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Genetic diversity of Persian walnut (*Juglans regia*) in the cold-temperate zone of the United States and Europe

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ABSTRACT

We compared the genetic diversity of *Juglans regia* L. growing in the cold temperate region of the eastern U.S. with *J. regia* growing in the cold-temperate and Mediterranean regions of Europe. Ten microsatellite (SSR) loci were used to assess the genetic relationships among 114 total trees originating from the Midwestern USA (n = 34), Hungary (n = 30), Slovakia (n = 28), and Italy (n = 22). All SSR loci were highly polymorphic with an average of 7.4 alleles per locus. All 114 trees were confirmed to be unique genotypes. Cluster analysis using Neighbor-joining (NJ) placed genotypes according to their geographic origin. STRUCTURE software confirmed the results of the NJ analysis and produced three main groups consistent with the geographic origins of the samples. According to Nei's genetic identity, samples from Slovakia and Hungary showed the highest similarity (0.94), while samples from the USA and Hungary had the lowest similarity (0.37). The genetic diversity of *J. regia* from the USA and Eastern Europe was relatively high compared to Italian samples. We found little genetic similarity between *J. regia* sampled from the eastern USA with *J. regia* currently growing near the Carpathian Mountains. There was strong evidence for a bottleneck in the U.S. population, but no evidence of inbreeding.

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1. Introduction

Interest in growing *J. regia* in the cold temperate region of North America dates back to British Colonial times (Grimo, 1979). Early seed sources were brought to North America by German immigrants in the 18th and 19th centuries along with germplasm from Eastern Europe, the Himalayan Mountains, and northern China (Grimo, 1979; Goodell, 1984). More recently, large quantities of seed were imported from the Carpathian Mountains of southern Poland by the Reverend Paul Crath (Grimo, 1979). These introductions proved especially cold hardy and led the common name "Carpathian walnut" to be applied to all cold hardy Persian walnuts growing in cold temperate regions of North America (McDaniels, 1977; Goodell, 1984; Beddes et al., 2011). Crath planted and selected trees in the USA and Canada from the 1930's to the 1950's (Wertis, personal communication, 2015). Unfortunately, the number of original accessions, their pedigree, and any records of their diversity are absent.

J. regia hybridizes with *J. nigra* L. to produce a vigorous F1 hybrid known as *J. × intermedia* (McKay, 1965). These hybrids are being bred and deployed for timber production in Europe (Aleta, 2004) and the United States, where the Hardwood Tree Improvement and Regeneration Center (HTIRC) maintains a number of *J. × intermedia* accessions. The wood of *J. intermedia* is nearly indistinguishable from black walnut in terms of grain and texture, although its heartwood is lighter in color. The cold hardiness of these hybrids exceeds the most cold-hardy Carpathian, which can tolerate temperatures down to −37 °C without injury (Mitra et al., 1991; Domoto, 2002; Barkley, 2007). Nevertheless, limited cold hardiness in the most elite germplasm restricts its commercial cultivation in North America to the temperate Mediterranean climate of California. The addition of new, cold-hardy Persian walnut germplasm could help fill the need for more cold tolerant cultivars for landowners in cold-temperate parts of North America, as well as the development of more cold-tolerant hybrids for timber production.

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Simple-sequence-repeats (SSRs) are highly polymorphic, neutral markers that have proven useful for the identification of parentage, for measuring pollen flow, and for discerning the population structure of *Juglans* (Dangl et al., 2005; Victory et al., 2006; Aradhya et al., 2010; Pollegioni et al., 2011; Ebrahimi et al., 2011) as well as for many other species (Bai et al., 2007; Losada et al., 2010; Billot et al., 2013; Nicolai et al., 2013; Wang et al., 2015). In this study we used SSR markers to determine and compare the genetic diversity and genetic similarity of *J. regia* from cold temperate USA with samples from two locations in Eastern Europe and cold sensitive *J. regia* from Italy. Our objectives were: (1) to determine the genetic similarity of North American Carpathian *J. regia* to European Carpathian germplasm, and (2) to characterize the genetic diversity of HTIRC *J. regia* germplasm to aid breeding of improved walnut for cold-temperate North America.

2. Materials and methods

2.1. Plant materials

Thirty-four unique grafted and seedling *J. regia* accessions were provided from the HTIRC at Purdue University *Juglans* germplasm collection and from members of the Indiana Nut and Fruit Growers Association. Thirty-three elite trees derived from northeast and central Hungary were provided from the gene bank collection of the Research Institute for Fruit in Budapest, Hungary. Twenty-five naturalized trees collected from local orchard and as feral trees in the lowlands in Slovakia were also sampled. Genotypes from Hungary and Slovakia represented present-day native Carpathian walnut. Finally, 22 cold-sensitive Italian genotypes reported by Foroni et al. (2007) were included to contrast with the cold-hardy populations (Table 1).

2.2. Genomic DNA extraction, PCR amplification and PCR product analysis

Genomic DNA was extracted from leaves (Hungary and Slovakia samples) using a CTAB buffer (Doyle and Doyle, 1987). DNA was extracted from twigs (U.S. samples) using a modification of the CTAB buffer protocol as described in Ross-Davis and Woeste (2008). PCR was performed using primer pairs to amplify each of 10 SSR loci (WGA1, WGA9, WGA27, WGA32, WGA69, WGA89, WGA118, WGA202, WGA276 and WGA321), originally described by Woeste et al. (2002) and Dangl et al. (2005). PCR conditions followed Pollegioni et al. (2014). Allele size was scored using Genemapper software (Applied Biosystems). Data for the Italian population was originally published by Foroni et al. (2007).

2.3. Genetic diversity analyses

Genetic diversity was based on the total number of observed alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E). The effective number of alleles (N_E), Shannon's information index (I), Nei's genetic distance, Nei's genetic similarity and identity (Nei, 1972), allelic richness (Ra) and the occurrence and frequency of private allele (Pa) were computed for each sample group using POPGENE version 1.31 software (Yeh et al., 1997), CERVUS version 2.0 software (Marshall et al., 1998) and Hp-rare software (Kalinowski, 2004). The statistical significance of F_{IS} was tested using Arlequin 3.11 software (Excoffier et al., 2005).

Mega software v.6 was used for Neighbor-joining cluster analysis based on Nei's genetic distance (Tamura et al., 2012). The program STRUCTURE 2.3.4 was used to analyze the genetic structure of the sampled populations (Pritchard et al., 2000). This program assigns individuals to a number (K) of genetically homogeneous groups based on a Bayesian estimate in accordance with the expected Hardy–Weinberg proportions assuming the absence of linkage disequilibrium between the loci in each group. For the analyses with STRUCTURE, we used a standard model with admixture, allele frequencies were assumed to be correlated. We allowed a burn-in period of 100,000 repeats followed by 250,000 Markov Chain Monte Carlo (MCMC) replications (Marinoni et al., 2013). Ten replicate runs were performed for each possible value of K (where K has values ranging from 1 to 10). The model developed by Evanno et al. (2005), based on the second order rate of change in the log probability (ln ΔK) of data between successive K values (the *ad hoc* ΔK test), was used to clarify values of K. The results from STRUCTURE were processed with the software STRUCTURE HARVESTER v.0.6.1 (Earl and VonHoldt, 2012) to obtain the most likely K value.

2.4. Bottleneck analysis

Reduction of Persian walnut genetic diversity caused by a sudden reduction in population size was tested with Bottleneck 1.2.02 software (Piry et al., 1999). Three assessment models were used: stepwise mutation model (SMM), two-phase mutation model (TPM), and infinite allele model (IAM). Significance was tested using Wilcoxon's sign-rank test under the assumption of mutation-drift equilibrium (Cornuet and Luikart, 1996). We also used Bottleneck software to analyze mode-shift, which is a qualitative description of distribution of allele frequencies of a population (Piry et al., 1999). Three models were used to test for bottlenecks because SSRs markers rarely conform to strict SMM (Cornuet and Luikart, 1996). Pollegioni et al. (2009) reported that the TPM was the most accurate model of mutational mechanisms of the microsatellite loci in their study. Busch et al. (2007) suggested the TPM and SMM are more appropriate for use with microsatellites. As a final test for bottlenecks, we used M-ratio test in the software M_P_VAL and the result compared with critical value (Mc). M-ratios below Mc reveal a bottleneck (Garza and Williamson, 2001). Mc was calculated for three values of θ (0.1, 1, and 10) as suggested in the software. Defaults were accepted for all software parameters.

3. Results and discussion

3.1. Genetic diversity

All 10 SSR loci were polymorphic (Table 2), and a total of 74 alleles were detected. The total number of alleles per locus ranged from 4 (WGA89) to 13 (WGA32), with a mean of 7.4. The average number of alleles per locus (7.4) was higher than the 5.1, 5.5, 5.5 and 6.2 reported by Ebrahimi et al. (2011), Foroni et al. (2007), Pollegioni et al. (2014) and Pollegioni et al. (2011) respectively, but lower than the value (about 10) reported by Aradhya et al. (2010). The number of effective alleles was lower than the total number of alleles, ranging from a minimum of 2.50 (WGA89) to a maximum of 6.70 (WGA69); the average was 4.52. The observed heterozygosity (Ho) varied from 0.43 (WGA27) to 0.80 (WGA276) (Table 2), and the Ho was less than the H_E for every locus. The average H_O and H_E were 0.61 and 0.75, respectively. Overall, the genetic variability as measured by Shannon's information index was high (mean = 1.60). Shannon's information index (I) averaged across all loci was 1.60 (Table 2), slightly lower than the values reported by Chen et al. (2014) (I = 1.73) and Vahdati et al. (2015) (I = 1.79) from China and Iran, respectively.

The average number of alleles across all four populations (N_A) was 4.75 (Table 3); the samples from Slovakia had the highest number (5.50), and Italian samples had the lowest average number (3.60). Expected heterozygosity ranged from 0.57 for Italian samples to 0.69 for Slovak samples. The lowest H_O recorded was for Hungarian samples (0.55) while the highest value (0.66) was

Table 1
Country and regions where *J. regia* samples originated.

ID	Region	Country	ID	Region	Country	ID	Region	Country
1	Caserta	Italy	42	Indiana	USA	83	Miskolc	Hungary
2	Caserta	Italy	43	Indiana	USA	84	Erd	Hungary
3	Caserta	Italy	44	Indiana	USA	85	Erd	Hungary
4	Caserta	Italy	45	Indiana	USA	86	Erd	Hungary
5	Caserta	Italy	46	Indiana	USA	87	Bratislava	Hungary
6	Caserta	Italy	47	Indiana	USA	88	Bratislava	Hungary
7	Caserta	Italy	48	Indiana	USA	89	Bratislava	Hungary
8	Caserta	Italy	49	Indiana	USA	90	Bratislava	Slovakia
9	Caserta	Italy	50	Indiana	USA	91	Nitra	Slovakia
10	Caserta	Italy	51	Indiana	USA	92	Nitra	Slovakia
11	Caserta	Italy	52	Indiana	USA	93	Nitra	Slovakia
12	Caserta	Italy	53	Indiana	USA	94	Bratislava	Slovakia
13	Caserta	Italy	54	Indiana	USA	95	Nitra	Slovakia
14	Caserta	Italy	55	Indiana	USA	96	Nitra	Slovakia
15	Caserta	Italy	56	Indiana	USA	97	Nitra	Slovakia
16	Caserta	Italy	57	Budapest	Hungary	98	Levice	Slovakia
17	Caserta	Italy	58	Budapest	Hungary	99	Levice	Slovakia
18	Caserta	Italy	59	Budapest	Hungary	100	Levice	Slovakia
19	Caserta	Italy	60	Budapest	Hungary	101	Levice	Slovakia
20	Caserta	Italy	61	Budapest	Hungary	102	Levice	Slovakia
21	Sorrento-peninsula	Italy	62	Budapest	Hungary	103	Trnava	Slovakia
22	Sorrento-peninsula	Italy	63	Budapest	Hungary	104	Trnava	Slovakia
23	Indiana	USA	64	Budapest	Hungary	105	Trnava	Slovakia
24	Indiana	USA	65	Budapest	Hungary	106	Trnava	Slovakia
25	Indiana	USA	66	Budapest	Hungary	107	Trnava	Slovakia
26	Indiana	USA	67	Debrecen	Hungary	108	Trnava	Slovakia
27	Indiana	USA	68	Debrecen	Hungary	109	Trnava	Slovakia
28	Indiana	USA	69	Debrecen	Hungary	110	Trnava	Slovakia
29	Indiana	USA	70	Debrecen	Hungary	111	Trnava	Slovakia
30	Indiana	USA	71	Debrecen	Hungary	112	Trnava	Slovakia
31	Indiana	USA	72	Debrecen	Hungary	113	Trnava	Slovakia
32	Michigan	USA	73	Debrecen	Hungary	114	Trnava	Slovakia
33	Illinois	USA	74	Debrecen	Hungary			
34	Missouri	USA	75	Debrecen	Hungary			
35	Indiana	USA	76	Debrecen	Hungary			
36	Indiana	USA	77	Debrecen	Hungary			
37	Indiana	USA	78	Debrecen	Hungary			
38	Indiana	USA	79	Miskolc	Hungary			
39	Indiana	USA	80	Miskolc	Hungary			
40	Indiana	USA	81	Miskolc	Hungary			
41	Indiana	USA	82	Miskolc	Hungary			

Table 2
Allelic diversity indicators for 10 SSR loci over all *J. regia* samples.

Locus	(N)	Size range (bp)	N_A	N_E	HW	Shannon index (*I*)	H_O	H_E
WGA1	114	180–196	6	3.67	NS	1.48	0.60	0.73
WGA9	114	231–250	6	3.98	NS	1.49	0.64	0.75
WGA27	114	192–235	6	3.02	**	1.30	0.43	0.67
WGA32	114	120–229	13	4.60	**	1.76	0.59	0.78
WGA69	114	159–199	8	6.70	NS	1.97	0.51	0.85
WGA89	114	211–221	4	2.50	NS	1.06	0.50	0.60
WGA118	114	183–206	5	2.88	NS	1.23	0.61	0.65
WGA202	114	249–295	9	6.50	NS	1.99	0.73	0.84
WGA276	114	156–195	11	6.50	NS	2.02	0.81	0.85
WGA321	114	222–275	6	4.80	*	1.66	0.67	0.80
mean		–	7.4	4.52		1.60	0.61	0.75

Note: N = Sample Size, Size range = Allele size range (base pairs), N_A = No. of alleles, N_E = Number of effective alleles, HW = Exact test of Hardy-Weinberg equilibrium with a significance at $p \leq 0.01$. *I* = Shannon's Information Index, Frequency estimated, H_O = Observed Heterozygosity, H_E = Expected Heterozygosity.

Table 3
Genetic diversity indices, tests of Hardy-Weinberg equilibrium, and tests of population bottlenecks for *J. regia* from different regions.

Region	na	*Ho*	*He*	*Fis*	χ^2	*P*	Ra	Pa	IAM	SMM	TPM	Mode-shift
Italy	3.6	0.60	0.57	−0.05	21.3	0.16	3.68	0.30	0.083	0.492	0.56	Normal
USA	3.9	0.66	0.62	−0.06	16.1	0.12	3.92	0.82	0.003	0.003	0.04	Shifted
Hungary	5.30	0.55	0.67	0.17	20.5	0.25	4.40	0.41	0.044	0.70	0.332	Normal
Slovakia	5.50	0.62	0.69	0.10	20.6	0.30	4.50	0.54	0.030	0.70	0.614	Normal
Mean	4.75	0.61	0.65	–	19.6	0.20	4.12	0.5	–	–	–	–

Note: Genetic diversity index: number of alleles (na), observed Heterozygosity (*Ho*), expect Heterozygosity (*He*), inbreeding value (*Fis*). Hardy-Weinberg: χ^2 = Hardy-Weinberg equilibrium, Probability (p), Ra = Allelic Richness, Pa = Private Allele. Probability of heterozygosity excess in bottleneck test: IAM = Infinite Allele Model, TPM = two-phase mutation model, SMM = stepwise mutation model.

recorded for U.S. samples. No population deviated significantly from Hardy-Weinberg expectations (Table 3). The U.S. population had lower allelic richness (3.92) than the Eastern Europe populations (~4.40), but the frequency of private alleles in the U.S. population (0.82) was higher than the frequency of private alleles in any other sampled population. Inbreeding (*F*is) varied from 0.17 (Hungary) to −0.06 in the USA population (Table 3).

All of the diversity indices showed the genetic diversity of the U.S. *J. regia* was comparable to European samples (Table 3). The average per-locus observed heterozygosity of US samples

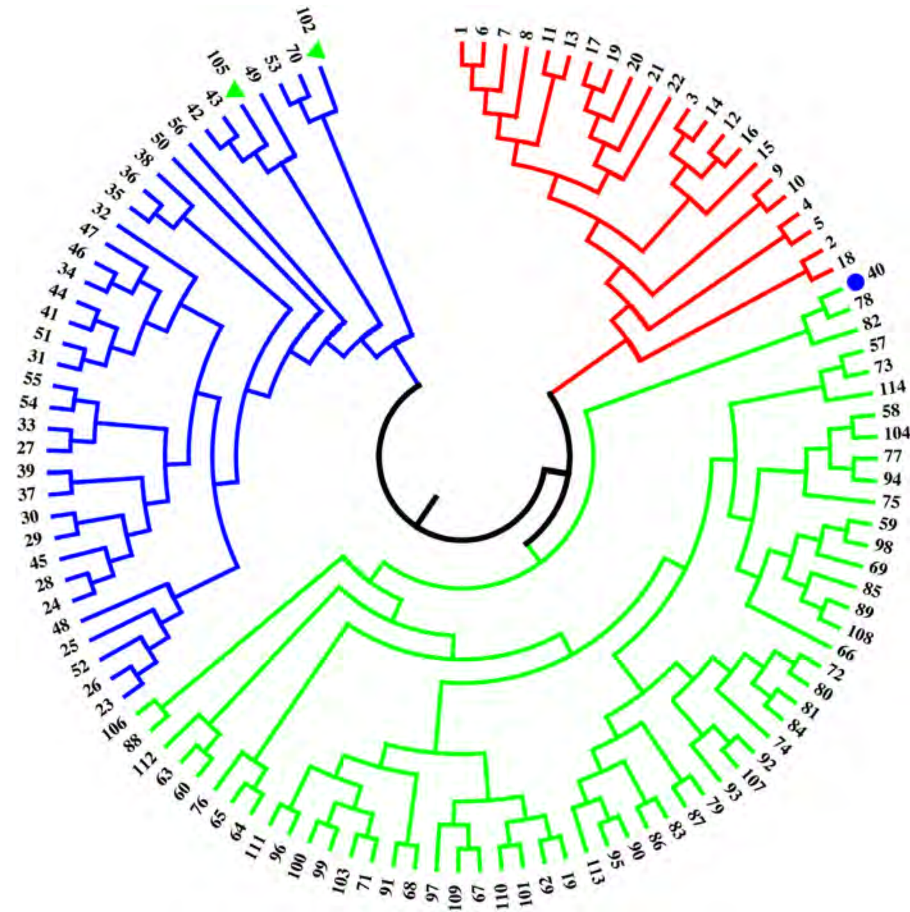

Fig. 1. Genetic differentiation of 114 *J. regia* samples using Neighbor-Joining methods.

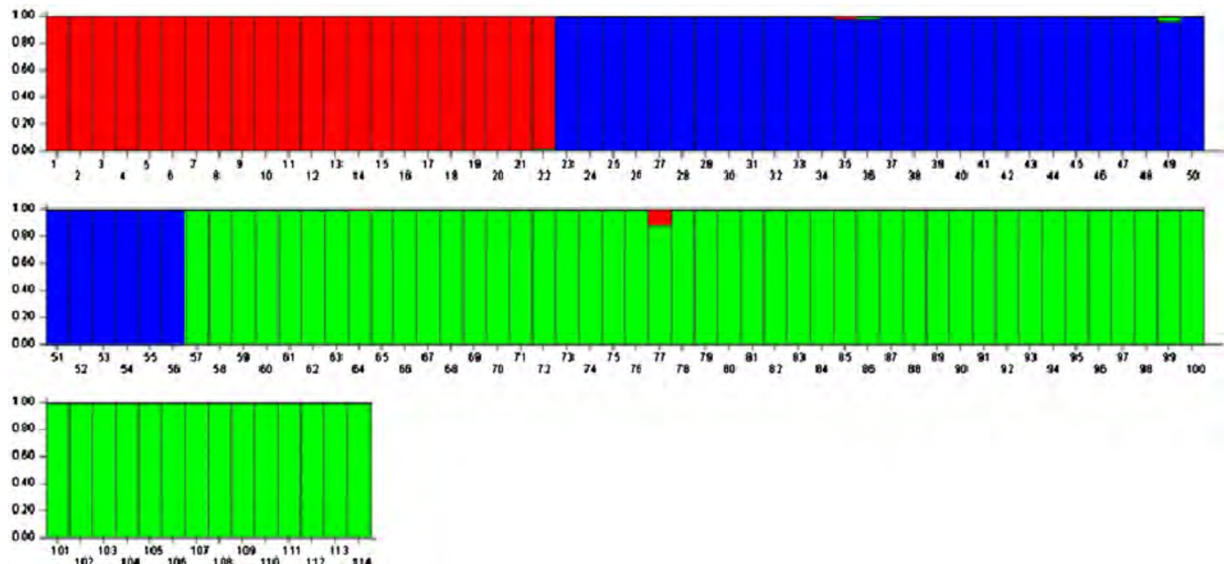

Fig. 2. Pattern of individual assignments into subsets (*K* = 3) using the STRUCTURE model. Each individual is shown by a vertical line that is assigned to three colored segments, according to its estimated membership probabilities (*Q*). The numbers indicate the sample ID numbers from Table 1.

(H_O = 0.61) was nearly identical to the value reported by Dangl et al. (2005) for non-cold-tolerant cultivars and elite germplasm (H_O = 0.60) and by Aradhya et al. (2010) for Carpathian germplasm accessions (H_O = 0.63), but lower than the value reported by Foroni et al. (2005) for a mixed population of Italian and French cultivars and Italian landraces (0.71). In an extensive sample of walnut populations throughout Iran, Ebrahimi et al. (2011) reported heterozygosity comparable to the value for Italian landrace Persian walnuts (H_O = 0.72). Other investigators analyzing the genetic diversity of non-cultivated *J. regia* found H_O values similar to those from the US samples, including Pop et al. (2013) (H_O = 0.61) and Chen et al. (2014) (H_O = 0.62). Thus, our results indicate that *J. regia* in Eastern North America is as diverse as *J. regia* in European and North American breeders' collections, comparable to many collections of wild germplasm from Asia, and show no loss of heterozygosity. The absence of inbreeding in the samples from USA probably indicates that founder effects were minimal, and/or that the samples reflect an "isolate breaking effect" that occurs when previously isolated populations, such as Polish Carpathian trees, cross with other imported populations of *J. regia*. A similar process was proposed by Foroni et al. (2007) in Italy to explain patterns of genetic diversity within the 'Sorrento' landrace.

3.2. Population differentiation and structure

Neighbor-Joining divided accessions into three main groups; the North American samples were all distinctly different from those from Eastern Europe or Italy (Fig. 1). The same three populations were clearly evident in the analysis using STRUCTURE software (Fig. 2). Of the 114 walnut samples, 113 shared >90% membership with one of three populations. The first population consisted of all samples from Italy. All U.S. samples were classified as population 2. The third population consisted of samples from Hungary and Slovakia. The lowest Q value observed was for sample 69 from Hungary (0.88) (Table 1), which showed some similarity to Italian samples.

Clear and near total separation of populations by origin has not been observed typically in walnut (Foroni et al., 2007; Pop et al., 2013) or other crops (Escribano et al., 2007; Wunsch and Hormaza 2002; Soriano et al., 2005). The neighbor-joining cluster analysis (Fig. 1) shows more complexity than the STRUCTURE analysis in the assignment of individuals to geographic groups as the former reflects a low amount of admixture within sample sites. In general, however, the STRUCTURE analysis and NJ tree show the same strong genetic division among sampled sites (Fig. 1). Our results agreed with Aradhya et al. (2010) who compared Carpathian samples to various Asian populations. Their Carpathian samples from the USA were comprised of two tightly-clustered sub-clades that were distinct from the Asian and derived (breeding) populations.

3.3. Bottleneck analysis

Bottleneck software calculates whether allelic diversity in a population has been reduced faster than heterozygosity under the assumption of mutation-drift equilibrium (Piry et al., 1999). Mutation-drift equilibrium is not a common feature of real populations, especially those of crop plants, which have been dispersed, admixed, and selected by humans and natural forces. Bottleneck software is also confounded by population structure (Chikhi et al., 2010), which was clearly evident in our samples. As a result, our analysis of bottlenecks in the USA samples should be interpreted with caution, although other authors have used this approach to evaluate bottlenecks among samples of domesticated tree species (Cornille et al., 2012). Based on analysis of mode-shift, only the U.S. samples showed evidence of a genetic bottleneck (Table 3). This result may have been driven by the relatively large samples size from USA and the combination of high heterozygosity with relatively small number of alleles among the U.S. samples (Table 3). Results of bottleneck analysis differed depending on which mutation model was employed. The U.S. samples were the only ones for which heterozygosity excess was detected under all three (IAM, TPM and SMM) models. Under the IAM model, all the populations showed significant heterozygote excess; under the SMM model, the U.S. population showed a significant bottleneck signal but the Italian population and combined Eastern European populations did not. Similarly, under the assumptions of TPM, only the U.S. population showed a significant signal of a bottleneck (Table 3). The TPM model assumes that most mutation changes result in an increase or decrease of one repeat unit but mutations of large magnitude can also occur (Busch et al., 2007), and several studies conclude TPM is the most appropriate model for microsatellite mutation (Piry et al., 1999; Busch et al., 2007; Pollegioni et al., 2009). We also tested for bottlenecks using the M-ratio test under a range of θ. This test relies on different sets of assumptions than other tests, and it indicated the presence of a bottleneck in all four populations, irrespective of θ. Domesticated species (or those under strong human selection) often show evidence of bottlenecks (Miller and Gross, 2011) or loss of diversity, and bottlenecks were even observed in wild populations of *J. regia* (Pollegioni et al., 2014), but the observed and expected heterozygosities of the breeding populations we studied were comparable to wild populations, so we do not see evidence for the importance of bottleneck effects in the North American *J. regia*.

4. Conclusion

Our results show that Midwestern USA *J. regia* germplasm is genetically distinct from existing Eastern European germplasm. Neither selection for cold hardiness nor founder effects appear to have severely depressed the genetic diversity of the population. Genetic similarity between Hungarian and Slovak *J. regia* genotypes may reflect previous exchange of germplasm among breeders and other walnut growers. Persian walnut germplasm better adapted to the colder regions of both North America and northern Europe could be attained through expanded collection of cold hardy *J. regia* and by sharing germplasm. The cold-hardiness of the eastern US Persian walnut could easily be supplemented by the addition of cold-hardy selections from Eastern Europe which, based on our study, are not closely related to genotypes already present in the Midwest USA. Exchange *of J. regia* between North America and north Eastern Europe should be fruitful in both directions. Additional cold hardy *J. regia* germplasm will be useful for expanding the range of cultivation for the species as a nut crop and for production of hybrid *J.* × intermedia for timber production.

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