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ORIGINAL PAPER

Intra- and interspecific hybridization in invasive Siberian elm

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Abstract Hybridization creates unique allele combinations which can facilitate the evolution of invasiveness. Frequent interspecific hybridization between the Siberian elm, *Ulmus pumila*, and native elm species has been detected in the Midwestern United States, Italy and Spain. However, *Ulmus pumila* also occurs in the western United States and Argentina, regions where no native elm species capable of hybridizing with it occurs. We examined whether inter- or intraspecific hybridization could be detected in these regions. Nuclear markers and the program STRUCTURE helped detect interspecific hybridization and determine the population genetic structure in both the native and the two non-native ranges. Chloroplast markers identified sources of introduction into these two non-native ranges. No significant interspecific hybridization was detected between *U. pumila* and *U. rubra* in the western United States or between *U. pumila* and *U. minor* in Argentina and vice versa. However, the genetic findings supported the presence of intraspecific hybridization and high levels of genetic diversity in both non-native ranges. The evidence presented for intraspecific hybridization in

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the current study, combined with reports of interspecific hybridization from previous studies, identifies elm as a genus where both inter- and intraspecific hybridization may occur and help maintain high levels of genetic diversity potentially associated with invasiveness.

Keywords Genetic diversity · Interspecific hybridization · Intraspecific hybridization · Invasiveness · Multiple introductions · Population genetic structure · *Ulmus*

Introduction

Hybridization and introgression can potentially facilitate the evolution of invasiveness, particularly in plants (Ellstrand and Schierenbeck 2000; Le Roux and Wieczorek 2009; Hovick and Whitney 2014). Hybridization can lead to evolutionary novelty via the creation of new genotypes, increased heterosis, larger pools of standing genetic variation and reduced genetic load (Whitney and Gabler 2008; Schierenbeck and Ellstrand 2009; Blair and Hufbauer 2010). In fact, hybrid zones often represent regions with high genetic variation and unique allele combinations where selection may be intense and evolution rapid (Keim et al. 1989; Abbott and Brennan 2014). Hybridization between native and non-native plant species is common and, in Germany alone Bleeker et al. (2007) identified 134 hybrid plant taxa resulting from hybridization between 81 non-native and 109 native plant species. In addition, Schierenbeck and Ellstrand (2009) reported 35 examples from 16 plant families where hybridization preceded invasiveness. Specific examples include sunflowers where introgressive hybridization between species facilitated adaptation to a broader range of habitats (Rieseberg et al. 2007). In *Rhododendron*, hybridization and introgression between the native *R. catawbiense* and the introduced *R. ponticum* enhanced cold tolerance in several non-native *R. ponticum* populations (Milne and Abbott 2000).

Besides interspecific hybridization, where gene flow occurs between species, intraspecific hybridization has more recently been shown to increase genetic diversity and facilitate the evolution of invasiveness (Kolbe et al. 2004; Williams et al. 2005; Culley and Hardiman 2009; Schierenbeck and Ellstrand 2009). Intraspecific hybridization takes place when gene flow occurs among genetically distinct populations or varieties within a species. With intraspecific hybridization, novel genotypes can be created by mixing individuals from genetically distinct populations that had previously been isolated geographically or by mixing different cultivars that were bred to adapt to different environments or for different traits (Schierenbeck and Ellstrand 2009; Rius and Darling 2014). Intraspecific hybridization leading to invasiveness has been detected in the perennial bunchgrass, *Brachypodium sylvaticum* (Rosenthal et al. 2008). In the callery pear, *Pyrus calleryana,* a Chinese tree commonly planted as an ornamental in the United States, crossing between distinct horticultural cultivars has been shown to create individuals that escaped cultivation and became invasive in the wild (Culley and Hardiman 2009).

Although numerous studies have examined herbaceous invasive species, few studies have investigated the invasion biology of woody species (Richardson and Rejmánek 2011). More than 700 woody species are considered invasive, with large economic and ecological impacts worldwide (Rejmánek and Richardson 2013). Siberian elm, *Ulmus pumila*, is one of these woody invasive species. Following the negative impact of Dutch elm disease on many native elm species, *U. pumila* was introduced in many countries because of its high tolerance to the disease (Leopold 1980; Mittempergher and Santini 2004). It

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has since naturalized and become invasive in some states in the United States and parts of Canada (Kartesz and The Biota of North America Program 2015; USDA, NRCS 2011), Mexico (Todzia and Panero 1998), Argentina (Mazia et al. 2001; Zalba and Villamil 2002), Spain (Cogolludo-Agustín et al. 2000; Cabra-Rivas et al. 2015) and Italy (Brunet et al. 2013; Bertolasi et al. 2015). In the Midwestern United States, Spain, and Italy, interspecific hybridization has been detected between *U. pumila* and the countries' native elms (Cogolludo-Agustín et al. 2000; Zalapa et al. 2009, 2010; Brunet et al. 2013; Elowsky et al. 2013). Hybrids between *U. pumila* and the native elm species, red elm or *U. rubra* are common in the Midwestern United States (Zalapa et al. 2009, 2010) and hybrids between *U. pumila* and field elm *U. minor* occur in Spain and Italy (Cogolludo-Agustín et al. 2000; Brunet et al. 2013). The presence of interspecific hybridization has been associated with invasiveness in these countries (Cogolludo-Agustín et al. 2000; Zalapa et al. 2009, 2010; Brunet et al. 2013). Moreover, *U. pumila* is found across a wider range of environments in the eastern and Midwestern United States (USDA, NRCS, 2011), where the tree seems to have adapted to more mesic conditions relative to its native range (Zalapa et al. 2010).

Although hybridization among elm species is extensive in the Midwestern United States and southern Europe and could have influenced the invasiveness of *U. pumila*, distribution maps of elm species indicate the absence of native elm species capable of hybridizing with *U. pumila* in the western United States and Argentina (Demaio et al. 2015; Kartesz and The Biota of North America Program 2015). Therefore, the presence of interspecific hybrids in these regions could only result from the naturalization of descendants of planted hybrid trees. Alternatively, intraspecific hybridization could have boosted genetic variability and facilitated the success of *U. pumila* in the western United States and Argentina. Intraspecific hybridization necessitates multiple introductions of *U. pumila* in the non-native range from genetically differentiated source populations from the native range. It also requires subsequent breeding and mixing of these differentiated genotypes in the non-native range. Multiple introductions are supported by different lines of evidence. Leopold (1980) reported three original introductions of *U. pumila* from China to the United States at the beginning of the twentieth century. Following its introductions, the planting of *U. pumila* was highly promoted by tree nurseries, especially in the Great Plains regions of the United States due to its vigorous growth even under dry climatic conditions (Leopold 1980). To meet the resulting demand, tree nurseries introduced additional elm material from unknown native origins (Webb 1948). In Argentina, *U. pumila* was introduced from the United States in 1928 and was quickly accepted as a forestry tree in the central region (Moore 1960) where it soon became naturalized (Cozzo 1968; Neher and Roic 1972). The early date of introduction reduces the probability that hybrids between *U. pumila* and *U. rubra* could have been introduced from the United States into Argentina. Further introductions occurred in 1950 from Italy to Argentina (Poduje 1972), and more unrecorded introductions could have taken place as few records exist. These multiple introductions increase the probability that trees from geographically distinct regions were introduced into Argentina and the western United States.

The current research examines whether hybridization occurs in Argentina and the western United States. We first tested whether interspecific hybrids could be identified; these hybrids would represent naturalized descendants of hybrid originally planted in the areas. We then examined whether intraspecific hybridization occurred in Argentina and the western United States. We looked for evidence of multiple introductions of *U. pumila* from genetically differentiated regions of the native range and for subsequent admixture in the non-native range. We also quantified and compared the genetic diversity of *U. pumila* between its native and non-native ranges. This study determines whether inter- or intraspecific hybridization can be detected in regions where no native elms capable of hybridizing with *U. pumila* occur and examines its impact on genetic diversity in these regions.

Methods

The species and populations sampled

Ulmus pumila L. (Ulmaceae), is a diploid, wind-pollinated tree native to temperate regions of east-central Asia (Wu et al. 2003; Wesche et al. 2011). The limits of its native distribution are central Mongolia, southern-central and south-eastern Russia, and

western China (Wesche et al. 2011). Moreover, two western outposts exist in the Altay and in the Tien Shan (Wesche et al. 2011). *Ulmus pumila* leaf material was obtained from 30 native populations (seven Chinese, 16 Mongolian, and seven Russian populations), and 41 non-native populations (11 Argentinean and 30 western United States populations) (Table S1, Supplementary Material; Fig. 1). In the native range, our sampling strategy focused on northern populations because they had not been extensively sampled in previous studies (Zalapa et al. 2008a). The minimum distance between populations was 5 km but the majority of populations were more than 10 km and up to several 100 km apart. Trees selected within a population were at least 5 m apart to avoid collection of clones produced by root suckers. We did not collect originally planted trees in the non-native ranges but collected from populations that had established in these areas.

Samples and genotyping

DNA extractions of leaf tissue were performed using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions except for an extended elution time of ten minutes to increase final DNA concentrations as recommended by Drábková et al. (2002). We used nuclear microsatellite markers (nSSR) to examine interspecific hybridization and quantify population genetic structure and genetic diversity (Table 1). Nuclear microsatellites were ran on leaf samples collected from 10 trees in each of 55 populations. These populations included the 14 native populations from Mongolia and China as well as the 30 non-native populations from the western United States and 11 from Argentina (Table S1, Supplementary Material). In addition, we utilized chloroplast microsatellite markers (cpSSR) to identify potential sources of introductions (Table 1). These markers are non-recombining with predominantly maternal inheritance in angiosperms and are suitable to reconstruct introduction routes (Hufbauer 2004). Non-recombining markers such as chloroplast SSRs vary less than nuclear SSRs and fewer individuals are needed to determine the main haplotype composition of a population using these (Provan et al. 1999; Hufbauer 2004). We therefore ran chloroplast markers on three individual trees from each of the 71 populations except one population in the native range (Mongolia) which was represented by a single specimen from the Moscow State University herbarium. To test whether 3 samples represented the haplotype diversity of the population, we ran 10 samples from randomly selected populations and did not find much differences in haplotype composition (Table S1, Supplementary Material).

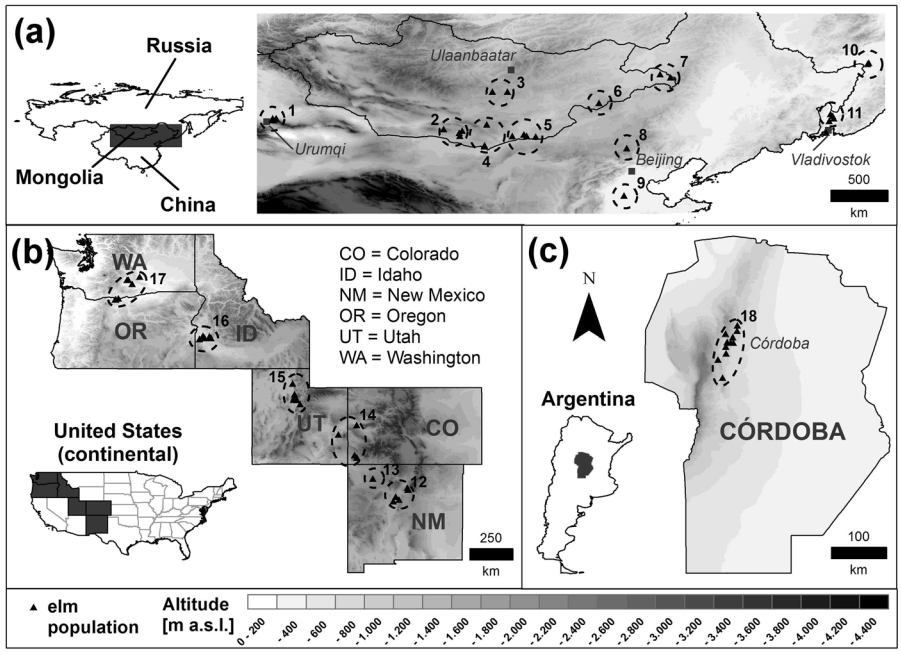

Fig. 1 Locations of the *Ulmus pumila* populations collected in (**a**) the native range (30 populations), and both non-native ranges in (**b**) the western United States and (**c**) Argentina (30 and 11 populations, respectively). Populations are characterized by *black triangles*. Populations less than 200 km apart were grouped into regions (numbered 1–18; symbolized by *dashed circles*)

Table 1 Summary of the different samples used for the various genetic analyses reported in this study

What was tested?	Samples used	Analyses used
Interspecific hybridization (nSSR)		
U. minor × *U. pumila*	Pure parental species: for *U. minor* 38 reference samples and for *U. pumila* 202 reference samples (65 from Asia and 137 from United States). The presence of hybrids was tested on 108 *U. pumila* samples collected in Argentina (11 populations) and 294 in the western United States (30 populations)	STRUCTURE (Pritchard et al. 2000)
U. rubra × *U. pumila*	Genetically pure *U. rubra* (n = 105) and *U. pumila* (n = 202) reference samples. The presence of hybrids was tested on 108 *U. pumila* samples from Argentina and 294 from the western United States	STRUCTURE (Pritchard et al. 2000)
Intraspecific hybridization		
Multiple introductions (cpSSR)	Haplotypes were obtained for 238 *U. pumila* samples; 47 from Argentina, 104 from the western United States and 87 from the native range	Haplotype identification (Eliades and Eliades 2009)
Population genetic structure (nSSR)	Within the native range, we used the 130 *U. pumila* samples collected for this study. Analyses were also ran using an additional 65 pure *U. pumila* reference samples with Asian origin (i.e. Asian reference samples). In the non-native ranges 108 *U. pumila* samples from Argentina (n = 108) and 294 from the western United States (n = 294) were utilized	STRUCTURE (Pritchard et al. 2000)
Analysis of molecular variance (nSSR)	195 *U. pumila* samples from the native range (130 + 65 samples described above) Sixty-two samples were collected in the eastern, 73 in the northern and 60 in the western regions (see text for details)	Hierarchical analysis of molecular variance (Excoffier et al. 1992)
Genetic diversity (nSSR)	For the native range we did the calculations using only the 130 samples from the native range and then adding the 65 pure *U. pumila* reference samples. We also examined genetic diversity within each of the three geographic regions described earlier (eastern, northern and western) with the 195 *U. pumila* samples (130 + 65). For the non-native ranges, we used 108 *U. pumila* samples collected in Argentina and 294 from the western United States	Observed and expected heterozygosity (Nei 1978) Kosman diversity index (Kosman and Leonard 2007) Mean number of alleles

Genetically pure reference samples used to test for interspecific hybridization included *Ulmus minor* (n = 38), *U. rubra* (n = 105) and *U. pumila* samples (n = 202; with 65 samples from Asia and 137 from the United States) (Table S2, Supplementary Material). Samples of *U. pumila* collected for this study included 130 samples collected in Mongolia and China (14 populations); 108 samples collected in Argentina (11 populations) and 294 individuals collected in the western United States (30 populations) (Table S1, Supplementary Material)

Nuclear markers (nSSR)

We initially tested twelve nuclear microsatellite loci previously isolated by Zalapa et al. (2008b) (UR101, UR123, UR138, UR153, UR158, UR159, UR173b and UR175), Collada et al. (2004) (Ulmi1-21, Ulmi1-98 and Ulmil-165) or Whiteley et al. (2003) (Ulm3) on a subset of samples. Five of these loci (UR101, UR173b, Ulmi1-21, Ulmi1-98 and Ulm3) were rejected due to a lack of consistent amplification. Each of the seven remaining forward primers was labeled at the 5′ end with the fluorochromes 6-Fam or Hex. Four of these loci were multiplexed in a PCR reaction (UR153 and Ulmil-165 6-Fam and UR158 and UR159 Hex) while the other 3 primer pairs were ran singly. The PCR reactions were performed in a 25 µl total volume, containing 10 ng DNA, 0.5 µl or 0.8 µl of each forward and reverse primer (5 pmol/µl; Metabion, Martinsried, Germany), 2.5 µl dNTPs (2 mM; Fermentas, St. Leon-Rot, Germany), 1 U Taq DNA polymerase (Fermentas), 2.5 µl incubation mix T. Pol with 1.5 mM $MgCl_2$ (MP Biomedicals, Eschwege, Germany) and 17.8 µl (16.6 µl for multiplex PCR) H_2O (bidistilled). For cycling conditions, we followed the protocol for 'PCR profile a' of Collada et al. (2004). Amplification products were diluted 1:5 and separated on a MegaBace 1000 system (Amersham Bioscience, Uppsala, Sweden) using MegaBace ET-ROX 400 (Amersham Bioscience) as the size standard. We used GeneMarker v.1.95 (SoftGenetics LLC) to identify alleles with marker panels and reference samples from past *U. pumila* studies to ensure continuity between the allele sizes observed in the current and former research efforts. Eighteen samples did not amplify at any of the loci even after repeated DNA extractions and PCR. Genotypes were obtained for 130 individuals from the native range and 402 from the non-native ranges, 294 from the western United States and 108 from Argentina. These genotypes will be used to detect interspecific hybridization and determine the levels of genetic differentiation and genetic diversity in native and non-native ranges.

Chloroplast markers (cpSSR)

We tested eight universal cpSSR primers, two (trnL-trnF and trnL) designed by Taberlet et al. (1991) and six (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10) by Weising and Gardner (1999) using the PCR conditions described below and recommended by K. Prinz (Friedrich-Schiller-University Jena, Germany; personal communication). Multiplex PCRs with two different labeled primer pairs (HEX and 6-FAM fluorescent dyes), and no overlapping size ranges between the corresponding amplification products were performed in final volumes of 15 µl containing 10 ng of template DNA, 1 U HOT FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia), 1.5 µl HOT FIREPol® 10 × Buffer B2, 1.5 µl $MgCl_2$ (25 mM), 2 µl dNTPs (2 mM; Fermentas, St. Leon-Rot, Germany), 0.5 µl of both forward primers (5 pmol/µl; Metabion, Martinsried, Germany), 0.5 µl of both reverse primers (5 pmol/µl) and 6.8 µl H_2O_{bidest}. PCR was carried out in a Mastercycler® epgradient (Eppendorf, Hamburg, Germany) with an initial activation step at 95 °C for 15 min followed by 30 cycles with 1 min denaturation at 94 °C, 1 min annealing at 50 °C as well as 1 min elongation at 72 °C, and a final elongation step for 20 min at 72 °C. Amplification products (1:10 diluted) were separated on a MegaBace 1000 system (Amersham Bioscience, Uppsala, Sweden) using MegaBace ET-ROX 400 or ET-ROX 900 as the size standard. GeneMarker v1.95 was used for genotyping. Because some samples did not amplify, even after repeated DNA extraction and PCR, our final dataset consisted of 238 samples with some populations having fewer than three samples (Table S1, Supplementary Material). The haplotype of each of the 238 trees from the 71 populations was determined by analyzing the cpSSR genotype data with the software Haplotype Analysis (Eliades and Eliades 2009). This software identifies haplotypes by combining the size variants at the investigated cpSSR loci (Eliades and Eliades 2009). Haplotype data helped to detect whether multiple introductions have occurred in the non-native ranges.

Interspecific hybridization in non-native range (nSSR)

Although no native elm species known to hybridize with *U. pumila* occur in the sampled areas of the western United States (Kartesz and The Biota of North America Program 2015), hybrids between *U. rubra* and *U. pumila* commonly occur in the Midwestern United States (Zalapa et al. 2009, 2010). These hybrids

could have been planted and later become naturalized in the western United States. We therefore tested for the presence of hybrids between *U. pumila* and *U. rubra* in the western United States. *Ulmus minor* was planted in Argentina (Martin 2008) and we therefore looked for the presence of potential hybrids between *U. pumila* and *U. minor* in Argentina. To be thorough, we also looked for hybrids between *U. pumila and U. minor* in the western United States and between *U. pumila* and *U. rubra* in Argentina. Nuclear microsatellite data were used for these analyses.

To facilitate the detection of potential interspecific hybrids in the non-native *U. pumila* populations, we used genetically pure individuals of the two respective parental elm species as reference populations. For example, to test for hybrids between *U. pumila* and *U. rubra*, we used reference samples of *U. pumila* (202 individuals) and *U. rubra* (105 individuals) determined to be genetically pure in previous studies using species-specific alleles and Bayesian classification (Zalapa et al. 2009, 2010). Of the 202 *U. pumila* reference samples, 65 came from Asia and 138 represent pure individuals from naturalized populations in the Midwestern United States (Zalapa et al. 2009, 2010) (Table S2, Supplementary Material). To test for hybrids between *U. pumila* and *U. minor*, we used the 202 reference samples of *U. pumila* just described, and reference samples of *U. minor* which consisted of 38 putatively pure genetic *U. minor* trees collected in this study from areas in Europe where no other elm species known to hybridize with *U. minor* occur (Table S2, Supplementary Material). The presence of hybrids was examined in the 108 naturalized *U. pumila* individuals collected from 11 populations in Argentina and in the 294 naturalized *U. pumila* individuals collected from 30 populations in the Western United States (Table S1, Supplementary Material). To ensure compatibility in the genetic data generated in this study and in previous studies (Zalapa et al. 2009, 2010), 15 randomly chosen samples from our dataset were analyzed under the same laboratory conditions as the reference samples and genotypes were compared.

We used the program STRUCTURE (version 2.3.3; Pritchard et al. 2000) to assign individuals to pure species or hybrids. Because we have two parental species, we expected the optimal value of K to consist of two genetic clusters (K = 2). We first confirmed this assumption by testing values of K from one up to the number of populations in the respective groups using the STRUCTURE HARVESTER software (version 0.6.92; Earl and vonHoldt 2012) and selected the optimum K following the method of Evanno et al. (2005). For each STRUCTURE analysis, we used an admixture model with 100,000 burn-in iterations, 500,000 Markov chain Monte Carlo repetitions, and 20 replicates at each level. The programs CLUMPP (version 1.1.2; Jakobsson and Rosenberg 2007) and DISTRUCT (version 1.1; Rosenberg 2004) were used to visualize the STRUCTURE results. We then tested for the presence of hybrids between *U. pumila* and *U. rubra* and between *U. pumila* and *U. minor* both in the western United States and in Argentina (four comparisons). The program STRUCTURE generates an admixture coefficient (*q*) which represents the proportion of an individual's genotype that originates from each of the K genetic clusters. We ran the program with the option ANCESTDIST which computed the 95% posterior probability for each q value, equivalent to a 95% confidence interval. Following Blair and Hufbauer (2010), individuals were classified as hybrids if their *q* value was <0.90 and their probability interval did not include 1.0. Finally, species-specific alleles identified in the reference data sets described above helped confirm the identification of hybrid individuals.

Multiple introductions in non-native range (cpSSR)

To determine whether multiple introductions could have occurred in the non-native ranges, we examined and compared the haplotypes of trees sampled in the non-native ranges to the trees sampled in the different regions of the native range. Tree haplotypes were generated using cpSSR for 238 trees from 71 populations with an average of 3 individuals per population (Table S1, Supplementary Material). The haplotypes present in each population were tabulated and the geographical distribution of haplotypes at a regional scale was obtained by combining populations that were less than 200 km apart. This resulted in 18 distinct groups or regions and the proportion of the different haplotypes within each group was calculated and plotted as a pie graph using R version 3.1.3

(R Core Team 2015). The presence of specific haplotypes was then compared between the native and the two non-native ranges to identify the potential geographic source of a haplotype.

Population genetic structure (nSSR)

We used the program STRUCTURE to determine whether genetic differentiation of populations occurred in the native range and to examine the population genetic structure and level of genetic differentiation in the non-native ranges. These analyses used nuclear microsatellite data with 10 individuals per population. In the native range, we first used the program STRUCTURE to examine the genetic structure of the 14 populations collected from Mongolia and China (n = 130). We then added samples (n = 65) previously genotyped by Zalapa et al. (2008a, 2009, 2010) to increase the sampling of the native range. These additional native samples consisted of individuals from the reference *U. pumila* dataset (hereinafter referred to as Asian reference samples) and analyses were performed with and without the Asian reference samples. Based on the levels of admixture for the two genetic clusters identified by STRUCTURE, we identified three geographic regions within the native range, thereafter called the eastern, southern and western groups. We performed a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) comparing these three groups.

In the non-native ranges, we first examined the genetic structure separately for the 30 populations from the western United States and the 11 populations from Argentina before combining all populations (both native and non-native ranges) into an overall analysis. For the overall analyses, we included only the 14 native populations (n = 130) in one case and added the Asian reference samples in the other (n = 195). In each case, we looked for the optimum value of K by testing values of K from one up to the number of populations in the respective groups using the STRUCTURE HARVESTER software and selected the optimum K following the method of Evanno et al. (2005). For all analyses, we followed Gilbert et al. (2012) and used an admixture model with correlated allele frequencies, 100,000 burn-in iterations, 500,000 Markov chain Monte Carlo repetitions and 20 replicates per run. The programs CLUMPP and DISTRUCT provided graphical visualization of the STRUCTURE results.

Genetic diversity (nSSR)

Genetic diversity was measured in populations where 10 trees were sampled. For the native range, genetic diversity was first measured only for the 14 *U. pumila* populations sampled in this study (n = 130), before adding the Asian reference samples (n = 195). We also calculated genetic diversity within the eastern, southern and western groups described earlier. We examined overall genetic diversity in the native range and in each of the two non-native ranges using the Kosman diversity index (KW) (Kosman and Leonard 2007), expected (H_e) and observed (H_0) heterozygosity (Nei 1978) as well as mean number of alleles (N_a). The KW index was selected because *U. pumila* has the potential for clonal growth (Meusel et al. 1965). The Kosman assignment-based approach considers an individual genotype as a fixed combination of alleles instead of a set of independent alleles as is typical of allele frequency based calculations (i.e. heterozygosity measures; Nei 1978). Such potential associations between alleles are more likely to occur in organisms with asexual or mixed modes of reproduction relative to outcrossed organisms (Kosmann and Leonard 2007). Values of KW diversity for the native and each of the two non-native ranges were calculated using the VAT software and its extension (Schachtel et al. 2012; www.tau.ac.il/lifesci/departments/plant_s/members/kosman/VAT.html). Dissimilarities between nSSR genotypes, as prerequisite for the KW diversity calculation, were estimated according to Kosman and Leonard (2005) for diploids with codominant makers. Expected (H_e) and observed (H_0) heterozygosity as well as mean number of alleles (N_a) were calculated using GenAlEx (v.6.5b; Peakall and Smouse 2012) and will facilitate comparisons with previous studies.

Results

Interspecific hybridization in non-native range (nSSR)

One potential hybrid between *U. pumila* and *U. rubra* was detected in Argentina after testing 108 individuals using the program STRUCTURE (Fig. 2a). The

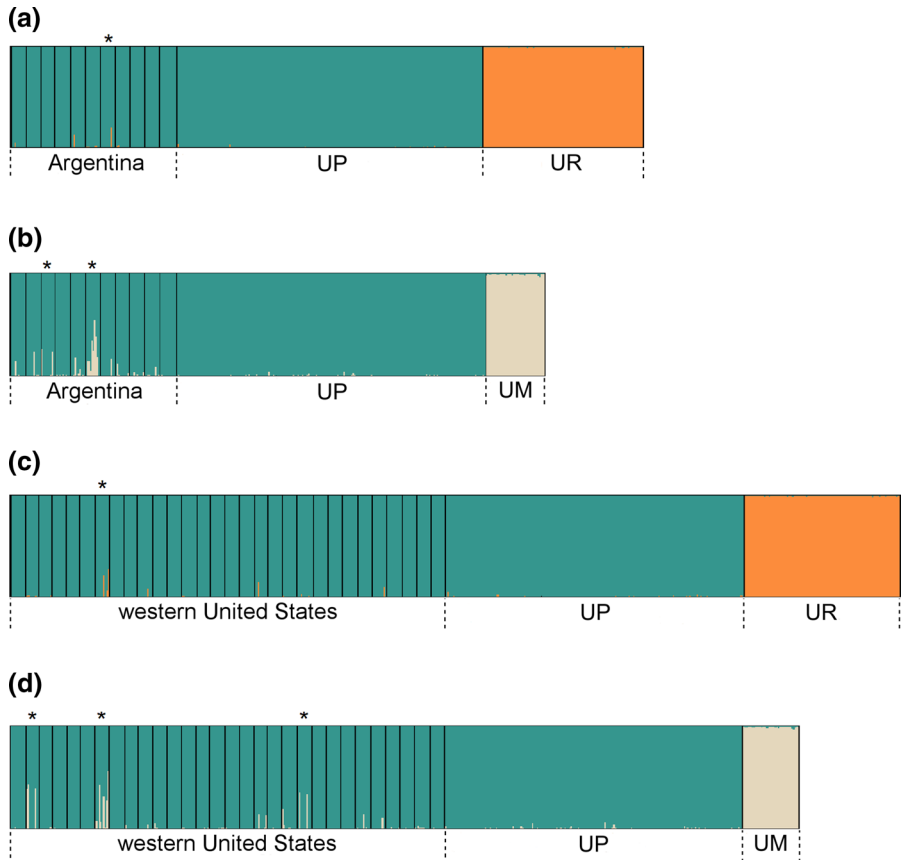

Fig. 2 STRUCTURE results for the detection of hybrids between *Ulmus pumila* and *U. rubra* or *U. pumila* and *U. minor* in the non-native ranges. Interspecific hybridization was tested between (**a**) *U. pumila* and *U. rubra* and (**b**) *U. pumila* and *U. minor* in 11 populations from Argentina (n = 108); and (**c**) *U. pumila* and *U. rubra* and (**d**) *U. pumila* and *U. minor* in 30 populations from the western United States (n = 294). UP represents the 202 pure *U. pumila* reference samples (65 from Asia and 137 from the United States), UR the 105 pure *U. rubra* reference samples and UM the 38 pure *U. minor* reference samples. The *asterisks above the bars* indicate non-native populations where putative interspecific hybrids were identified

potential hybrid individual had a *q* value of 0.80 (relative to pure *U. pumila*) and did not carry *U. rubra* specific alleles. Of the 115 alleles identified across all reference samples, we found 17, 22 and 26 species-specific alleles for *U. minor*, *U. pumila* and *U. rubra*, respectively (Table S3, Supplementary Material). The program STRUCTURE also identified two populations in Argentina with potential hybrids between *U. pumila* and *U. minor* (Fig. 2b) but only one individual in each of these two populations was classified as a potential hybrid based on Blair and Hufbauer's (2010) criteria (Table S1, Supplementary Material). One individual had a *q* value of 0.45 (relative to pure *U. pumila*) and would likely represent a first-generation hybrid (F_1; hybrid classification according to Brunet et al. 2013). The second potential hybrid individual with a *q* value of 0.74 would most probably indicate a first-generation backcross. *Ulmus minor* specific alleles were found in one of the two Argentinean hybrids.

In the western United States two potential *U. pumila* and *U. rubra* hybrids were detected among 294 tested individuals (Fig. 2c). The two hybrids had respective *q* values of 0.79 and 0.72 and did not carry any *U. rubra* specific alleles. Nine individuals were identified as potential hybrids between *U. pumila* and *U. minor* in the western United States. These hybrids were distributed among three populations (4, 2 and 3 individuals per population) (Fig. 2d). The *q* values for these nine individuals varied between 0.43 and 0.73 and most of them represented potential F_1 hybrids. *Ulmus minor* specific alleles were found at one or two loci in six of the nine potential hybrid trees.

Multiple introductions in non-native range (cpSSR)

We detected 15 different haplotypes across the 238 samples with varying frequencies in the three ranges (Table 2; Fig. 3). Pairwise comparisons of haplotypes indicated differences at one and up to four loci (Table S4, Supplementary Material). Nine haplotypes were detected in the native range, seven in the non-

Table 2 *Ulmus pumila* haplotype frequencies for the native Asian range and the two non-native ranges (Argentina and the western United States)

Haplotype	Asia	Argentina	United States
A	0.16	–	0.02
B	0.38	0.60	0.75
C	0.17	0.04	0.14
D	0.07	–	–
E	0.08	–	–
F	0.01	–	–
G	0.10	0.09	–
H	0.02	–	–
I	0.01	–	–
J	–	0.17	–
K	–	0.04	–
L	–	0.04	–
M	–	0.02	0.05
N	–	–	0.04
O	–	–	0.01

native Argentinean range, and six in the western United States range (Table 2; Fig. 3). Five haplotypes were private in the native range (haplotypes D, E, F, H and I) (Table 2). The non-native Argentinean range had three private haplotypes (J, K, and L) and the western United States had two (N and O) (Table 2). Haplotype M was only detected in the two non-native ranges. The non-native ranges each shared three haplotypes with the native range; the western United States populations shared haplotypes A, B, and C with the native range while the Argentinean populations shared haplotypes B, C, and G (Table 2; Fig. 3). Haplotypes B and C were present in all three ranges (native and two non-native) with haplotype B being the most frequent haplotype (Table 2). The proportions of the different haplotypes in each of the 18 geographical regions are summarized in Table S5 (Supplementary Material) and the haplotypes present in each population are summarized in Table S1, Supplementary Material. The geographical distribution of the haplotypes indicated that regions 2, 4, and 5

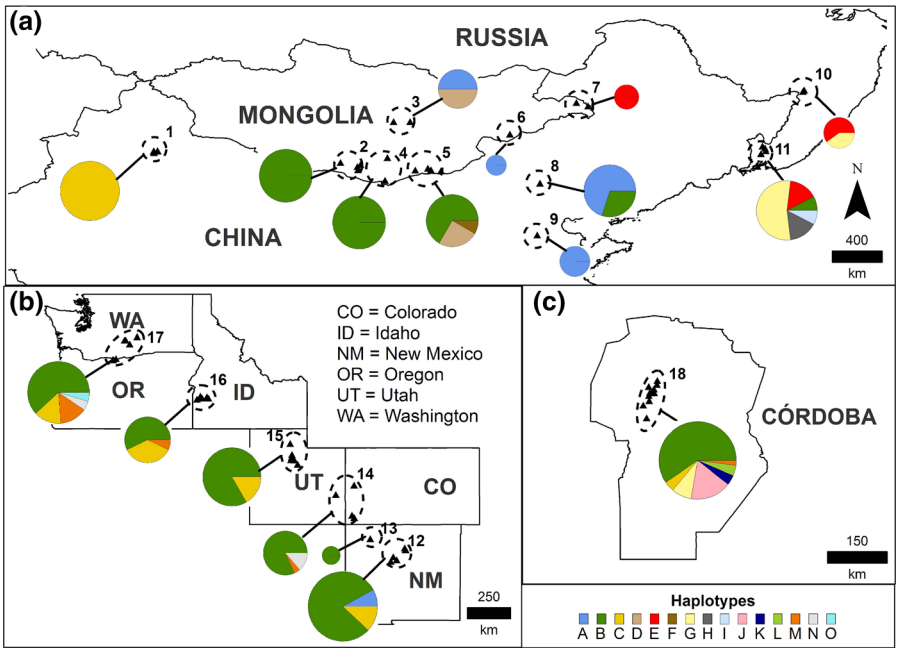

Fig. 3 The proportion of *Ulmus pumila* haplotypes in 18 geographic regions in its (**a**) native range and two non-native ranges, (**b**) Argentina and (**c**) the western United States. Seventy-one populations were sampled for this analysis and the 18 geographic regions were obtained after combining the sampled populations that were less than 200 km apart

in southern Mongolia and region 8 near Beijing, China were potential source regions for haplotype B (Fig. 3). Haplotype C was only detected in the western China portion of the native range (region 1; Fig. 3). Therefore, haplotypes B and C originated from different regions in the native range indicating the potential for multiple introductions in both non-native ranges (Table 2). In addition, haplotype A, shared between the United States and the native range, could have originated from northern or eastern Mongolia (regions 3, and 6), or from the areas surrounding Beijing, China (regions 8 and 9) (Fig. 3). Haplotype G, shared between Argentina and the native range, was only present in the Russian regions 10 and 11 (Fig. 3). Therefore, haplotypes B and G, both present in Argentina, also originated from two distinct regions in the native range. Because haplotypes were based on the combination of size variants at the cpSSR loci, it was not possible to present a haplotype network or report the number of base pair changes between haplotypes.

Population genetic structure (nSSR)

In the native range, two genetic clusters (K = 2) were identified by the program STRUCTURE (Fig. 4a). The two clusters persisted whether only the 14 *U. pumila* populations collected for this study were considered (n = 130) or whether the Asian reference samples were also included in the analysis (n = 195) (Table 1; Fig. 4a). When considering only the 14 populations (blue dots in Fig. 4a), populations from western China consisted mainly of one genetic cluster while populations from Mongolia and northern China were mostly from the second genetic cluster. However, many of the populations included in the Asian reference samples exhibited a greater level of admixture (red triangles on Fig. 4a).

Moreover, when all 195 samples were examined, populations could be separated into a western, northern and southern group based on their geographical location and levels of admixture for the two genetic clusters (Fig. 4a). Results of the hierarchical AMOVA for these three groups detected 7% of the genetic variation among groups, 9% among populations and 83% among individuals within populations.

When native and non-native populations were analyzed together, the program STRUCTURE identified two genetic clusters, and detected substantial admixture within non-native Argentinean and western United States populations (Fig. 4b, c). A similar pattern of admixture was observed whether only 130 or 195 Asian samples were included or not in the analyses (Fig. 4b, c).

When populations from each of the two non-native ranges were analyzed separately, the program STRUCTURE identified three genetic clusters (K = 3) within both the Argentinean and western United States ranges (Fig. 4d). Populations within each of the two non-native ranges exhibited considerable admixture (Fig. 4d). Delta K plots used to determine the optimum number of genetic clusters for the different analyses described in this section are presented in Fig. S1, Supplementary Material.

Genetic diversity

We identified 78 alleles in the 55 populations examined for genetic diversity using nSSR (52 alleles in Asia; 53 in Argentina and 69 in the western United States). The KW values were similar between the two non-native ranges (KW = 0.66 for Argentina and KW = 0.67 for the western United States). The KW value for the native range was 0.66 when only the 14 native populations were examined and 0.65 when the Asian reference samples were also included. Expected (H_e) and observed (H_0) heterozygosity values were also similar between the two non-native ranges with $H_e = 0.56$ and $H_o = 0.52$ for Argentina and $H_e = 0.56$ and $H_o = 0.50$ for the western United States. Expected and observed heterozygosity values in the native range were $H_e = 0.54$ and $H_o = 0.44$ when Asian reference samples were excluded and $H_e = 0.54$ and $H_o = 0.45$ when including the Asian reference samples. The mean number of alleles was $N_a = 9.86$ in the western United States and $N_a = 7.57$ in Argentina. In the native range, $N_a = 7.43$ without the Asian reference samples and $N_a = 8.57$ with the reference samples. Finally, when comparing the three groups within the native range, genetic diversity was slightly greater in the northern group (KW = 0.67; $H_e = 0.56$; $H_o = 0.47$; $N_a = 7.43$), followed by the eastern group (KW = 0.59; $H_e = 0.53$; $H_o = 0.52$; $N_a = 7.14$), and lastly the western group (KW = 0.50; $H_e = 0.41$; $H_o = 0.35$; $N_a = 5.00$).

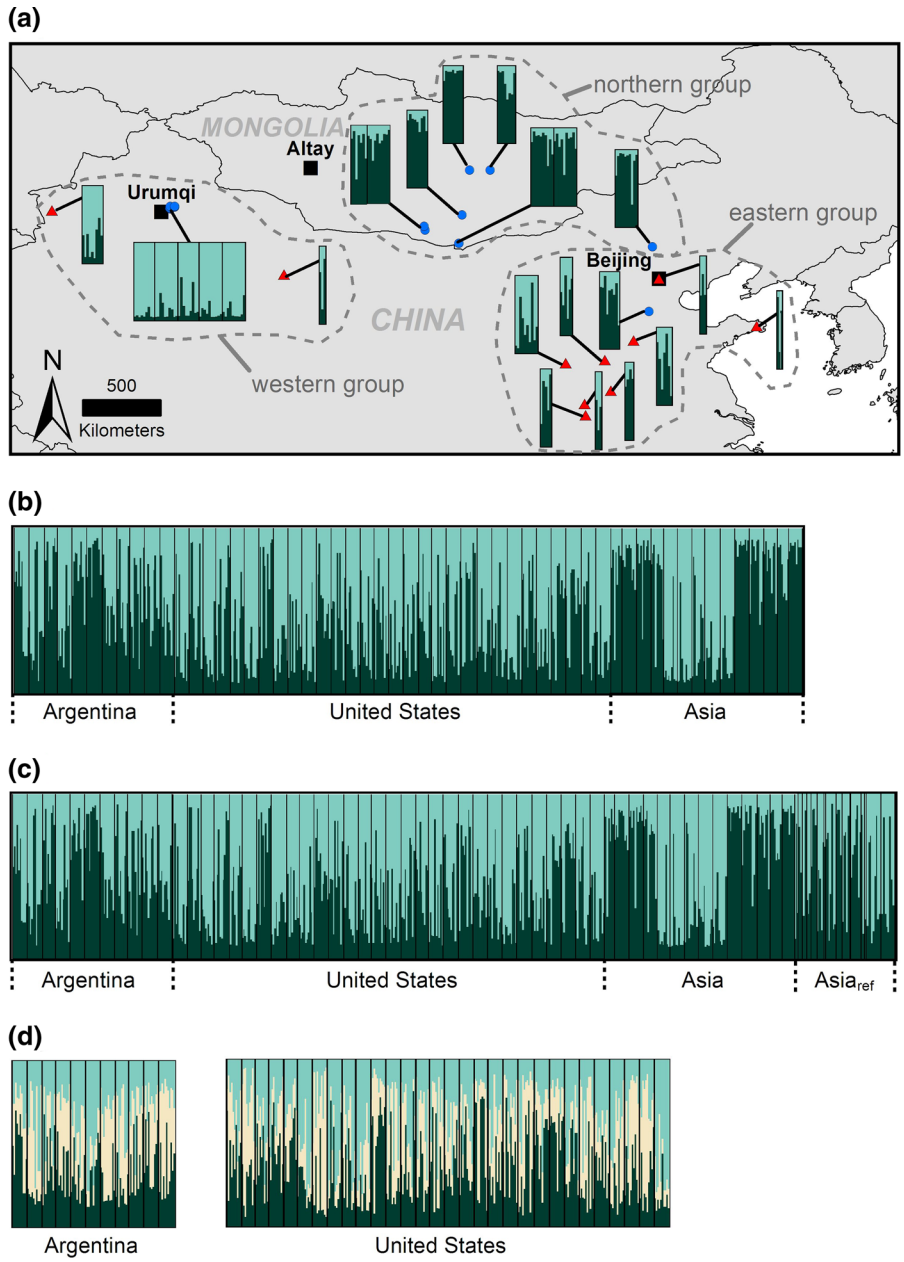

Fig. 4 Population genetic structure based on the program STRUCTURE for (**a**) populations in the native range; (**b**) populations from the two non-native ranges and the 14 native populations (*blue dots*; Asia); (**c**) populations from the two non-native ranges, the 14 native populations and the Asian reference samples (*red triangles*); (**d**) the non-native populations from Argentina (11) or the western United States (30 populations). The STRUCTURE analyses revealed two genetic clusters (K = 2) for cases (**a**), (**b**), and (**c**) and three genetic clusters (K = 3) for cases (**d**). The native populations were grouped into a western, northern or eastern group (**a**) based on the admixture level of the two genetic clusters

Discussion

Interspecific hybridization is rare in the western United States and Argentina. Only two putative hybrid individuals between *U. pumila* and *U. rubra* were detected in the western United States (n = 294) and one in Argentina (n = 108), with no putative hybrids carrying *U. rubra* private alleles. Similarly, we

observed only nine *U. pumila* x *U. minor* hybrids in the western United States and two in Argentina. Moreover, the majority of the putative hybrid trees were F_1 hybrids suggesting a lack of introgression in these regions. These results contrast sharply with the preponderance of interspecific hybridization and patterns of introgression observed between *U. pumila* and *U. rubra* in the Midwestern United States (Zalapa et al. 2009, 2010) and between *U. pumila* and *U. minor* in Italy and Spain (Cogolludo-Agustín et al. 2000; Brunet et al. 2013). The major difference between the western United States and Argentina regions relative to the Midwestern United States, Italy and Spain is the absence of native elm species capable of hybridizing with *U. pumila* in the former regions. The lack of *U. rubra* and *U. minor* trees in the western United States and Argentina, combined with our sampling of naturalized *U. pumila* individuals, suggests that the putative hybrids descended from hybrid ornamental trees originally planted over the landscape. We conclude, interspecific hybridization did not facilitate the spread of *U. pumila* in the western United States and Argentina.

A first step in detecting intraspecific hybridization is to determine the presence of multiple introductions in the non-native ranges. The haplotype data, based on chloroplast data (cpSSR), indicated the presence of multiple haplotypes in the native and non-native ranges. Most importantly, the haplotypes shared between either the western United States or Argentina and the native range originated from diverse regions within the native range. For example, haplotypes A, B and C are shared between the western United States and the native range. While haplotype C is found in the western portion of China, haplotype A occurs in the eastern part of China while most of haplotype B is detected in the northern region of the native range. Therefore, multiple introductions of single *U. pumila* haplotypes originating from different regions of the native range can help explain the diversity of haplotypes observed in the non-native western United States. A similar pattern exists for Argentinia. These findings conform with the documented multiple introductions events of *U. pumila* into the United States (Webb 1948; Leopold 1980) and Argentina (Moore 1960; Poduje 1972). Multiple introductions associated with invasiveness have been observed in other plant species including the South African Ragwort (*Senecio inaequidens*) in Europe (Lachmuth et al. 2010).

The multiple introductions must have originate from genetically distinct populations in the native range before intraspecific hybridization can occur. The haplotype data support genetic differences among regions in China. For example, haplotype C is only found in the western part of the range (haplotype region 1) (Fig. 3) while haplotype D occurs only in the northern part (regions 3 and 5) and haplotype G in the eastern part (region 7). Genetic differences among populations are further supported by the nuclear data. The STRUCTURE analyses indicated differences among the western, northern and eastern parts of China and Mongolia. STRUCTURE identified two genetic clusters with one cluster dominating in the western part and the second cluster in the northern part. Both clusters were admixed in the eastern part of the native range. Finally, the AMOVA results indicated some degree of genetic differentiation among the three regions in the native range with 7% of the genetic variation occurring among regions. The evidence therefore supports some genetic differentiation between different geographical regions in the native range and multiple introductions of genotypes from these distinct regions into each of the two non-native ranges.

Lastly, to demonstrate intraspecific hybridization in the non-native ranges, individuals must have bred and created novel recombinant genotypes (Culley and Hardiman 2009). In both non-native ranges, the STRUCTURE analyses indicated a high degree of admixture whether populations in Argentina or the United States were examined separately or together with the Asian populations. Moreover, while two genetic clusters were identified in the native range, when examined separately both non-native ranges indicated the presence of three genetic clusters. This finding supports the concept that novel recombinant genotypes may be present in the non-native ranges. In addition, populations in Argentina and the western United States were more likely to contain multiple haplotypes relative to populations in the native range (Fig. 3). The chloroplast data suggest potential intraspecific hybridization between the B and C haplotypes in the western United States, which correspond to the northern and western groups within the native range. In Argentina, intraspecific hybridization between the B and C and B and G haplotypes may

have occurred. The presence of multiple introductions from genetically different regions of the native range, and the detection of admixture of these genotypes in the non-native ranges support the presence of intraspecific hybridization in both the western United States and Argentina.

The intraspecific mixing of unique genetic clusters and resulting intraspecific hybridization could have provided a boost of genetic variability facilitating the success of *U. pumila* in western United States and in Argentina. Such increase in genetic diversity resulting from intraspecific hybridization has been hypothesized in other invasive species such as the wetland grass *Phalaris arundinacea* (Lavergne and Molofsky 2007) and the Cuban lizard *Anolis sagrei* (Kolbe et al. 2004). We observed high genetic diversity levels in *U. pumila* in both the western United States and Argentina, comparable to the level of genetic diversity in the native range. Several new alleles were observed for the nuclear loci (nSSR) in the non-native ranges, 5 private alleles in Argentina and 11 in the western United States, but only one of the new alleles was shared between the two non-native regions (data not shown). Moreover, several private haplotypes (cpSSR) were present in the two non-native ranges. Three private haplotypes (J, K, and L) were observed in Argentina, two (N and O) in the western United States, and the common haplotype (M) in both of non-native ranges. These private haplotypes could represent haplotypes produced post-introduction through interspecific or intraspecific hybridization. They might also indicate that additional haplotypes from native regions were not sampled in this study but served as introduction sources.

Intraspecific hybridization and the high level of genetic diversity could have facilitated the evolution of invasiveness of *U. pumila* in the western United States and Argentina (Ellstrand and Schierenbeck 2000). Noticeably, previous studies of native and non-native *U. pumila* indicated enhanced germination (Hirsch et al. 2012) and growth performance (Hirsch et al. 2016) in non-native compared to native individuals. Such traits could have facilitated the establishment of *U. pumila* in a wider range of environments in the non-native range and facilitated the evolution of invasiveness in this plant species. Combining our results with previous studies of hybridization in elm, therefore supports the presence of hybridization, inter- or intraspecific, in all regions where *U. pumila* has become invasive (Cogolludo-Agustín et al. 2000; Zalapa et al. 2009, 2010; Brunet et al. 2013).

In conclusion, in addition to previously observed interspecific hybridization, our data support intraspecific hybridization in geographic areas where no native elm species capable of hybridizing with *U. pumila* are present. Hybridization, both intra- and interspecific, can create high levels of genetic diversity which may facilitate the evolution of invasiveness. Prompted by the interesting observations herein, future studies utilizing newer marker technologies and further sampling of *U. pumila* and its hybrids in native and non-native ranges should increase our knowledge of the post-introduction mechanisms that have resulted in its global invasiveness. Perhaps more importantly, such studies using the *Ulmus* system could provide important contributions to the field of invasion ecology by broadening current understanding of the relationship between hybridization, both inter- and intraspecific, and the evolution of invasiveness.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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