of China 2002) are also shown. B: A picture of alligatorweed at the main collection site at Peng Zhuang that near Muli gate growing in the clean waters of the upper Xiaoqing River.

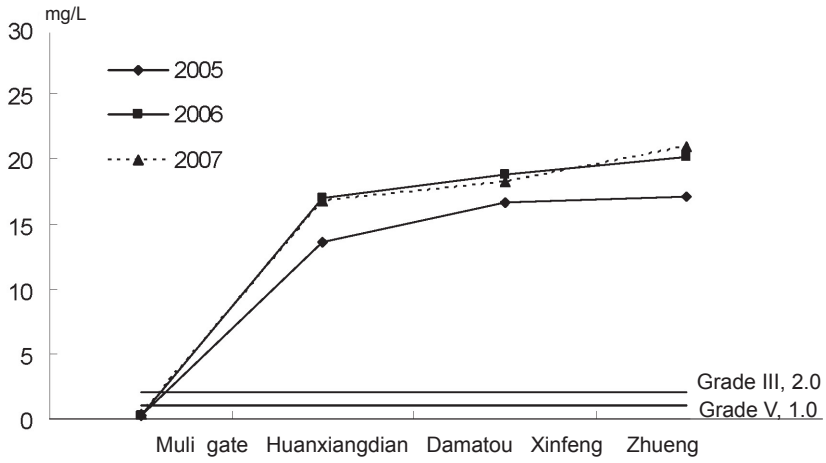

Figure 4. A: The values of ammonia nitrogen of the four monitoring sites of Muli gate, Huanxiangdian, Damatou and Xinfeng Zhuang in the upper Xiaoqing River. The limit values of Grade III and Grade V of "Environmental Quality Standard for Surface Water (GB3838-2002)" (State Environmental Protection Administration of China 2002) are also shown. B: A picture of alligatorweed nearly covering the Zhaowanghe a small flood discharge and irrigation waterway between the Xiaoqing River and the Yellow River in eastern Jinan. This site is between the Huanxiangdian and Damatou monitoring sites and is heavily polluted.

branches was 22-33 with an average of 27.01 ± 2.25. The number of adventitious roots per main branch was 13-25 with an average of 17.11 ± 0.84. The number of branches from each main branch ranged from 3 to 9 with an average of 5.71 ± 0.62, with most secondary branches having additional tertiary branches. The number of flowers in each main branch and its branches was 3-10 with an average of 6.52 ± 0.61.

The number of nodes, adventitious roots and secondary branches of the longest main branch was 30, 25 and 7, respectively. The longest secondary branch was 185 cm in length, and the number of its nodes, adventitious roots and tertiary branches was 20, 13 and 3, respectively. The number of main branches per linear meter ranged from 376-511, with an average of 436.52 ± 55.33.

Hydrochory is an important means of propagule transport for plants (Nisslon et al. 2010), and dispersal by fragmentation is particularly effective for aquatic plants (Riis and Sand-Jensen 2006). The stems of the alligatorweed are hollow, buoyant, and easily broken (Julien et al. 1992), which contributes to their dispersal ability and the invasiveness of this species in aquatic environments. The invasiveness of plants has a close relationship with their reproductive ability (Barret 1983), and alligatorweed can reproduce by asexual means and can grow into a new plant from a short stem (Lin and Qiang 2004).

The population of alligatorweed in the upper Xiaoqing River has a very high likelihood of invading the middle and lower Xiaoqing River, and also has a likelihood of invading other rivers, for example Yellow River, that have hydro connections with Xiaoqing River. The updates and revision for the previous distribution map, and certain basic characterization of alligatorweed stands in the upper Xiaoqing River will be helpful to understand and predict the status and spread of the alligatorweed population in northern China, and also helpful to make a reasonable management strategy for the weed in northern China.

Local Impacts

Alligatorweed was found to have invaded two of the five quadrants of vegetable land surveyed, however the infestations were not severe and the local farmers did not consider the weed to have damaged vegetable production or to be a problem weed. No alligatorweed was found in any of the rice production areas or in fish ponds in the upper Xiaoqing River. Indeed, without special introduction, most local farmers were not familiar with it.

The main impact of the weed in the upper Xiaoqing River is the restriction of flood flow in the rainy seasons. Since 2005, the management office of the Xiaoqing River of Jinan removes and controls this weed in waterways by mechanical methods, and the flood discharge is no longer a problem.

Although alligatorweed was not found to cause serious damage to agriculture in the upper Xiaoqing River, the losses are significant in south China. The results show that the weed reduces vegetable production by 5% to 15% on average, and may cause losses over 20% (Yin 1992). It can reduce yields in sweet potato and rice by 63% and 45% respectively (Liu and Huang 2002). Over 200 hectares of fish ponds were abandoned due to alligatorweed infestations in the neighboring suburbs of Chongqing city (Zhang et al. 1993).

The agriculture of Shandong plays an important role in China. The output of grain crops of the province account for 8.27% of the national total output, and rank second in China (Ministry of Agriculture of China 2007). Areas along the Yellow River and Xiaoqing River are the main regions for agriculture in Shandong. Presently, no obvious negative agricultural impact was found in this study, however the presence of well-established populations in the region warrant both weed control efforts and education of local populations to the potential agricultural impacts and weed control measures in Shandong, and other provinces of northern China.

ACKNOWLEDGMENTS

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