

ORIGINAL ARTICLE

The extent of hybridization and its impact on the genetic diversity and population structure of an invasive tree, *Ulmus pumila* (Ulmaceae)

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Abstract

Ulmus pumila is considered an invasive tree in 41 of the United States. In this study, we examined the extent of hybridization in naturalized populations of *U. pumila*, its impact on genetic diversity and genetic structure and its potential role in explaining the invasion process of *U. pumila*. Genetic analyses indicated widespread hybridization with native *Ulmus rubra* in naturalized *U. pumila* populations. Hybridization increased the genetic diversity of *U. pumila* populations and affected their genetic structure. The level of genetic diversity in 'mature' accessions, many of which may represent original plantings throughout the USA, was high and similar to the diversity of East Asian accessions. Hybridization with the native red elm may play an important role in the success of Siberian elm as an invader in temperate regions of the USA.

Introduction

Biological invasions by exotic species together with loss of wildlife habitat, pollution and land-use change all pose serious threats to biodiversity and increase the risk of species extinctions (Sakai et al. 2001; Ruiz and Carlton 2003; Mooney et al. 2005; Clout and Russell 2007). Invasive species also can have significant impacts on human health and our economic, cultural and ecological well being (Mack et al. 2000; Pimentel 2002; Colautti et al. 2006; Pfeiffer and Voeks 2008). Invasive and colonizing species have been the subjects of considerable interest for decades (Baker and Stebbins 1965; Cox 2004), especially concerning the role of natural selection in facilitating species adaptation to new environments (Allendorf and Lundquist 2003; Blair and Wolfe 2004; Holt et al. 2005). More recently, attention has been given to the role of genetic and evolutionary processes as key features in determining whether and how invasive species establish and spread (Sakai et al.

2001; Huey et al. 2005; Sax et al. 2005; Wolfe et al. 2007; Kanarek and Webb 2010; Gomulkiewicz et al. 2010).

Levels of genetic diversity can affect the potential for spread of an invasive species (Sakai et al. 2001). High levels of genetic diversity in the early process of invasion may result from a single introduction of a large number of individuals or from multiple introductions from genetically divergent source populations (Sakai et al. 2001; Allendorf and Lundquist 2003). By introducing new genes into an invasive species, increasing the level of heterozygosity or adding new gene combinations, hybridization may overcome the low levels of genetic diversity associated with the introduction of a small number of individuals (Londo and Schaal 2007; Moody and Les 2007; Wolfe et al. 2007), facilitate the adaptation process and further serve as a stimulus for the evolution of invasiveness (Ellstrand and Schierenbeck 2000; Vila et al. 2000; Hedge et al. 2006).

Ulmus pumila L. (Siberian elm), native to East Asia, was introduced into the United States on at least three

separate occasions in the early 1900s (Leopold 1980) with subsequent widespread planting, especially on the Great Plains, to serve as windbreaks and shelterbelts (Webb 1948; Ward 1985). *Ulmus pumila* is highly tolerant of Dutch elm disease (DED) and has been used as a source of disease resistance genes in many elm breeding programs (Smalley and Guries 2000). Currently, *U. pumila* is considered an invasive tree in 41 of the United States (USDA, NRCS 2002; Ding et al. 2006; Zalapa et al. 2009), with naturalized and/or invasive populations also reported in Canada (Brouillet et al. 2006), Mexico (Todzia and Panero 1998), Argentina (Zalba and Villamil 2002) and Spain (Cogolludo-Agustin et al. 2000). *Ulmus pumila* has become one of the top ten new American invasive plant species of Asian origin and one of the most problematic woody species alongside Russian olive and tamarisk (USDA, NRCS 2002; Ding et al. 2006).

Little is known about the genetic factors that have allowed *U. pumila* to prosper in so many different habitats and to become an invasive species. In its native Asia, *U. pumila* occurs in semi-arid steppe habitats of China, Mongolia and parts of the present-day Russian Federation (Dulamsuren et al. 2009). In the USA, *U. pumila* is now common in the semi-arid western states while naturalized populations in the eastern and mid-western USA occur across a wide range of environments (USDA, NRCS 2002). The range of *U. pumila* in the eastern and mid-western USA is more or less coincident with the range of the native, DED-susceptible red elm, *Ulmus rubra*. Hybridization with *U. rubra* could have provided a stimulus for the evolution of invasiveness of *U. pumila* at least in some parts of the USA, possibly by increasing the genetic diversity and adaptability of naturalized *U. pumila* populations.

We have recently reported natural hybridization between *U. pumila* and *U. rubra* and the occurrence of an asymmetric pattern of introgression toward *U. pumila* in Wisconsin (Zalapa et al. 2009). While this former study examined populations with morphologically identifiable hybrids between these two species, in this study, we collected from naturalized *U. pumila* populations that contained morphologically typical *U. pumila* individuals. Our goal was to describe the population structure of naturalized *U. pumila* populations, but the unexpectedly high level of hybridization found in these populations permitted us to further quantify the extent of hybridization in *U. pumila* populations and its impact on the genetic diversity and genetic structure of these populations. In addition to collecting morphologically typical *U. pumila* individuals from populations in Wisconsin, Illinois and South Dakota, we also obtained leaf samples from mature trees growing in towns and villages across 28 states in the USA (hereafter referred to as 'mature' trees although their origins are uncertain) to provide a sample of the genetic diversity

representative of that contained in the various but poorly documented original introductions in the USA.

Our first objective in this study was to quantify the extent of hybridization in naturalized morphologically typical *U. pumila* populations and to examine if hybridization could also be detected in the earlier US introductions ('mature' trees). Our second objective was to examine the impact of hybrids on the genetic diversity and genetic structure of naturalized morphologically typical *U. pumila* populations. Our final objective was to estimate the level of genetic diversity in the earlier US introductions ('mature' trees) and compare this to the level of genetic diversity of *U. pumila* samples from East Asia (maintained in the UW elm arboretum near Madison, WI). Understanding the extent of hybridization in US populations and its impact on the genetic diversity and genetic structure of *U. pumila* populations may shed light on the potential role played by hybridization in the invasion process of *U. pumila* in the USA.

Materials and methods

Plant material

To establish a genetic profile of *U. pumila* in its native range, 53 accessions were sampled from a collection maintained at the University of Wisconsin-Madison elm arboretum (Columbia County, WI) established from seed collected in 10 provinces of the People's Republic of China (PRC) between 1981 and 1990. These accessions were previously used to characterize the genetic diversity of *U. pumila* (Zalapa et al. 2008), but in this study, we increased sampling to include 33 more accessions of known origin: 19 from the PRC; 9 from the former USSR; one from Korea; and four accessions provided by Dr George Ware of the Morton Arboretum, Lisle, IL (Table 1).

To examine the extent of genetic diversity in early US introductions of *U. pumila* and compare it with that of our arboretum accessions of East Asian origin, leaf samples were collected from 37 mature, morphologically typical *U. pumila* trees from throughout the USA (PUS; Table 1). To ensure that samples were 'typical' *U. pumila*, we asked collectors to discriminate between *U. rubra* and *U. pumila* using the criteria of Rehder (1940) and Wyman (1951) (Table 2).

In addition, we sampled 20 'mature' *U. pumila* trees in southern Wisconsin, and we used an additional 52 herbarium specimens collected throughout Wisconsin between 1948 and 2001 (PWI; Table 1).

To examine the genetic diversity and genetic structure of naturalized morphologically typical *U. pumila* populations, we collected leaf samples from 12 to 30 individuals in each of eight naturalized *U. pumila* populations located in Wisconsin, South Dakota and Illinois (Table 3). Two

Table 1. Locations and sample sizes of *Ulmus pumila* mature accessions collected in the United States (PUS and PWI) and of *U. pumila* (PEA) and *Ulmus rubra* (RU) reference accessions.

PUS	<i>U. pumila</i> – United States mature accessions (KY, KS, OR, CO, AZ, IA, OK, OH, PA, UT, TX, DE, GA, NJ, MN, AK, IL, IN, VA, WA, TN, SD, MO, MA, NV, LA, NY, MD)	37
PWI	<i>U. pumila</i> – Wisconsin mature accessions (state-wide collections from 30 counties; 20 living trees + 52 UW-Herbarium specimens)	72
PEA	<i>U. pumila</i> – Accessions from East Asia: 72 China (15 Henan, 13 Shanxi, 10 Hebei, 7 Xinjiang, 6 Hubei, 5 Beijing, 5 Heilongjiang, 3 Ganzu, 3 Shandong, 2 Liaoning, 2 Guizhou, and 1 Shaanxi), 9 Russia, 1 Korea, and 4 Morton Arboretum	86
RU	<i>U. rubra</i> – UW-Herbarium historic specimens collected 1890–1965. 20 specimens collected before 1960 (UW-Herbarium, Madison, WI)	25

Table 2. Diagnostic leaf, twig and fruit characters for discriminating between *Ulmus rubra* and *Ulmus pumila* (after Rehder 1940 and Wyman 1951).

Traits	<i>U. rubra</i>	<i>U. pumila</i>
Leaves	Obovate-oblong, 10–20 cm long; doubly serrate margin; rough above, densely pubescent beneath; petioles 0.4–0.8 cm long	Elliptic-lanceolate, 2–7 cm long; simply serrate margin; smooth above, glabrous beneath; petioles 0.2–0.4 cm long
Buds	Large, rust-brown pubescent	Small, black glabrous
Twigs	Pubescent and scabrous; red-brown to orange	Pubescent while young; slender, grayish or gray-brown
Seed	Broadly elliptic, 1–2 cm long, slightly notched; pubescent in center	Sub-orbicular, 1–1.5 cm long, closed notch; seed slightly above middle

Table 3. Location and number of sample collected from putative *Ulmus pumila* naturalized populations in the United States.

Populations	Location	Latitude and longitude	Sample size
PSD	Conata, SD	Lat. 43.44°N, Long. 102.12°W	12
PIL	Marengo, IL	Lat. 42.09°N, Long. 89.01°W	20
PCW	Madison, WI	Lat. 43.07°N, Long. 89.21°W	23
PJN	Janesville, WI	Lat. 42.41°N, Long. 88.58°W	27
PCG	Cottage Grove, WI	Lat. 43.02°N, Long. 89.12°W	23
PMM	Fitchburg, WI	Lat. 42.57°N, Long. 89.22°W	30
PSL*	Wisconsin Dells, WI	Lat. 43.32°N, Long. 89.46°W	19
PAB*	Blooming Grove, WI	Lat. 43.04°N, Long. 89.15°W	17

*Individuals previously used in Zalapa et al. (2009) including, PSL = 12 *U. pumila* and 7 hybrids, PAB = 11 *U. pumila* and 6 hybrids.

of these naturalized populations had been described previously (Shady Lane or PSL and Blooming Grove or PAB; Zalapa et al. 2009). Twenty-five *U. rubra* historic herbarium specimens (RU; 1890–1965) previously described in Zalapa et al. (2009) were also included to permit identification of hybrids between *U. pumila* and *U. rubra* in our samples (RU; Table 1).

DNA isolation and PCR

Thirteen microsatellite loci were used in this study: UR123, UR141, UR153, UR158, UR159, UR173a, UR175, UR188a, Ulmi1-98, Ulmi1-165, ULM2, and ULM3 (Zalapa et al. 2008). Nine of these loci have species-specific alleles that permit identification of hybrids between *U. pumila* and *U. rubra* (Fig. S1) (Zalapa et al. 2009). Total genomic DNA was isolated from approximately

0.5 cm² of leaf tissue using a DNeasy kit (QIAGEN, Valencia, CA, USA). PCRs were performed in 15 µL total volume using 1.5 µL 10× PCR buffer, 1.8 µL 25 mM MgCl₂, 2.4 µL dNTPs (1.25 mM of each dATP, dGTP, dTTP, and dCTP), 1.0 µL 5 µM primer, 2 µL 10 ng/µL genomic DNA, 1 U *Taq* DNA polymerase (Lucigen, Mid-dleton, WI, USA), and 6.2 µL H₂O. Thermocycling conditions consisted of an initial melting step (94°C for 3 min), followed by 30 cycles of 94°C for 15 s, 55°C/60°C for 90 s, and 72°C for 2 min, and a final elongation step (72°C for 20 min), followed by an indefinite soak at 4°C. For herbarium samples, 1% PVP and 1% BSA were added to the PCR protocol. Microsatellite allele genotyping using fluorescent labeled primers (5' end 6-FAM fluorophore; IDT, Inc., Coralville, IA, USA) was performed at the University of Wisconsin Biotechnology Center DNA Sequence Facility using an ABI 3730 fluorescent sequencer

(POP-6 and a 50-cm array; Applied Biosystems, Foster City, CA, USA) and a Gensize Rox 650 ladder (GENPAK Ltd., Brighton, UK). Alleles were scored using GeneMarker Software version 1.5 (SoftGenetics, State College, PA, USA). PCRs were repeated on approximately 12% of the samples and we obtained 99% accuracy.

Identification of *U. pumila* and hybrid individuals

To detect hybrid individuals between *U. pumila* and *U. rubra* in both the 'mature' material (PUS and PWI) and eight naturalized populations, we used 12 markers with 66 *U. rubra* and 42 *U. pumila* species-specific alleles (no overlapping allele sizes between species) obtained from 125 *U. rubra* individuals collected in wild populations and 86 *U. pumila* accessions from the east Asian collection maintained at the University of Wisconsin-Madison elm arboretum (Zalapa et al. 2009 and therein). Individuals possessing 100% species-specific alleles for either *U. rubra* or *U. pumila* were considered members of that respective species. A first-generation hybrid (F_1) was identified as one heterozygous for species-specific alleles at all loci; a backcross individual (BC) had at least one locus fixed for species-specific alleles for one of the two parental species, while all other loci were heterozygous for the species-specific alleles. As an approximation, we classified individuals with over 50% and below 85% of specific alleles for a single species (expected mean 75%) as first-generation backcross (BC_1) and individuals with between 88% and 96% as second-generation backcross (BC_2) (expected mean 87.5%).

We used three other methods to confirm our manual classification of hybrid individuals: the Bayesian clustering algorithms available in the program STRUCTURE (v. 2.2) (Pritchard et al. 2000), the Bayesian algorithms available in the program NewHybrids v. 1.1 beta (Anderson and Thompson 2002), and principal coordinates analyses (PCoA). We performed a PCoA for the genetic profile of each individual using GeneALEX 6.0 (Peakall and Smouse 2006). As the Bayesian methods and the PCoA utilized both species-specific and shared alleles at each locus to detect hybrids, small discrepancies among such methods and our manual classification using species-specific alleles are expected (Vaha and Primmer 2006). The 25 herbarium specimens of *U. rubra* (RU; Table 1) and the 86 accessions of *U. pumila* (PEA; Table 1) were used as reference populations (pure parental species) in these analyses.

The program STRUCTURE (Pritchard et al. 2000) calculates an admixture coefficient (q) for each genotyped individual, where q represents the proportion of an individual's genotype that originates from each reference population. Therefore, while q -values for the two parental species are expected to be close to 1, first-generation

hybrids (F_1) are expected to have q -values of 0.5. An *a priori* value of $K = 2$ accounted for the two parental species, when we used the genetic admixture analysis, and correlated allele frequencies model of the program STRUCTURE. We used eight genotypic classes when running the program NewHybrids: the two parental species, F_1 and F_2 generations, and first- and second-generation backcrosses to each of the parent. We performed several iterations for the Bayesian analyses to ensure that independent chains converged to the same values. Finally, PCoA clustering was conducted based on three groups (*U. rubra*, *U. pumila*, or hybrids) predefined based on the manual classification of hybrids discussed earlier.

Genetic diversity

Genetic diversity measures were estimated within the East Asia (PEA) accessions, within the US and WI accessions (Table 1), and for the eight naturalized populations in the USA (Table 3). Genetic diversity was examined for all individuals before excluding the hybrids (only pure *U. pumila* individuals included). The observed (N_a) and effective (N_e) number of alleles, number of alleles with frequency >0.05 ($N_{a \text{ Freq.}} > 0.05$), number of private alleles ($N_{a \text{ private}}$), levels of observed (H_O) and expected (H_E) heterozygosity, Shannon's information Index (I), and fixation index (F) were calculated for each locus using GeneALEX 6 (Peakall and Smouse 2006). Within each population, we examined the contribution of the hybrids to estimates of genetic diversity. In order to approximate the likely levels of genetic diversity in the early introductions of *U. pumila* relative to the genetic diversity in the Asian populations of origin, we compared the genetic diversity found in the 'mature' and herbarium materials (PUS and PWI) to that of the accessions recently collected in East Asia (PEA).

Population structure

The PEA, PUS and PWI populations were contrasted in order to determine levels of genetic differentiation between East Asia and the USA (Table 1). We separately examined the genetic structure of the eight naturalized *U. pumila* populations (Table 3). To determine the contribution of hybrid individuals to the genetic structure of *U. pumila* populations, we first examined the degree of genetic differentiation among populations using all individuals before considering only the typical *U. pumila* individuals (excluding hybrids). The hybrid individuals were identified using the manual classification method described above. An analysis of molecular variance (AMOVA) determined how observed genetic diversity was partitioned within and among populations (Excoffier

et al. 1992). The degree of genetic differentiation among populations was also estimated using F_{ST} , calculated over all populations in a group and then between all pairs of populations using GeneAEx 6 (Peakall and Smouse 2006). Because the F_{ST} and AMOVA procedures assume a hierarchical level of organization (Dyer and Nason 2004), we also used a Bayesian clustering method available in the program STRUCTURE (v. 2.2) (Pritchard et al. 2000) to infer whether there were clear genetic discontinuities in multilocus genotype data independent of the populations from which the individuals were sampled. We ran STRUCTURE using 10^6 Markov chain Monte Carlo iterations with 50 000 burn-in iterations and 10 replicates per run. We used the 'admixture model' in which each individual draws a fraction of its genome from each of K subpopulations, and the case of 'no prior population information'. The most likely true value of K was estimated using Bayes' rule as specified in Pritchard et al. (2000) and the ΔK method proposed by Evanno et al. (2005). We graphically represented the admixture coefficients for each individual in each population before combining all individual assignments for each population.

Results

Identification of *U. pumila* genotypes and hybrid individuals

The addition of 33 accessions to our previously characterized 53 East Asian *U. pumila* accessions (PEA; Table 1;

Zalapa et al. 2008) resulted in the detection of 10 new alleles (frequencies of $\geq 2\%$) for a total of 81 alleles in our *U. pumila* reference population. We detected only three new alleles with the set of nine primers used by Zalapa et al. (2009).

Using manual classification, we identified 220 pure *U. pumila* individuals and 60 hybrids between *U. pumila* and *U. rubra* among all the samples collected for this study (Fig. 1A). Thirty-two of the 60 hybrids were classified as F_1 hybrids and 28 as backcrosses based on their genetic profiles (Fig. 1A). All backcrosses indicated introgression toward *U. pumila* with 17 of them identified as BC_2 . Interestingly, hybrid individuals were observed in 14% of the 'mature' trees (PUS and part of PWI) and 15% of the samples from the herbarium collection (part of PWI). The majority of these hybrids were classified as advanced backcrosses (BC_2) (Fig. 1A). In addition, we estimated that most of the naturalized populations contained a large proportion of hybrids (4–53%; Fig. 1A). Although a few BC_2 individuals were detected in the South Dakota population and some BC_1 individuals were identified in four of the naturalized populations, the great majority of hybrids identified represented F_1 individuals. We did not detect any genotypically pure *U. rubra* or backcross introgressions toward *U. rubra* in any of the naturalized populations. The absence of *U. rubra* among our samples is not surprising given that we collected individuals that appeared morphologically typical of *U. pumila*. In addition, DED eliminates most natural

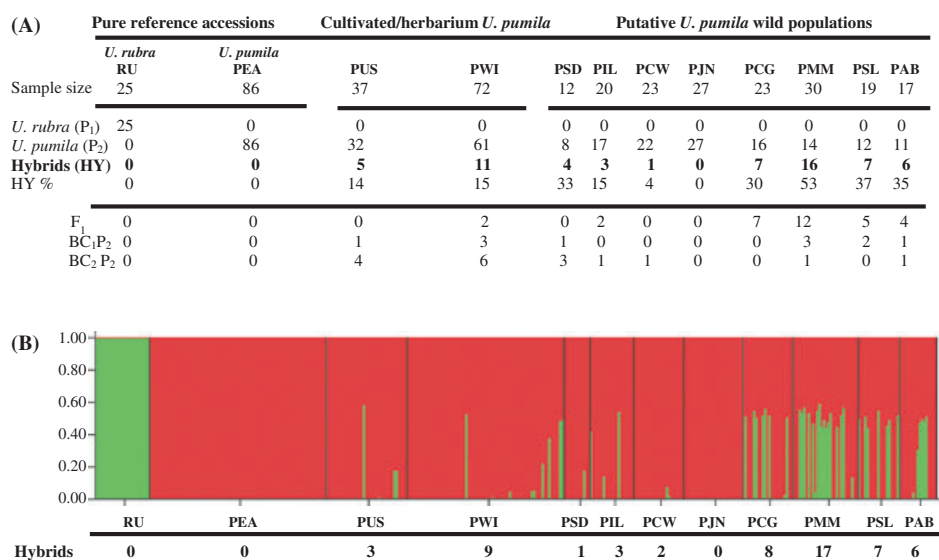


Figure 1 Classification of putative *Ulmus pumila* individuals collected from throughout the United States. (Panel A) Identification using species-specific alleles in 13 microsatellite markers. (Panel B) Taxon designations tested by Bayesian admixture ($K = 2$; Panel B). In the STRUCTURE plots each individual is represented by a thin vertical line divided into $K = 2$ colored segments that represent the individual's estimated membership fractions in these two clusters. Black lines separate individuals from different populations.

U. rubra trees at an early age, but hybrids with *U. pumila* are often highly tolerant and survive infection (Lester and Smalley 1972).

Our different methods of hybrid classification yielded slightly different (but expected) results because the Bayesian methods consider gene frequencies at both species-specific alleles and shared alleles at each locus to detect hybrids while only species-specific alleles are used in the manual classification (Vaha and Primmer 2006). Compared to the 220 *U. pumila* and 60 hybrids identified using the manual classification, the program STRUCTURE identified 225 *U. pumila* individuals and 55 hybrids (Fig. 1B), classified eight advanced backcrosses as *U. pumila* (Fig. 1B), and detected three additional hybrid individuals, although such individuals possessed only *U. pumila* specific alleles. The PCoA analyses confirmed the presence of various hybrids although assignments were not as precise as the manual classification in identifying hybrid types (i.e. F₁ versus backcrosses; Fig. 2A). The PCoA analysis suggested an introgression pattern toward *U. pumila* with various levels of intro-

gression. Manual classification yielded slightly different hybrid assignments than did the program NewHybrids, which identified 34 F₁ individuals (as opposed to 32 F₁ individuals determined manually), six as BC₁ (instead of 11 manually) and 11 BC₂ (instead of 17 manually) (Fig. 2B). NewHybrids determined that two BC₁ were F₁ hybrids, three BC₁ were classified as BC₂, and nine BC₂ were assigned to pure *U. pumila* (Fig. 2B).

Genetic diversity

Across all US collections and including the hybrids ($n = 280$; PUS, PWI, and eight naturalized populations), we detected 152 alleles, with 42 alleles specific to *U. pumila*; 66 specific to *U. rubra*, 29 alleles shared between *U. rubra* and *U. pumila* and 15 new alleles (Appendix S1). Less than half as many alleles were detected (78 alleles) when hybrids were excluded from the analysis ($n = 220$) (40 *U. pumila* alleles, 28 shared, and 10 new). Clearly, hybridization increased the level of

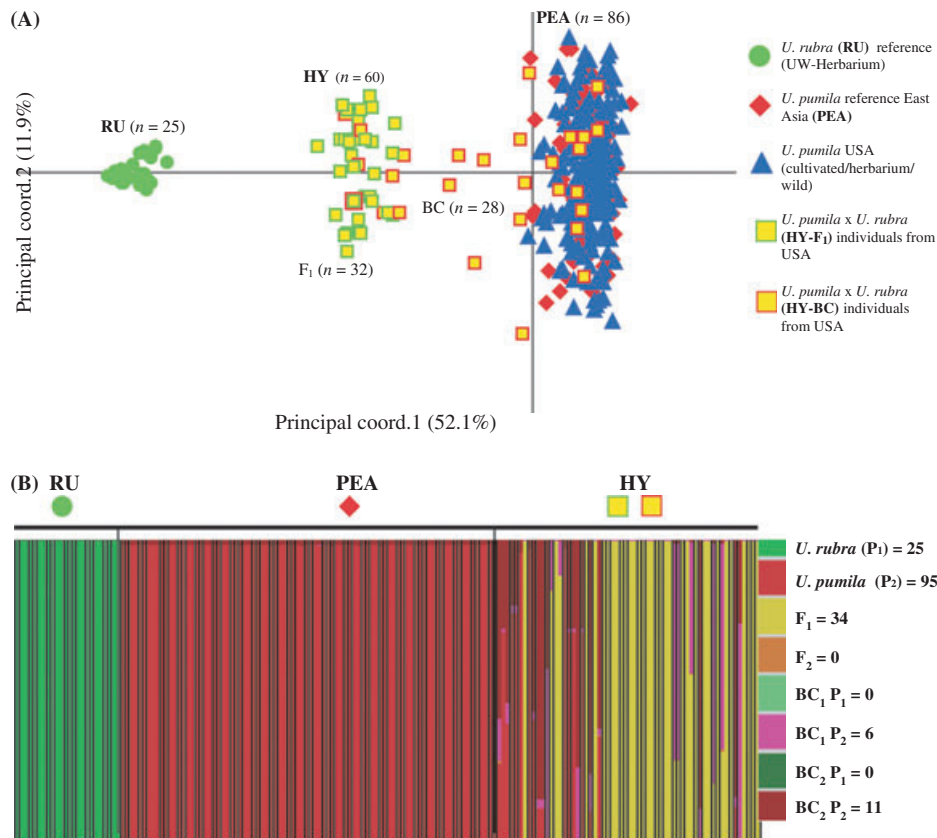


Figure 2 Principal coordinates analyses (P. coord. 1 and 2; Panel A) of pure *Ulmus pumila* and hybrids (*U. pumila* × *Ulmus rubra*) individuals identified in the United States using species-specific alleles in 13 microsatellite markers (Panel A) and taxon designations tested by Bayesian NewHybrids (Panel B). In the NewHybrids plots, each individual is represented by a thin vertical line divided into eight colored segments that represent the individual's estimated membership fractions to each of the eight cross types.

genetic diversity in present-day US populations because 143 alleles were identified in the 60 hybrid individuals (39 *U. pumila* alleles, 66 *U. rubra*, 29 shared and 9 new). The alleles not previously identified in either species may result from limited sampling of parental species or represent alleles that originated in the sampled populations via mutation.

The level of genetic diversity in the naturalized *U. pumila* populations was substantial (Table 4). Excluding hybrids, we observed between three and four alleles per locus per population and moderate levels of observed heterozygosity (range of 0.34–0.44 over all eight naturalized populations) (Table 4). Little inbreeding was detected in adult trees in these naturalized populations and the slightly negative inbreeding coefficients in several of these populations suggest an excess of heterozygotes in these populations (*F*-values; Table 4). The presence of hybrids clearly increased the genetic diversity of naturalized populations, and the increase in the number of alleles and effective alleles per population was related to the proportion of hybrids detected in a population (Table 4; Fig. 1A). The presence of hybrids also increased the levels of heterozygosity and reduced estimates of the inbreeding coefficient (more negative *F*-values; Table 4). Such trends are expected given that many of the trees in these naturalized populations are *F*₁ hybrids and are thus heterozygous at all species-specific loci.

In order to compare the genetic diversity that may have existed at the time *U. pumila* was introduced into the USA, we compared the genetic diversity of the PEA population (East Asia) to the PUS and PWI populations (US) (Table 1). When hybrids were excluded, the genetic diversity of the East Asian accessions (PEA) was similar (e.g. *I* = 0.9 vs 0.9) to the level of diversity characteristic of contemporary US populations (PUS and PWI) (Table 4). We observed slightly more alleles (*N*_a) in PEA but similar effective numbers of alleles (*N*_e), levels of observed (*H*_O) and expected heterozygosity (*H*_E) and fixation indices (*F*) (Table 4). Hybrids increased the genetic diversity of the US populations and rendered the US populations slightly more diverse relative to the East Asian accessions. *Ulmus pumila* from both East Asia and the USA appear genetically variable and heterozygous with little evidence of inbreeding (Table 4).

Population structure

The AMOVA for the eight naturalized populations indicated that most of the genetic diversity occurred within populations and this was true whether hybrids were included or not in the analyses: 93% within and 7% among populations when hybrids were included and 94%

Table 4. Genetic diversity characteristics of *Ulmus pumila* populations (not bold: including hybrids; bold: excluding hybrids) based on 13 microsatellite loci.

Parameter	US accessions				Wild populations							
	PEA	PUS	PWI	PSD	PIL	PCW	PJN	PCG	PMM	PSL	PAB	
Hybrid %*	0	14	15	33	15	4	0	30	53	37	35	
Sample size	86 (86)	37 (32)	72 (61)	12 (8)	20 (17)	23 (22)	27 (27)	23 (16)	30 (14)	19 (12)	17 (11)	
No. alleles	81 (81)	75 (63)	90 (70)	51 (42)	63 (47)	52 (49)	55 (55)	68 (39)	103 (48)	72 (41)	75 (47)	
No. private alleles	10 (13)	7 (1)	3 (2)	0 (0)	0 (0)	1 (2)	1 (2)	4 (0)	14 (1)	5 (0)	0 (0)	
<i>N</i> _a	6.23 (6.23)	5.77 (4.85)	6.92 (5.38)	3.92 (3.23)	4.85 (3.62)	4.00 (3.77)	4.23 (4.23)	5.23 (3.00)	7.92 (3.69)	5.54 (3.15)	5.77 (3.62)	
<i>N</i> _a Freq > 5%	3.08 (3.08)	3.46 (3.08)	3.15 (3.08)	3.08 (3.23)	3.54 (2.85)	2.46 (2.46)	2.62 (2.62)	3.15 (2.54)	4.23 (3.15)	3.77 (2.62)	4.08 (2.62)	
<i>N</i> _e	2.62 (2.62)	2.81 (2.67)	2.83 (2.66)	2.44 (2.32)	2.38 (2.25)	2.02 (2.00)	2.53 (2.53)	2.50 (2.08)	3.52 (2.47)	2.93 (2.25)	3.18 (2.50)	
<i>I</i>	0.90 (0.90)	1.00 (0.90)	1.02 (0.90)	0.89 (0.78)	0.90 (0.74)	0.73 (0.71)	0.80 (0.80)	1.03 (0.66)	1.39 (0.82)	1.13 (0.70)	1.17 (0.81)	
<i>H</i> _O	0.37 (0.37)	0.41 (0.40)	0.43 (0.39)	0.47 (0.43)	0.43 (0.38)	0.35 (0.34)	0.36 (0.36)	0.51 (0.35)	0.70 (0.44)	0.58 (0.37)	0.58 (0.44)	
<i>H</i> _E	0.40 (0.40)	0.45 (0.42)	0.44 (0.41)	0.44 (0.40)	0.43 (0.37)	0.36 (0.36)	0.39 (0.39)	0.51 (0.36)	0.62 (0.41)	0.54 (0.36)	0.54 (0.40)	
<i>F</i>	0.08 (0.08)	0.10 (0.01)	0 (0.01)	-0.04 (-0.02)	-0.03 (-0.03)	0.08 (0.09)	0.03 (0.03)	-0.05 (0)	-0.14 (-0.06)	-0.11 (-0.03)	-0.09 (-0.06)	

*Pure *U. pumila* individuals in each population were inferred using species-specific markers following Zalapa et al. (2009).

Multilocus averages: *N*_a, observed number of alleles; *N*_e Freq. > 5%, number of alleles with frequency > 0.05; *N*_a private, number of private alleles; *N*_e, effective number of alleles; *I*, Shannon index of diversity; *H*_O, observed heterozygosity; *H*_E, expected heterozygosity; *F*, fixation index.

Table 5. Analysis of molecular variance for eight naturalized *Ulmus pumila* populations (estimates not bold) and for a subset of these samples determined to be genotypically pure individuals within each population (in bold).

Source of variance	d.f.	MS	Variance components	Total variance (%)	Stat.	Value
Among populations	7*	13.2	0.24	7	F_{ST}	0.07
	7	8.03	0.17	6		0.06
Within populations	334	6.46	3.23	93		
	246	5.12	2.56	94		
Total	731	18.51	3.19			
	611	13.03	2.76			

*Pure *U. pumila* individuals in each population were inferred using species-specific primers following Zalapa et al. (2009).

within and 6% among when hybrids were excluded (Table 5). Similar results were obtained across all US collections: 95% of the genetic variation within and 5% among when hybrids were included and 97% within and 3% among in the absence of hybrids.

The overall F_{ST} -value for the eight naturalized populations was 0.07 with hybrids, 0.06 when excluding hybrids, and 0.05 across all US collections with hybrids (0.03 when hybrids were excluded). The level of genetic differentiation between the Asia (PEA) and US accessions was low (pairwise F_{ST} for PEA–PUS = 0.023 and for PEA–PWI = 0.013) and became even lower when hybrids were excluded (pairwise F_{ST} for PEA–PUS = 0.017 and for PEA–PWI = 0.011). There was little genetic differentiation between the *U. pumila* individuals collected throughout the USA (PUS) and those collected in Wisconsin (PWI) (pairwise F_{ST} for PUS–PWI = 0.003) including hybrids (0.002 when hybrids were excluded). The greatest levels of genetic differentiation were found among some

of the naturalized populations, although only moderate levels of genetic differentiation were observed (pairwise F_{ST} -range 0.024–0.149 in the absence of hybrids). When hybrids were included in the analyses, the level of genetic differentiation increased for 13 of the pairwise cases, decreased for eight cases and remained unchanged for the other seven (Table 6). Overall, the PCG, PMM and PJN populations were the most differentiated while PAB and PSD were the least differentiated from all others, and this pattern did not change when hybrids were included in the calculations (Table 6).

When only the PEA, PUS and PWI populations were considered, STRUCTURE identified a single genetic cluster ($K = 1$) which confirmed the genetic similarities between the East Asian accessions and the US populations and between the samples collected throughout the USA and those restricted to Wisconsin. When we contrasted only the eight naturalized US *U. pumila* populations with the hybrids included in the analysis, STRUCTURE identified as many genetic clusters as there were populations ($K = 8$; Fig. 3A); with hybrids excluded, four genetic clusters were inferred (Fig. 3B). Significant admixture levels were observed between populations as each population and individuals within each population were comprised of these four genetic clusters. A single cluster made up more than half of the composition of the PCG, PMM and PJN populations although a different cluster predominated in each one of these three populations (Fig. 3B).

Discussion

Our sampling strategy in the USA targeted morphologically typical *U. pumila* individuals, but our genetic method identified a surprisingly large number of *U. pumila* × *U. rubra* hybrid individuals and even classified some individuals from the ‘mature’ accessions as

Table 6. Pairwise genetic differentiation (F_{ST}) among *Ulmus pumila* naturalized populations including hybrids (upper diagonal) and excluding hybrids (below diagonal).

Population	PSD	PIL	PCW	PJN	PCG	PMM	PSL	PAB	Mean F_{ST} all	Mean F_{ST}
PSD		0.048*	0.027	0.027	0.072	0.069	0.042	0.029	0.045	0.033
PIL	0.04		0.070	0.047	0.118	0.068	0.062	0.025	0.063	0.062
PCW	0.011	0.075		0.056	0.089	0.099	0.061	0.048	0.064	0.051
PJN	0.016	0.041	0.058		0.129	0.112	0.071	0.055	0.071	0.067
PCG	0.073	0.149	0.088	0.128		0.08	0.048	0.056	0.085	0.093
PMM	0.042	0.051	0.055	0.07	0.127		0.051	0.034	0.073	0.065
PSL	0.027	0.053	0.033	0.054	0.075	0.072		0.009	0.049	0.046
PAB	0.019	0.024	0.037	0.047	0.068	0.041	0.008		0.037	0.035

Mean F_{ST} all represent average pairwise F_{ST} -values for each population including hybrids. The Mean F_{ST} -value excludes hybrids. The non-mean F_{ST} -values in bold are significantly different from 0.

*Pure *U. pumila* individuals within each population were inferred using species-specific primers following Zalapa et al. (2009). Test of genetic differentiation using AMOVA ($P < 0.05$ in bold).

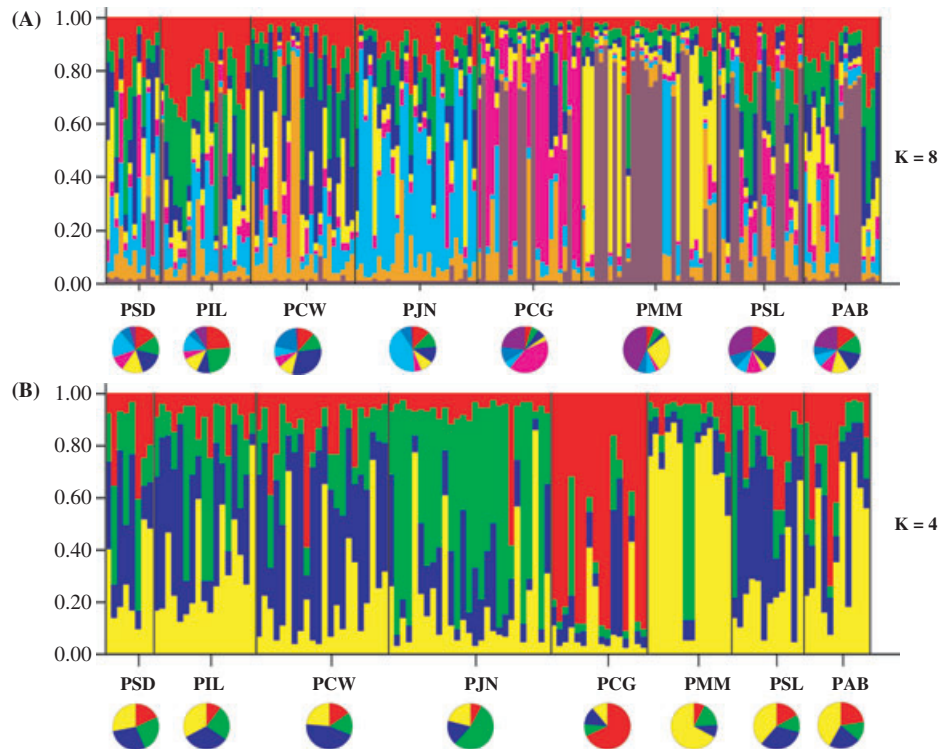


Figure 3 STRUCTURE analysis of eight naturalized *Ulmus pumila* populations (Panel A = hybrids included and Panel B = hybrids excluded) in the United States using 13 microsatellite loci. In the STRUCTURE plots each individual is represented by a thin vertical line divided into K -colored segments that represent the individual's estimated membership fractions in each of the K clusters. Black lines separate populations (labeled at the bottom). The pie graph for each population represents the overall relative contribution of each of the K clusters to that population (Panel A, $K = 8$ and Panel B, $K = 4$).

hybrids. The origins of such 'mature' trees are unknown but at least some were deliberately planted while others likely represent 'volunteers' that freely established in disturbed landscapes. Like many successful invasive woody plants, *U. pumila* is precocious and can produce seed in as few as 10 years (Lester and Smalley 1972; Ware 1995; Smalley and Guries 2000). Our finding of backcross individuals among the 'mature' trees suggests that hybridization between *U. rubra* and *U. pumila* may have begun soon after the first *U. pumila* introductions a century ago and continued on a more widespread scale.

We did not expect that most naturalized populations sampled would contain hybrids (seven of eight) or that a sizeable proportion of hybrid individuals would occur within each population (4–53%) although we previously had suspected the existence of natural hybrids in some of these populations. Our data indicate widespread hybridization between *U. pumila* and *U. rubra* across diverse landscapes, at least in Wisconsin. It also confirms that leaf morphology is an unreliable indicator of pure or hybrid individuals in these elms (Fig. S2). The majority of hybrids in naturalized populations were F_1 progeny and not backcrosses, suggesting recurring hybridization

between *U. pumila* and *U. rubra* in the wild. The presence of some first- (BC_1) and second-generation (BC_2) backcrosses toward *U. pumila* further supported that hybridization between the two elm species has, in fact, occurred over several generations, probably beginning in the 1930s when *U. pumila* was first widely planted in the USA (Ware 1995; Smalley and Guries 2000). Additional later-generation backcross progeny may exist given the time period involved since initial introduction, but more markers would be needed to evaluate this possibility. We did not observe any backcrossing toward *U. rubra* but this may not be surprising as we biased our collection toward morphologically typical *U. pumila* trees. The observed introgression biased toward one of the parental species, *U. pumila* in this case, is a pattern that we have previously documented (Zalapa et al. 2009), and which has been observed in other plant species, including trees (Keim et al. 1989; Bacilieri et al. 1996).

Naturalized *U. pumila* populations were quite genetically diverse, with high levels of heterozygosity and low levels of inbreeding. Although the number of alleles per locus was lower than in the East Asian accessions, at least when hybrids were excluded, sample sizes per population

were also smaller. The relatively high heterozygosity and low levels of inbreeding reflect the fact that most elm species are self-incompatible (Santini et al. 2008). The excess heterozygosity in some of the naturalized populations could occur if inbred individuals do not survive to adulthood as has been found in other plant species (Herlihy and Eckert 2002). Although we observed significant levels of admixture within our naturalized US populations, there were also significant levels of differentiation among some of these populations.

Hybrids contributed 66 *U. rubra* alleles overall to the *U. pumila* populations. The larger number of alleles specific to *U. rubra* relative to *U. pumila* (66 vs 42) observed in this study was expected as our previous study found more ($\sim 2\times$) alleles in *U. rubra* relative to *U. pumila* populations using the same set of primers (Zalapa et al. 2009). Populations with hybrids had more alleles per locus and greater levels of heterozygosity, but the increase in heterozygosity is not surprising as the majority of hybrids are F_1 s and therefore heterozygous at all species-specific loci. The presence of hybrids increased the level of genetic differentiation among naturalized populations for some pairwise comparisons while it decreased it or did not change it for others. However, the change in level of genetic differentiation in our pairwise comparisons was not necessarily related to the proportion of hybrids in these populations. Our data suggest that hybridization strongly affects the level of genetic diversity observed within *U. pumila* populations and the distribution of genetic diversity within and between these populations.

The US populations were drawn largely from urban and landscape trees (PUS and PWI), many of which may represent original plantings throughout the USA. These two US populations were not only genetically very similar to each other, but they also resembled the accessions from East Asia (PEA). The program STRUCTURE placed all three populations within the same genetic cluster while the F_{ST} -measures calculated for pairwise comparisons of these populations were low. We observed little genetic differentiation between the East Asian accessions and US populations. In addition, the level of genetic diversity within the US populations (PUS and PWI) was quite similar to the level of genetic diversity in the East Asian accessions (PEA), especially when hybrids were excluded. The genetic similarity observed here and the high level of genetic diversity within both the US populations and East Asian accessions suggest a pattern of multiple introductions of *U. pumila* into the USA. This interpretation does not support Webb (1948) that seeds from as few as eight trees made up the source of most plantings in the USA. However, wind dispersal of both pollen and seeds in this species promotes high levels of mixing within and among populations such that seeds from a few trees can contain

a large proportion of the genetic diversity in a population (Zalapa et al. 2008). Therefore, a few modest introductions with diverse progeny arrays could explain the high genetic diversity observed in the 'mature' material. Future studies using non-nuclear markers may help elucidate whether the high level of genetic diversity observed in the 'mature' material resulted from a few modest introductions of genetically diverse progeny or from multiple introductions of genetically diverse material.

The high level of genetic diversity in the 'mature' material and the tolerance of *U. pumila* to DED could have facilitated the evolution of invasiveness in *U. pumila*. Moreover, the widespread hybridization observed in naturalized populations of *U. pumila*, at least in Wisconsin, suggests that hybridization may have also played a role in promoting invasiveness in this plant species. *Ulmus pumila* is likewise invasive in Europe and hybridizes with another elm species (*Ulmus minor*) in Spain where naturalized hybrid populations occur. Many hybrids 'appear to be nearer to *U. pumila* than *U. minor*' morphologically but the allozyme data could not identify levels of backcrossing (Cogolludo-Agustin et al. 2000). *Ulmus pumila* was introduced in Spain as early as the 16th century, so hybridization and the evolution of invasiveness may have a much longer history there. By further increasing genetic diversity and creating novel genotypes, hybridization appears to have facilitated the evolution of invasiveness in a number of introduced species (Ellstrand and Schierenbeck 2000; Vila et al. 2000; Hedge et al. 2006; Londo and Schaal 2007; Moody and Les 2007; Wolfe et al. 2007).

In that respect, *U. pumila* is common in parts of the semi-arid western USA typical of its native Asian habitats, but we do not expect hybridization there due to lack of native elm species in the area. Nonetheless, *U. pumila* can successfully colonize parts of the western USA even without forming hybrids. However, in the eastern and Midwestern regions of the USA, *U. pumila* has adapted to more mesic or lowland forest conditions and is found across a wider range of environments (USDA, NRCS 2002). Coincidentally, we observed hybridization between *U. pumila* and *U. rubra* in naturalized populations in six populations in Wisconsin and in one population each in Illinois and South Dakota and suspect that hybridization will be found to be common throughout the Midwestern and eastern USA whenever the two parental species come in contact (Zalapa et al. 2009). The increased heterosis and the creation of novel genotypes created via hybridization may have helped facilitate the adaptation of *U. pumila* to diverse habitats typical of the Midwestern and eastern regions of the USA. Future studies on the adaptability to various soil and moisture conditions of first- and later-generation hybrids relative to 'pure' *U. pumila* are needed to help corroborate this hypothesis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Microsatellite data for alleles at each of the nine species-specific loci used for the manual identification of *Ulmus pumila* × *Ulmus rubra* hybrid individuals.

Figure S2. Leaf phenotypes of *Ulmus pumila* and *Ulmus rubra* (top) and their natural hybrids (bottom). Leaf phenotypes are a poor indicator of cross-type.

Appendix S1. A total of 152 alleles from 13 microsatellite loci detected in 280 putative *Ulmus pumila* individuals collected throughout the United States from cultivated material, herbarium specimen and eight naturalized populations.

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