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Research

Microsatellites Uncover Multiple Introductions of Clonal Giant Reed (*Arundo donax*)

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Giant reed (*Arundo donax*) is an invasive weed that is native to the Old World. Tens of thousands of hectares of riparian habitat in the Rio Grande Basin (RGB) in Texas and Mexico have been heavily affected by invasions of *Arundo*. Additionally, many other watersheds across the southwestern United States have also been affected. Giant reed is being targeted for biological control because it displaces native vegetation and consumes water that could potentially be used for agricultural and municipal purposes, especially in areas with limited access to water. Finding the best-adapted insects for biological control involves locating the origin(s) of this plant. To narrow down the proximal source(s) of invasion of giant reed in the RGB, 10 microsatellite markers were developed. An analysis of 203 Old World and 159 North American plants, with an emphasis on the RGB, indicated a reduction in the allelic diversity in the introduced range compared with the Old World. Clonal assignment, neighbor joining, principal coordinates analyses, and STRUCTURE analyses were consistent and implied multiple introductions in North America, with one (likely clonal) lineage responsible for the invasion of the RGB, northern Mexico, and other parts of the southwestern United States. Although no identical matches with the RGB lineage were found in the Old World, several close matches were found on the Mediterranean coast of Spain.

Nomenclature: Giant reed, *Arundo donax* L.

Key words: Biological control, clonal, invasive species, Mediterranean, Rio Grande Basin, RGB.

The process of developing biological controls can be facilitated with information about the origins of target weeds and their genetic diversity in the introduced range. The determination of the original source(s) of invasive genotypes and ecotypes is important because phytophagous insects can be locally adapted to their host plants (Goolsby et al. 2006a), implying that control agents may need to be closely matched geographically, genetically, and physiologically to the target invasive organism to be effective. Additionally, when multiple genotypes of an invasive weed are present in an invasion, they can vary in tolerance and resistance to biological control agents. For example, different genotypes of cordgrass (*Spartina alterniflora* Loisel.) had varying tolerances to *Prokelisia marginata* Van Duzee, a planthopper employed as a biocontrol agent (Garcia-Rossi et al. 2003). Thus, screening of potential biocontrol agents from multiple source locations might be necessary.

It can be quite challenging to gather data appropriate to determine origins of invasive weeds definitively (Estoup and Guillemaud 2010), but it is often possible to detect whether multiple introductions are probable or not based simply on the structure of genetic variation and identity of alleles. Microsatellite loci are particularly useful in this regard because they can be polymorphic enough to detect fine-scale population-level differences (Ahmad et al. 2008; Goolsby et al. 2006a,b).

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Management Implications

Giant reed is a clonal, rhizomatous grass that has invaded tens of thousands of hectares of riparian habitat throughout the Rio Grande Basin (RGB) and other parts of the southwestern United States. In this paper, we used microsatellites to determine the original population source(s) of the invasive *Arundo donax* in the RGB to locate biocontrol agents from the Old World. Biological control is deemed the best long-term option for control of giant reed. Chemical and mechanical control of *A. donax* is expensive, especially in heavily affected areas. Although *A. donax* is clonal, some genetic variation was found throughout the RGB. We also discovered multiple introductions in the United States, but only one lineage is responsible for the invasion in the RGB. This indicates that a limited sampling of biocontrol insects might be effective in controlling *A. donax* along the Rio Grande. Additionally, these biocontrol agents might also be effective in controlling giant reed in others areas where this lineage has been introduced, such as California and Mexico.

Giant reed (*Arundo donax* L.) is a perennial C_3 grass that has become invasive in many parts of the world (see Study System, below). It is an excellent candidate for biological control because *Arundo* is an Old World genus with no close relatives in the Americas. Additionally, *A. donax* appears to be asexual in the New World and thus has limited evolutionary potential to evolve resistance to control agents. Finally, several insect species are known to be monophagous on giant reed in the Old World (Boland 2006; Tracy and DeLoach 1998).

The objectives of this study were to use microsatellite data from New World (introduced) and Old World (native) accessions to (1) determine levels and geographic pattern of genetic diversity in introduced and native populations, (2) evaluate whether spread within local areas occurs mainly through clonal propagation or through sexual reproduction, (3) evaluate whether genotypes of *A. donax* appear to have been introduced to North America once or multiple times, and (4) narrow down the geographic origin(s) of North American populations.

Materials and Methods

Study System. The body of existing literature supports a hypothesis that *A. donax* had its ultimate origin in Asia, was subsequently introduced into the Mediterranean, then was spread worldwide (Ahmad et al. 2008; Bell 1997; Dudley 2000; Frandsen 1996; Mariani et al. 2010; Perdue 1958). Giant reed was likely introduced in North America during colonization by the Spanish, possibly for use as a lightweight building material and roof thatching (Frandsen 1996; Goolsby and Moran 2009; Goolsby et al. 2009). By the early 1800s, *A. donax* was already abundant in the Los Angeles River Basin of California (Bell 1997; Dudley 2000; McGaugh et al. 2007).

Arundo donax is a polyploid with documented chromosome counts of 60, 72, 108, and 110 (Connor and Dawson 1993; Gould and Shaw 1983). It is considered to be asexual in the New World and throughout much of its global distribution, where viable seeds have not been observed (Bell 1997; Boland 2006; Mariani 2010; Perdue 1958; Sharma et al. 1998). Seed sterility is the result of a failure of the megaspore mother cell to divide (Bhanwra et al. 1982). However, rare germination from seed has been documented in India and the Middle East (Bell 1997; Boland 2006; Mariani 2010; Perdue 1958; Sharma et al. 1998). *Arundo donax* is known to spread vegetatively through fragmentation of the stems and rhizome and through layering (Boland 2006). *Arundo donax* is considered one of the world's 100 worst invasive alien species (Lowe et al. 2000).

In the United States, the Southwest is experiencing the most severe economic and ecological problems with *A. donax*, particularly in riparian areas in Texas and California (Tracy and DeLoach 1998). *Arundo donax* can grow up to several inches a day and can be very aggressive, displacing native vegetation, such as cottonwoods and willows, in riparian habitats (Bell 1997; Perdue 1958). Along with the destruction of native habitat, invasion by *A. donax* leads to the loss of biodiversity. For example, it has been shown that overall biodiversity, and arthropod diversity in particular, are lower in stands of *A. donax* than in native vegetation (Herrera and Dudley 2003). Giant reed has also been implicated as the main cause of the extinction of the Rio Salado darter (*Etheostoma segrex* Norris and Minckley), a small endemic fish of the Cuatro Ciénegas nature reserve in northern Mexico (McGaugh et al. 2007). Cuatro Ciénegas is a UNESCO Biosphere Reserve, and *A. donax* is one of major threats to this ecologically unique area (McGaugh et al. 2007). Another direct consequence of giant reed infestation is an increase in the risk of catastrophic fires (Bell 1997). In particular, it is thought that the biomass produced by *A. donax* could increase the frequency and intensity of wildfires during the Santa Ana wildfire season in Southern California (Bell 1997; Scott 1993). However, the most important consequence of invasion by *A. donax* could be its water usage (McGaugh et al. 2007). The potential impact of water loss is most critical in areas with limited water resources, such as the Rio Grande Basin (RGB). Nearly 600 river kilometers of the Rio Grande have been invaded by giant reed, with the heaviest infestations between the Texas/Mexico border cities of Laredo and Del Rio (Everitt et al. 2005; Moran and Goolsby 2010a,b).

Recent efforts to identify potential biocontrol agents for *A. donax* have uncovered four highly specific phytophagous insects, including a wasp (*Tetramesa romana* Walker), an armored scale (*Rhizaspidiotus donacis* Leonardi), a chloropid fly (*Cryptonevra* spp.), and a leafsheath miner (*Lasioptera donacis* Coutin & Faivre-Amiot) (Goolsby and

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Table 1. A list of the countries sampled and the number of accessions tested from each region.

Country sampled	No. of accessions
New World	
United States	125
Texas/RGB	105
Southeast U.S.	12
California/Nevada	8
Mexico	29
Argentina	5
Old World	
Spain	132
Turkey	6
Israel	3
Greece	6
Italy	24
Portugal	11
Morocco	3
France	6
Algeria	12

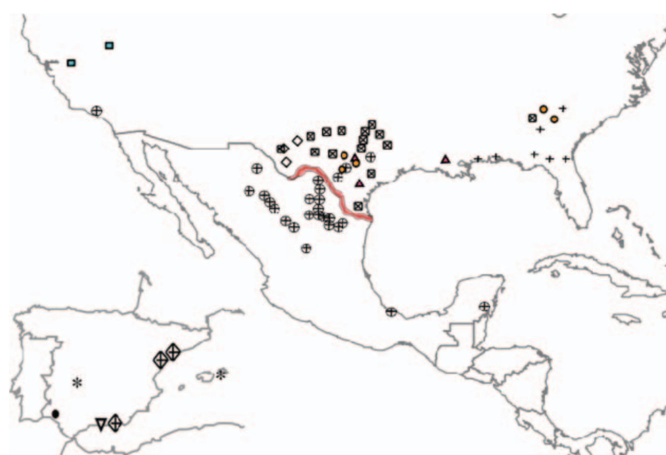

Figure 1. Distribution of groups I–VII in North America with an inset map of Spain locating the closest matches to the group I lineage. The colors and symbols correspond to the groups in Figures 3 and 4.

Moran 2009; Goolsby et al. 2009; Moran and Goolsby 2010a,b; Tracy and DeLoach 1998).

Sample Collections. On the basis of historical, botanical, and ecological evidence, our search for the proximal source(s) of *A. donax* invasions of the Rio Grande Basin was focused on Mediterranean Europe and North Africa. In total, 362 samples (203 Old World and 159 North American samples) collected from 2000 to 2009 were included in this study, with a major focus on the Mediterranean region and the RGB. Sample information and collection locations are available in Table 1 and in Supplemental File 1. Distributions of samples from North America are shown in Figure 1.

To evaluate whether clonal propagation or sexual reproduction dominates in local populations, three sites (two New World and one Old World) were sampled more intensively. Multiple sampling of plants in relatively close proximity was performed along a 35-km transect from Santa Barbara to the Ventura River near the California coast (5 plants), along a 13-km transect on the Rio Grande River near Eagle Pass, TX (14 plants), and in a cluster near Latran, Israel (6 plants). *Arundo donax* and *Phragmites australis* (Cav.) Trin. ex Steud. are both very large grasses in the subfamily Arundinoideae and appear very similar when not in flower. For this reason, several reference samples of common reed, *P. australis*, were also collected and tested by microsatellite amplification and genotyping to rule out any mis-collection of samples.

DNA Isolation. A 2.5-cm^2 piece of leaf tissue was macerated using the BioMasher homogenizer (BioMasher catalog no. 40010-050, Cartagen Inc.) with incubation in 500 μl of isolation buffer (200 mmol/L Tris pH 7.5, 250 mmol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid pH 8, 0.5% sodium dodecyl sulfate) for 1 h at 60 C followed by the DNA isolation procedure described in Pepper and Norwood (2001).

Microsatellite Amplification. The biotinylated capture protocol described in Terry et al. (2006) was used for microsatellite primer development for *A. donax* (Table 2). Forward primers were tagged with either a 5′ HEX or 5′ FAM fluorescent dye. Polymerase chain reactions were carried out using Phusion (Phusion High Fidelity DNA Polymerase [F-530], New England Biolabs) polymerase. The final reaction volume was 13 μl and consisted of 0.125 μl of Phusion, 2.5 μl of 10× concentration HF buffer, 8.025 μl of dH_20, 0.0625 μl of 25 mmol/L dNTPs, 1.25 μl of primer at a concentration of 10 pmol/μl, and 20 ng of DNA. The PCR conditions were 1 cycle of 98 C for 30 s, 30 cycles of 98 C for 10 s, 55–62 C for 30 s, and 72 C for 15 s, and a final extension step at 72 C for 5 min.

Before capillary electrophoresis, the products were electrophoresed on a 4% agarose gel and stained with ethidium bromide. On the basis of the intensity of the ethidium bromide–stained bands, the PCR products were diluted in a range from 1 : 10 to 1 : 75 with dH_2O. One microliter of diluted PCR reaction was then added to 9 μl of HiDi (HiDi Formamide, Applied Biosystems) formamide and 0.1 μl of Rox dye 400HD (Applied Biosystems) size standard dye from Applied Biosystems, multiplexed to include a HEX- and a FAM-labeled reaction, then run on an ABI 3130 XL DNA fragment analyzer. Peak sizes for

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Table 2. The primer sequences, annealing temperature, expected fragment size range, and GenBank accession number for each microsatellite primer pair.

Locus name	Primer sequences[a]	GenBank accession No.	Size range	Annealing temperature
			bp	C
Ad_G8	G8-F: CCATGTAGAGACAAATCGGAAAG G8-R: CAATAGTTCCAATATCAAACGTATCC	HQ224521	132–155	55
Ad_B7	B7-F: CTATGCATAAGTTTAGATCTACAACTAG B7-R: GGTTTTGGCGACAATAAGATAGTTC	HQ224523	134–196	55
Ad_A3	A3-F: CACAGGCGCGTGATTGACC A3-R: CAGATCCGACAGGACGAGATG	HQ224519	102–149	55
Ad_B1	B1-F: CATGGATGCCAACTCCTTCAAC B1-R: GGAAATACTTCATTCTAGTTAGAGATAGA	HQ224518	103–161	55
Ad_E10	E10-F: GCTTCATATGTTTTGTCCCAACTC E10-R : CAATCCTCGCCAGATCCAAGC	HQ224522	101–137	60
Ad_F8	F8-F: TAGTGGTGACATGTGGCTTACC F8-R: GGTTGGGCATGCACACGG	HQ224527	98–142	55
Ad4-13	Ad4_13F: TAGTAAGGCACCACGCAGCAC Ad4_13R: TTGGTCTAACTGAGAATGGGTTTC	HQ224526	90–125	55
Ad4-6	Ad4_6F:AGGAGTCCAATTTAACAGGAAAAGG Ad4_6R: GGGTTTCTCTCTAGTAGATTGATTAG	HQ224524	157–189	62
Ad_H1	H1-F: GCTGGACATAGAAGCCGTTTTTG H1-R: GCTTCCTTGGAAGTGAGGCAG	HQ224520	121–159	62
Ad5-4	Ad5_4F: CGCGTATGATCGTCCACCG Ad5_4R: ACGACCACACGCATTGCTCTG	HQ224525	90–144	55

[a] F, forward; R, reverse.

each fragment were called using a common algorithm implemented in Genescan 3.7 and Genotyper 3.7 (Applied Biosystems) and Peak Scanner Software v.1.0 from Applied Biosystems. To ensure that the fragment analysis programs scored the fragments identically, several samples were used to cross-calibrate the fragments for each program.

Data Analysis. Assessing the genetic diversity of clonal polyploid organisms like *Arundo* poses unique challenges because most conventional population genetic measures are not readily applicable because of locus duplication and the absence of sexual reproduction. GenoDive Population Genetics Program is a software package developed for the analysis of molecular data from polyploid organisms (Meirmans and Van Tienderen 2004). To quantify the genetic diversity in *A. donax* in native and introduced populations, the total number of alleles, the number of genotypes, Nei's genetic diversity (Div) and Shannon's corrected index (Shc) were calculated. The significance of differences in total number of alleles between the Old World and New World was evaluated using Fisher's Exact Test.

Determining whether individual ramets are part of the same genet (i.e., spread through clonal propagation) vs. close siblings that arise from sexual reproduction is difficult (Arnaud-Haond et al. 2007). In the complete absence of mutation, individuals from the same clone should harbor exactly the same multilocus genotype. In practice, however, technical errors in the genotyping and somatic mutation can lead to an observation of low levels of genetic diversity among individuals within a single clone, even in the complete absence of any sexual reproduction (Arnaud-Haond et al. 2007; Douhovnikoff and Dodd 2003; Meirmans and Van Tienderen 2004). To resolve the issue of clonal assignment, we employed a frequency distribution of pairwise distances to assign multilocus genotypes (that may be different solely because of somatic mutation or scoring errors) into putative clonal groups (Arnaud-Haond et al. 2007; Douhovnikoff and Dodd 2003; Meirmans and Van Tienderen 2004; Rogstad et al. 2002). We employed this method, as implemented by the GenoDive software package (Meirmans and Van Tienderen 2004), to create a frequency distribution of pairwise genetic distances using an infinite alleles model (IAM). When such a distribution is bimodal, individuals from the genetically closer pairwise distances are assumed to be clonemates, and a threshold number of genetic differences for clonal assignment is estimated (Meirmans and Van Tienderen 2004); this threshold value estimates an upper limit to the amount of mutation and genotyping error between clonemates.

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To evaluate whether genotypes of *A. donax* appear to have been introduced to North America once or multiple times and to narrow down the geographic origin(s) of North American populations, we employed several complementary analytical methods to detect and visualize patterns of genetic differentiation and clustering among *A. donax* accessions. These included NJ, PCO, and STUCTURE analyses of the genetic data matrix generated by GenoDive. Genetic differentiation of New World plants, as well as the association of certain clusters of New World plants with different Old World accessions, would be the expected hallmarks of multiple introductions from different Old World sources.

Because of the polyploid nature of *A. donax* and associated difficulties with assigning specific fragments as alleles at a specific locus, all fragment sizes were entered in GenoDive and were treated essentially as "allele phenotypes" (Saltonstall 2003). An individual clonal genetic distance measure was selected for the analysis with the IAM because this model is preferred with genetically or geographically related populations, or both (Weising et al. 2005). Individuals with missing data were excluded from the analysis. The resulting genetic matrix was entered into the R statistical package (The R Project for Statistical Computing) and a neighbor joining (NJ) analysis was performed using Analysis of Phylogenetics and Evolution (APE) v.2.5 (The R Project; Paradis 2004; R Development and Core Team 2009). A bootstrap analysis of the data set employing 1,000 replicates was carried out using APE. Additionally, R was used to carry out a principal coordinates analysis (PCO) on the genetic data matrix.

To address the geographic pattern of genetic differentiation in *A. donax*, a standard Mantel test was implemented in GenoDive using the individual clonal genetic distance and geographic distance measures as calculated by GenoDive from our GPS collection data (Meirmans and Van Tienderen 2004). Three different Mantel tests were done with 1,000 permutations each: (1) a New World test, (2) an Old World test, and (3) an overall test with the entire data set. STRUCTURE v.2.3.3 was also used to identify genetic clustering of New World *A. donax* (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000). To determine the correct number of K clusters in the New World, the procedure described in Evanno et al. (2005) was implemented. The total burn-in for the STRUCTURE analyses was set at 10,000 and was run for 100,000 iterations. STRUCTURE was implemented with the assumptions of no admixture and correlated allele frequencies.

Results and Discussion

Genetic Diversity. A total of 10 microsatellite primer pairs were used to amplify products from 362 samples (Tables 2 and 3). None of the 10 markers amplified in the *Phragmites* samples, thus eliminating the possibility of error due to misidentification. Each primer pair amplified two or three homeologous loci that were discernible by size (e.g., as many as four to six fragments were obtained per primer pair from a single individual), but it was not possible to assign fragments (alleles) conclusively to specific homeologous loci.

Table 3. The genetic diversity of *Arundo donax* worldwide compared with the diversity of *A. donax* in the introduced range.

	No. of alleles	
Marker	Old World	North America
A3	21	18
B7	29	16
F8	15	7
E10	19	15
Ad5-4	15	12
B1	24	13
G8	15	7
Ad4-13	17	9
H1	18	9
Ad4-6	18	13

Genetic diversity, as assessed by the total number of alleles, was lower in the introduced range compared with that in the Old World (Table 3), and this difference was significant (Fisher's Exact Test, $P = 0.0023$). All of the genotypic diversity measures, including Div and Shc showed that there has been a substantial reduction in the overall genetic diversity of the introduced *A. donax* in the New World compared with that of the Old World, strongly implying a limited number of introductions to the New World (Table 3). Genetic diversity was 0.243 for the New World and 0.929 for the Old World. Shannon's corrected index was 0.266 for the New World and 2.218 for the Old World.

Overall, this study found more genetic variation in *A. donax* than previous studies using other marker systems. Other studies of *A. donax* uncovered low genetic variation using isozymes and random amplified polymorphic DNAs (RAPDs) (Khudamrongsawat et al. 2004), RAPDs only (Lewandowski et al. 2003), sequence-related amplified polymorphisms, and transposable element–based markers (Ahmad et al. 2008), intersimple sequence repeats (ISSRs), and amplified fragment length polymorphisms (AFLPs) (Mariani et al. 2010).

Multiple sampling of plants in relatively close proximity was performed along a 35-km transect from Santa Barbara to the Ventura River near the California coast (5 plants), along a 13 km transect on the Rio Grande River near Eagle Pass, TX (14 plants), and in a cluster near Latran, Israel (6 plants) (Table 4). In these localized samplings, genetic

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Table 4. Comparison of the number of genotypes, Nei's genetic diversity (Div), evenness (Eve), and Shannon's corrected index (Shc) for the New World and the Old World. Also included is a test of clonality of three separate genets from three separate locations.

Population	Size (n)	No. of genotypes	Div	Eve	Shc
Old World vs. New World genotypic diversity					
New World	159	6	0.243	0.220	0.266
Old World	203	129	0.929	0.120	2.218
Within-population genotypic diversity					
Latran, Israel	6	1	0	1	0
California	5	1	0	1	0
Eagle Pass, TX	14	1	0	1	0

diversity indices showed that within each location, the sampled plants shared a single multilocus genotype. These results imply that our frequency of scoring errors was relatively low and indicate that all of the sampled plants in each geographic location were members of the same gamet, thus providing supporting evidence for the clonal nature of *A. donax* propagation in the New World.

Putative Clonal Groups. The frequency distribution of pairwise genetic distances generated by GenoDive showed a bimodal distribution, and the threshold for clonal assignment was set at seven mutational changes (Figure 2). Using this cut-off, a total of 134 clones were detected among the 362 global accessions, with only six clonal groups detected in the New World. One New World clonal lineage had a large geographic distribution that included Texas, Mexico, and California.

Although some presumptive clonemates had nearly identical genotypes, no two plants had an identical match at all loci. Potential sources of genetic diversity within clonally related groups include somatic mutation, sexual out-crossing (presumably rare), and inevitable errors in genotyping (Dewoody et al. 2006; Pompanon et al. 2005). We expected low genotyping error rates because we used the highly processive, blunt-end producing Phusion polymerase, which greatly reduces "stutter" and eliminates 3′ single-nucleotide addition, two of the major sources of error in microsatellite genotyping (Dewoody et al. 2006; Ginot et al. 1996; Pompanon et al. 2005). Furthermore, we obtained indirect evidence suggesting a very low genotyping error rate in our analysis of localized clusters of plants along the California coast, on the Rio Grande River near Eagle Pass, and in Latran, Israel. Within each locality, samples gave identical multilocus genotypes (i.e., no unexpected differences among the 25 plants sampled). Given that we used 10 primer pairs, which we estimate to have amplified a total of $\sim$ 25 loci (250 loci total), these results suggest that we may have achieved a very low error rate ($<$ 0.16%).

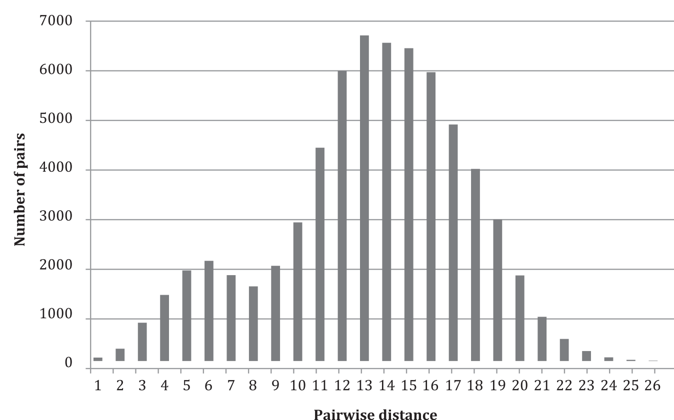

Figure 2. Histogram of the number of pairs of individuals of *Arundo donax* and clone classes at each pairwise distance generated by GenoDive.

An alternative source of variation within clones is somatic mutation. Among plants that reproduce asexually, microsatellite mutation rates vary from negligible to hypermutable depending on the individual species and locus examined (Cloutier et al. 2003; Lian et al. 2004; Mes et al. 2002; O'Connell and Ritland 2004). Between individuals in our data set that were assigned as clonemates, the average divergence was $\sim$ 4.6 mutational changes. Assuming $\sim$ 250 loci queried, and an $\sim$ 400-year period since the peak of Spanish colonization of North America, a microsatellite mutation rate $\sim 4.6 \times 10^{-4}$ per locus year would explain the observed divergence among putative clones. In the literature, there are no comparable estimates of somatic microsatellite mutation rates in organisms that reproduce by rhizomes. However, given that rhizomatous growth recurs annually (and continues throughout a very long growing season throughout much of the introduced range of *A. donax*), the number of cell divisions in the meristem initials per year could be comparable to the number of cell divisions in the apical meristems of annual plants, where estimates of microsatellite mutation rates range from 3.9×10^{-3} per locus generation in chickpeas (Udupa and Baum 2001), to 2.43×10^{-4} in wheat (Thuillet et al. 2002), and 7.7×10^{-4} in maize (Vigouroux et al., 2002). Thus, the observed genetic variation within putative clones could be entirely attributable to some

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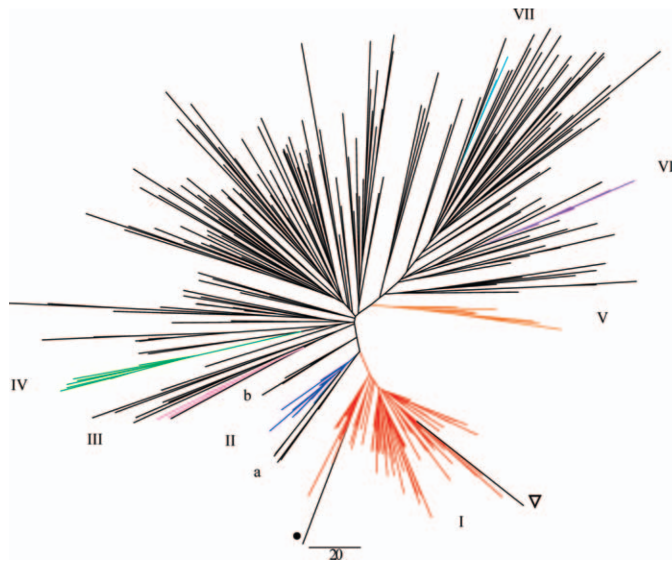

Figure 3. Neighbor joining (NJ) tree of individual genetic distance measure from GenoDive. Group I (red) includes plants from the RGB, Mexico, Argentina, and California. Group II (blue) contains plants mostly from Central Texas. Group III (pink) is composed of two plants from Texas and one individual from Louisiana. Group IV (green) includes only West Texas individuals. Group V (orange) is the most diverse group, containing plants from Texas, Georgia, and the Canary Islands. Group VI (purple) is from the southeastern United States, and group VII (cyan) is made up of one individual each from California and Nevada. The black lines indicate Old World collections. Groups a and b are made up of Spanish plants. Spanish plants from Almunecar (•) and Seville (∇) clustered with group I plants.

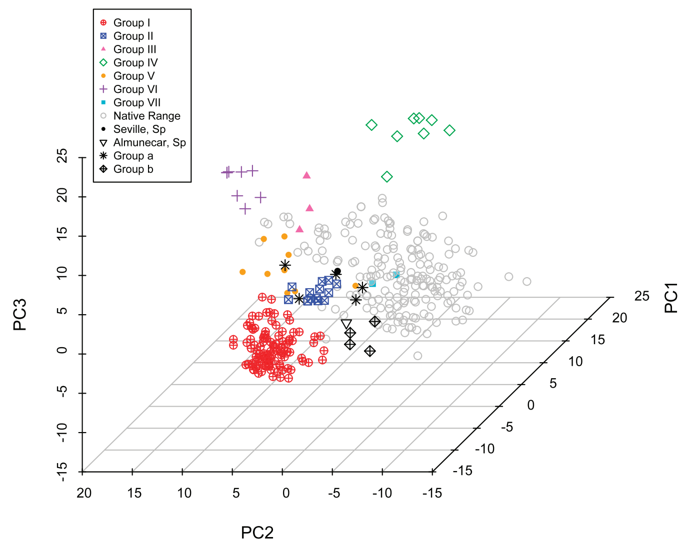

Figure 4. Scores plot of principal coordinates (PCs) 1 to 3. PC1 explained 9.95% of the variation, PC2 explained 4.17% of the variation, and PC3 explained 2.9% of the variation. Symbols and colors correspond to those in Figure 3.

combination of genotyping error and somatic mutation, without having to invoke sexual reproduction as an explanation.

NJ Analysis. The NJ tree (Figure 3) indicated as many as seven genetic clusters of *A. donax* in North America. These New World clusters were designated groups I through VII. Group I, the most geographically widespread group, included all accessions from Mexico, the RGB of Texas, and California. Group II consisted mostly of plants found in central Texas, along with one individual from Georgia, and two individuals from West Texas. Group III contained plants from two Texas locations and a single plant from Louisiana. Plants collected from West Texas composed group IV. Group V contained plants from Central Texas, Georgia, and the Canary Islands. Group VI was limited to plants collected from the southeastern United States. Group VII comprised one plant each from Putah Creek and California and Silver Springs in northern Nevada; collectively, these two accessions were the northernmost New World plants in our study.

Importantly, the New World groupings obtained in the NJ analysis corresponded exactly to the putative clonal assignments made by an examination of the distribution of pairwise genetic distances, with the exception of groups I and II, which appear distinct in the NJ topology but are combined in the clonal assignment test. Unfortunately, bootstrap support values above 50% were limited to small clades of plants that were in close geographic proximity (Supplemental Figure 1).

PCO Analysis. The PCO analysis (Figure 4) also supported multiple genetic clusters of *A. donax* in the New World. The first three axes were responsible for 17% of the variation found in the data set. Each of the groups from the NJ analysis (Figure 3) mostly formed distinct clusters in the PCO analysis, while the Old World plants were scattered throughout the plot (Figure 4). This was consistent with the high amount of genetic diversity found throughout the Mediterranean. Importantly, several accessions from Spain clustered closely with New World groups in the NJ and PCO analyses (Figures 3 and 4). The closest matches to group I (RGB) in the NJ tree were from Seville and Almunecar, Spain. Old World groups a and b, which include Spanish accessions from Menorca, Mengabril, Barcelona, Coloma, and the Rio Guadalmedina, also clustered closely to groups I (RGB) and II (central Texas) in both analyses (Figures 3 and 4).

Mantel Test. Mantel's r for the entire data set was 0.189 (P = 0.001) in the Old World plants only, r was 0.091 (P = 0.022), and the New World r was 0.222 (P = 0.004). The low values for Mantel's r in both Old World and New

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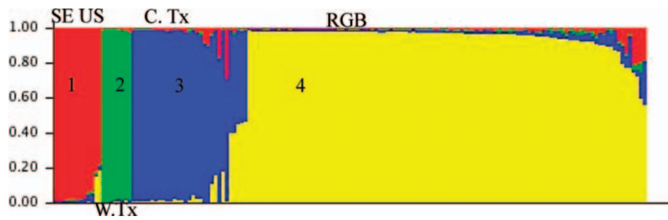

Figure 5. Probabilities derived from STRUCTURE that assign the 159 New World *Arundo donax* genotypes into four distinct genetic clusters that agree with the NJ analysis and the PCO analysis. The four genetic clusters correspond to the regions that were the most thoroughly sampled in North America.

World plants indicate a low correlation between genetic and geographic distances and may be due to anthropogenic activities such as human-assisted long distance transport of *A. donax*. The far greater age of the Old World populations would allow more time for both human transport and clonal divergence. Therefore, it is not surprising that Mantel's r is higher in the New World than in the Old World. In the NJ and PCO analyses of Old World plants, there were no genetic groupings corresponding to medium or large geographic areas (e.g., North Africa, the Middle East, or Iberian Peninsula). However, Old World plants that were collected from very nearby locations were typically closely related in genetic tests and grouped closely together in the NJ analysis. For example, the few groups that were supported by the bootstrap analysis in the NJ were generally collected from very proximal geographical locations (Supplemental Figure 1). Similarly, previous AFLP and ISSR analyses of *A. donax* and other *Arundo* species did not show a correlation between genetic and geographic distances in the Old World, suggesting a historical pattern of widespread dispersal, perhaps due to flooding or anthropogenic activities (Mariani et al. 2010).

STRUCTURE Results. The optimal value for K obtained from the STRUCTURE analysis of New World plants was 4. The major groups (I, II, IV, and VI) generally corresponded with those obtained in the NJ and the PCO analyses (Figure 5). The smaller divergent groups were included within the larger geographical clusters. Groups III and V were clustered with Group II, whereas Group VII was included with Group VI. The major clusters in the STRUCTURE analysis correspond to the individual groups that were the most heavily sampled in the New World. Thus, all three of the analytical methods we employed (NJ, PCO, and STRUCTURE) gave consistent results: there is clear genetic differentiation of New World accessions into distinct genetic clusters, thus indicating the introduction of multiple distinct genotypes.

Old World Origins of *A. donax* Invasion in the Rio Grande Basin. No exact genetic matches to Old World locations were found for any of the *A. donax* accessions surveyed in North America. However, a close match of Old World plants to the main RGB group was obtained from Almunecar, on the southern (Mediterranean) coast of Spain. Moreover, plants collected from the northeastern (Mediterranean) coast of Spain near Barcelona also grouped closely with the plants from the RGB (Figures 3 and 4). Importantly, this group of Spanish accessions and the RGB cluster of plants were all assigned to a single putative clone in the clonal assignment analysis.

Most of the accessions (98.2%) collected in the New World from group I (RGB) carried the private allele H1-145, and 75.5% had the globally rare B7-170. These markers are among the most polymorphic markers used in this study (Table 2), and individuals carrying both alleles are not seen anywhere outside of group I. The combination of these alleles in the vast majority of the RGB plants makes it an excellent hallmark for this group of plants. Outside of the RGB, H1-145 and B7-170 are found in plants from central Texas, California, several Mexican states, and Argentina. However, H1-145 was absent from any of the Old World plants surveyed, including clonemates of group I (RGB). This finding suggests that the precise population(s) of the origin of the RGB invasion (1) might not have been sampled, (2) might have been extirpated since it was transported to the New World, (3) might have undergone evolution at the H1 locus since introduction into the New World, or (4) some combination of the above scenarios.

Considered together, NJ, PCO, and STRUCTURE analyses all suggested that the group I invasion in the RGB was either the introduction of a single clone or, alternatively, single or multiple introductions of a set of very closely related clones from the western Mediterranean region. The clonal assignment test supports the model of a single introduction of a single clone. The limited, but nonetheless observed, genetic diversity within the RGB (group I) suggests the possibility that mutation at the microsatellite loci may have occurred in the ca. 400 yr since these plants were introduced. This would be consistent with the known rapid evolution of microsatellite markers (Avise 2004).

Delineation of Other Likely Introductions in North America. The observation of several well-differentiated clusters in North America, combined with the likely clonality of *A. donax*, imply multiple introductions from different sources. Unlike the RGB group I, the additional introductions into North America appear to be limited in scope. Group II was mostly centered near the Edwards Plateau of Central Texas. This lineage also contained plants from West Texas, South Texas, and Georgia (Figures 3 and 4). Group II contained one of the rare alleles (B7-170) also found in group I, and both NJ and PCO analyses

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(Figures 3 and 4) show that this group is closely related to group I. The absence of the H1-145 allele suggests that either (1) group II was a separate introduction from that of the group I plants, but from a genetically similar western Mediterranean (Spanish) origin, or (2) groups I and II diverged from each other soon after transport into the New World.

Group III is a small group of three plants found in Texas and Louisiana. In the NJ tree (Figure 3), these plants were grouped together, but in the PCO analysis (Figure 4) these plants did not form a distinct group. Group IV consisted solely of plants from West Texas and contained a private allele (B7-134). The closest matches to group III and group IV plants were found in the eastern Mediterranean in Israel. According to the PCO analysis (Figure 4), group IV is the furthest removed from all the other groups. Perdue (1958) stated that *A. donax* was introduced by the U.S. Department of Agriculture (USDA) into various regions of the United States from outside the Mediterranean in the 1940s. The limited extant range of group IV points to a recent introduction with little or no ongoing dispersal. Interestingly, group V contained putative clonemates from the Canary Islands, Central Texas, and Georgia. The Canary Islanders (Los Isleños) were considered by the Spanish to be hardy and adaptable, and were often the vanguard settlers in new Spanish Colonies (Weber 1994). The Canary Islands figure prominently in the history of central Texas as Isleños settlers arrived in Texas in 1731 to establish San Antonio (Dunmire 2004; Weber 1994). Individual ramets from group V were collected in Johnson City, Austin, and San Antonio. Two ramets from Georgia also clustered in the Canary group, suggesting an early introduction by Isleños that settled in the southeastern United States, then the Spanish colony of Florida (Weber 1994). Group VI plants were found solely in the southeastern United States. In the NJ and PCO analyses (Figures 3 and 4), these individuals form distinct groups that indicate a separate introduction and grouped with plants from Colepasso, Italy, and from Talavera, Spain. The group VII lineage was only observed in California and Nevada and are grouped with plants from Seville, Spain.

Summary and Implications. The genetic similarity—and possible clonality—of all *A. donax* in the RGB has implications for the ongoing biological control program. Although a precise origin has not been found for the RGB lineage, reduced genetic diversity in the RGB means that a very limited sampling of biocontrol agents from closely related Old World populations can potentially be used effectively to control the RGB plants. Several U.S states that were sampled had plants that were likely the result of additional introductions, with as many as five different putative lineages in Texas, two in California, and three in the southeastern United States. Interestingly, only group I (RGB) plants were found in all parts of Mexico that were sampled, including the Mexican states of Durango, Zacatecas, Veracruz, and Quintana Roo. The presence of only one genetic group in Mexico means that a limited sampling of biocontrol agents could be effective both in the RGB and other parts of Mexico. This finding is also important because the presence of diverse genotypes across Mexico would have complicated the development and release of biocontrol agents because of possible tolerant or resistant genotypes of *A. donax* that could mount a secondary invasion of the RGB.

Given its distribution, it is most likely that the RGB group of New World plants, group I, was introduced early in the colonization of the New World. The Rio Grande River was one of the major corridors of entry for the Spanish conquistadores and settlers into North America. Don Juan Oñate, a conquistador, was among one of the first settlers into modern day New Mexico, and he built settlements along the Rio Grande in the late 1500s (Dunmire 2004; Weber 1994). Mission San Juan Bautista, located across the river from Eagle Pass, Texas, was the final stop for colonists before entry into central Texas (Dunmire 2004; Weber 1994). The likely common origin of all *A. donax* in the RGB simplifies the collection and evaluation of biocontrol agents from Mediterranean Spain.

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