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CLARIFYING THE CONSERVATION STATUS OF NORTHERN CALIFORNIA BLACK WALNUT (*JUGLANS HINDSII*) USING MICROSATELLITE MARKERS

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ABSTRACT

The conservation status of the northern California black walnut (*Juglans hindsii* (Jeps.) Jeps. ex R. E. Sm.) has been a source of considerable confusion and controversy. Although not currently legally protected by either Federal or State Endangered Species Acts, this species is given conservation status by the California Native Plant Society and the California Department of Fish and Wildlife, and some California counties require mitigation for removal of individuals of this species, especially older trees. Despite the current widespread distribution in northern California and southern Oregon of trees that match *J. hindsii* morphologically, there are only three or four sites where the species is known to have occurred prior to extensive settlement of California by Europeans in the mid-19th century. This has led to the suspicion that trees found in other places may not be genetically pure *J. hindsii* but may instead be descendants of lineages that experienced past gene flow from one or more other species. In addition, despite its more distant relationship, the cultivated walnut (*J. regia* L.) readily hybridizes (as the male parent) with *J. hindsii*, producing morphologically identifiable 'Paradox' hybrids, which occur spontaneously and are widely planted as rootstocks and street trees. Finally, recent collections of *J. hindsii* from southern California have raised questions about the respective geographic distributions of *J. hindsii* and southern California black walnut (*J. californica* S. Watson). We analyzed genotypes at 10 microsatellite loci for 158 mostly wild *J. hindsii* trees, as well as some orchard waifs, from 10 counties in northern and southern California and one county in southern Oregon, including representatives of putative original native populations. We also sampled several Paradox hybrids, 10-20 standards for each of the five North American black walnut species, and six standards for *J. regia*. Bayesian cluster analyses with the program STRUCTURE revealed that at least 71.5% of the putatively wild *J. hindsii* represent genetically pure members of that species, while the remaining trees show evidence of past hybridizations with one or more of the other North American black walnut species. We found no evidence of introgression of *J. regia* into *J. hindsii*. The results suggest that individual *J. hindsii* trees should not have conservation status.

Key Words: hybridization, introgression, native species, naturalized species, Paradox walnut, protected species, rare species, SSR.

Two species of black walnut (*Juglans* sect. *Rhysocaryon*) are native to California: *J. hindsii* (Jeps.) Jeps. ex R. E. Sm., the northern California black walnut, and *J. californica* S. Watson, the southern California black walnut. Other North American black walnut species, including eastern black walnut (*J. nigra* L.), Arizona walnut (*J. major* [Torrey] A. Heller), and Texas black walnut (*J. microcarpa* Berland.), are occasionally cultivated in California. Persian (aka English) walnut (*J. regia* L, a member of *Juglans* section *Juglans*) is a major orchard crop in the state and occasionally escapes as a waif but is not considered naturalized (Baldwin et al. 2012). *Juglans hindsii* has been widely planted in California as a street tree and as a rootstock for *J. regia*.

Most species of *Juglans* are interfertile, and the pioneering horticulturist Luther Burbank conducted

crossing experiments and named two hybrids involving California walnuts, 'Royal' (*J. hindsii* × *J. nigra*) and 'Paradox' (*J. hindsii* × *J. regia*) (Burbank 1914; Howard 1945). Burbank first observed hybrids between *J. hindsii* and *J. regia* in 1878 (Howard 1945); he bestowed upon them the name 'Paradox" in 1893, in recognition of their rapid growth and low nut production, factors that have contributed to their popularity as shade trees. He was particularly impressed by the high quality of the wood, considering the primary economic promise of Paradox hybrids to lie in their potential value as timber trees. Subsequently, Paradox was found to be superior in several qualities to *J. hindsii* as a rootstock for *J. regia* (Smith et al. 1912; Catlin 1998), and, while both are still used, Paradox is now the most extensively used rootstock for *J. regia* in California (McGranahan and Catlin 1987; Kluepfel et al. 2012). Paradox hybrids occur spontaneously in areas where *J. hindsii* grows in proximity to *J. regia* orchards; they are readily distinguished from both parents by their intermediate leaf morphology and bark color and by their remarkable vigor. Like *J. hindsii*, Paradox hybrids have also been widely planted as street trees. Available evidence indicates that all species and hybrids of *Juglans* are diploids, with $2n = 32$ (Elias 1972).

Although there is no question that *J. hindsii* is native to California, there is considerable confusion and controversy over its indigenous range and conservation status. In addition to extensive plantings in urban areas and orchards, trees that match *J. hindsii* morphologically occur widely in unmanaged habitats, especially riparian areas, in central and northern California, where they are generally considered "naturalized" (Griffin and Critchfield 1972). Nonetheless, the species has been given rarity status by the California Native Plant Society (CNPS 2017) and California Department of Fish and Wildlife (CNDDB 2017), and, although it is not currently officially listed by either the State or Federal Endangered Species Acts (ESA), some California counties require mitigation for removal of individuals this species, especially older trees. Furthermore, under the California Environmental Quality Act, species that are not currently officially listed by either ESA still require actions during pre-project review, including surveys, disclosure of what is present and its significance, and proposed mitigation for any significant losses proposed during the project implementation. The designation of conservation status for *J. hindsii* rests on the fact that, despite its current widespread distribution, there are only three (Smith 1909; Smith et al. 1912), or possibly four (Jepson 1917) sites, in Contra Costa, Sacramento, and Napa Counties, where the species was confirmed to have occurred prior to extensive settlement of California by Europeans in the mid-19th century. These have generally been accepted as the only sites where the species should be considered indigenous, rather than having been planted intentionally or escaped from intentional plantings and naturalized (Kirk 2003). According to CNPS (2017), only one of these sites is considered viable and the species is threatened by changes in land use and by hybridization with *J. regia*. These assertions have led to widespread concern among individuals and agencies in northern California that trees occurring in other places may not be genetically pure *J. hindsii*, but instead be descended from lineages that experienced past gene flow from one or more other *Juglans* species. By contrast, Callahan (2008) considered it likely that *J. hindsii* occurs at multiple additional locations, including sites in southern Oregon, beyond those documented by Smith et al. (1912) and Jepson (1917). Results from analyses of restriction fragment length polymorphisms (RFLPs; Fjellstron and Parfitt 1994) revealed reduced genetic diversity in *J. hindsii* compared to other *Juglans* species, consistent with a past genetic bottleneck as would be expected if extant members of the species were derived from a relatively small number of ancestral populations.

The difficulty of pinpointing the original sites where *J. hindsii* occurred prior to the mid-19th century is compounded by the fact that extensive movement and planting of the species and various hybrids by people as well as changes in land use have resulted in the occurrence of trees in wild-looking places which in fact represent rootstocks from old orchards or other past intentional plantings. Thus, extant populations almost certainly represent a mixture of naturally occurring and intentionally planted stands, as well as of spontaneous and intentionally produced hybrids.

To date, two published studies have used molecular markers to address the question of genetic purity of *J. hindsii* trees from locations other than one of the putative native sites. The first (Potter et al. 2002) focused on investigating the genealogies of Paradox sources, the individual mother trees from which Paradox rootstock seedlings are obtained, using data from single nucleotide polymorphisms (SNPs) derived from nucleotide sequences from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (biparentally inherited) and three non-coding regions of plastid DNA (maternally inherited). Of 27 Paradox sources tested, 18 were found to be pure *J. hindsii* based on the markers used, while the remainder showed contributions from *J. nigra*, *J. major*, or *J. californica* as maternal and/or paternal parents, as well as *J. hindsii*, in their backgrounds. Similar results (i.e., that most *J. hindsii* are genetically pure, but some individuals show evidence of past gene flow from other species) have been observed in testing of additional trees, using the same markers, over the past decade (Potter, unpublished data).

The second published study (Kirk 2003) examined variation in ITS sequences of eight putatively naturalized (escapes from intentional plantings) populations of *J. hindsii* in the Sacramento Valley, as well as two of the putative native populations, and

representatives of the other species present in California. The results suggested the possibility of past gene flow from *J. major* into both native and naturalized populations of *J. hindsii*.

There are several limitations of the aforementioned studies. First, only one nuclear genomic region was sampled and because concerted evolution tends to homogenize ribosomal DNA, including ITS (Wendel et al. 1995), polymorphisms present in first-generation hybrids could be lost in later generations, precluding the possibility of detecting gene flow from other species as ancestors more remote than parents or grandparents. Second, not surprisingly, low levels of DNA sequence variability were observed among these closely related species, so conclusions were based on just six SNPs in the ITS and ten in the plastid DNA sequences. Finally, only a limited number of representatives of other species, especially *J. major* (five and three individuals, respectively, in Potter et al. 2002 and Kirk 2003) were included. This is not meant to suggest that these past studies and the markers they employed did not provide valuable information, but their value would be significantly enhanced by expanded sampling and additional evidence from other markers. In this regard, the microsatellite markers developed for *J. nigra* (Woeste et al. 2002; Robichaud et al. 2006) and successfully applied to studies of genetic diversity and cultivar identification in *J. regia* (Dangl et al. 2005) are particularly promising.

Two additional issues require further investigation. The first is that, although gene flow from *J. regia* to *J. hindsii* is considered unlikely due to male sterility and very low fruit set by Paradox hybrids, anecdotal reports that some Paradox individuals do produce large numbers of viable seeds (e.g., Robinson, Sierra Gold Nurseries, personal communication) cast some doubt on the certainty of this conclusion. Thorough genetic testing is needed to assess definitively whether gene flow from cultivated to wild walnuts is a concern. The second issue requiring clarification is the presence of spontaneous populations of *J. hindsii* in southern California, recorded from herbarium specimens collected in five counties (Los Angeles, Orange, Riverside, San Bernardino, and San Diego) over the past five years (Sanders, University of California Riverside Herbarium, personal communication; Consortium of California Herbaria 2017). Genetic testing is important for clarifying the identification and elucidating the origins of these populations.

In order to address persistent questions about the genetic identities of putatively wild walnut trees in California and southern Oregon, and to test the hypothesis that most of them are pure *J. hindsii*, we analyzed genotypes at 10 microsatellite loci for 158 putatively wild trees of *J. hindsii* from 10 counties in northern and southern California, and from one county in southern Oregon including representatives of putative original indigenous populations. We also included several Paradox hybrids, 10-20 standards for each of the five North American black walnut species, and six standards for *J. regia*.

Materials and Methods

Sampling

A total of 249 trees were sampled for this study, of which 167 were new field collections (Fig. 1, Supplemental Table S1) from localities in Jackson County, southern Oregon (13 trees) and 10 California counties (Alameda [n = 1]; Contra Costa [n = 64]; El Dorado [n = 5]; Los Angeles [n = 4]; Napa [n = 66]; Orange [n = 3]; San Bernardino [n = 2]; San Diego [n = 5]; Santa Clara [n = 1]; Sonoma [n = 3]). Most (158) of these trees were identified as *J. hindsii* at the time of collection based on morphology. Fifteen of the trees were collected from two of the putative original native populations (10 from Las Trampas Creek in Contra Costa County and 5 from Circle Oaks Drive at the Capell Creek/Wooden Valley site in Napa County; CNDDB 2017). We also included seven trees identified as Paradox hybrids and two samples from Los Angeles County identified as *J. californica*. The majority of these samples (134) were collected by Heath Bartosh; additional collections were contributed by Richard Riefner (14 collections from southern California), Frank Callahan (10 collections from Oregon), Dan Potter (five collections from El Dorado County), Brian Peterson (three collections from Oregon), and Chris Jannusch (one collection from Santa Clara County). Voucher specimens for samples collected by Bartosh, Riefner, and Potter were deposited in the herbarium of the Center for Plant Diversity at UC Davis (DAV).

In addition, we included 10-20 standards for each of the five North American black walnut species plus six standards for *J. regia* (Table S2). These species standards comprised 66 individuals from the collection of the USDA National Clonal Germplasm Repository (further details about these accessions are available at https://npgsweb.ars-grin.gov/gringlobal/search.aspx?), identified as *J. hindsii* (n = 10), *J. californica* (n = 16), *J. major* (n = 14), *J. microcarpa* (n = 16), *J. nigra* (n = 4), and *J. regia* (n = 6), as well as 16 accessions of *J. nigra* from the collections of the Hardwood Tree Improvement & Regeneration Center (HTIRC), USDA-Forest Service, Northern Research Station, associated with Purdue University, kindly provided by Keith Woeste and James McKenna. The majority of these individuals originated from seeds collected from wild trees. As described below, microsatellite marker genotypes suggested that 11 of these 82 species standards did not represent genetically pure members of the species to which they were assigned; those individuals were excluded from final analyses. Thus, for our final analyses, we

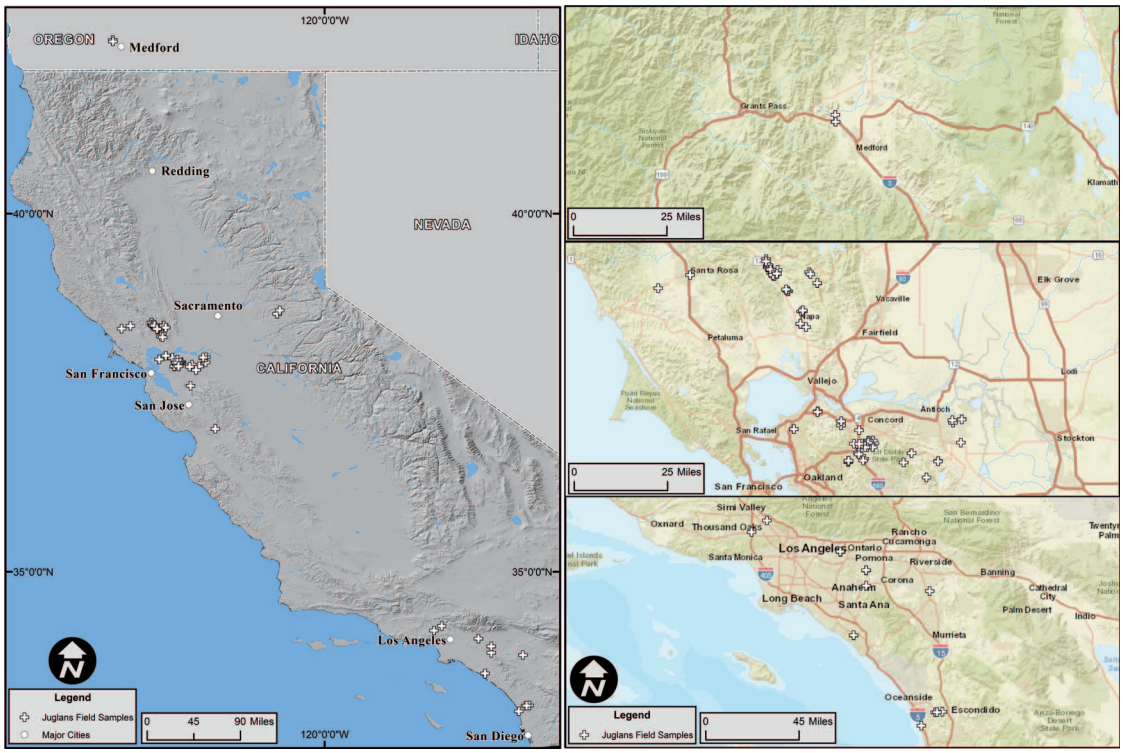

FIG. 1. Overview of all collection localities (left) and close-ups (right) of collection localities in southern Oregon (top), the San Francisco Bay Area (middle), and southern California (bottom) for the 167 field-sampled trees included in this study.

analyzed the genotypes of 238 trees (71 species standards + 167 field collected trees).

Genotyping

Collected samples consisted of young, fresh, green leaves that were dried using chemical desiccants (Bautista et al. 2008). Whole genomic DNA was extracted by a modified CTAB protocol adapted from Doyle (1991). Samples were analyzed at 12 microsatellite loci. These loci were originally developed from *J. nigra*, as described in Woeste et al. (2002); primer sequences are found in Dangl et al. (2005). Two loci (WGA202 and WGS349) produced multi-locus genotypes across several species and were excluded from final analyses. The 10 loci used in the final analyses are listed in Table 1. A standard set of PCR conditions was used for all samples (Dangl et al. 2005). The fragment separation and sizing were performed as previously described for *Vitis* species

TABLE 1. STATISTICS (NUMBER AND PERCENTAGE OF MISSING DATA POINTS, NUMBER OF ALLELES (N_a), NUMBER OF EFFECTIVE ALLELES (N_e), INFORMATION INDEX (I), OBSERVED HETEROZYGOSITY H_o), EXPECTED HETEROZYGOSITY (H_e), UNBIASED EXPECTED HETEROZYGOSITY (uH_e), AND FIXATION INDEX (F)) OF SSR LOCI AMPLIFIED FOR THIS PROJECT BASED ON THE FINAL 238 SAMPLES ANALYZED. Designations for SSR primer pairs follow Dangl et al. (2005).

Locus	Missing #	Missing %	N_a	N_e	I	H_o	H_e	uH_e	F
WGA 001	150	63.0	18	10.149	2.483	0.455	0.901	0.907	0.496
WGA 276	1	0.4	24	4.682	2.107	0.570	0.786	0.788	0.276
WGA 376	2	0.8	12	5.492	2.024	0.631	0.818	0.820	0.228
WGA 009	15	6.3	16	5.411	2.061	0.704	0.815	0.817	0.136
WGA 118	5	2.1	27	3.876	2.019	0.536	0.742	0.744	0.277
WGA 089	0	0.0	28	2.435	1.640	0.424	0.589	0.591	0.280
WGA 331	1	0.4	19	3.124	1.713	0.494	0.680	0.681	0.274
WGA 321	136	0.6	20	11.098	2.610	0.480	0.910	0.914	0.472
WGA 332	1	0.4	21	3.984	1.999	0.574	0.749	0.751	0.234
WGA 069	0	0.0	11	1.896	1.151	0.248	0.472	0.473	0.475
Mean	31.1	7.4%	19.6	5.215	1.981	0.512	0.746	0.749	0.315

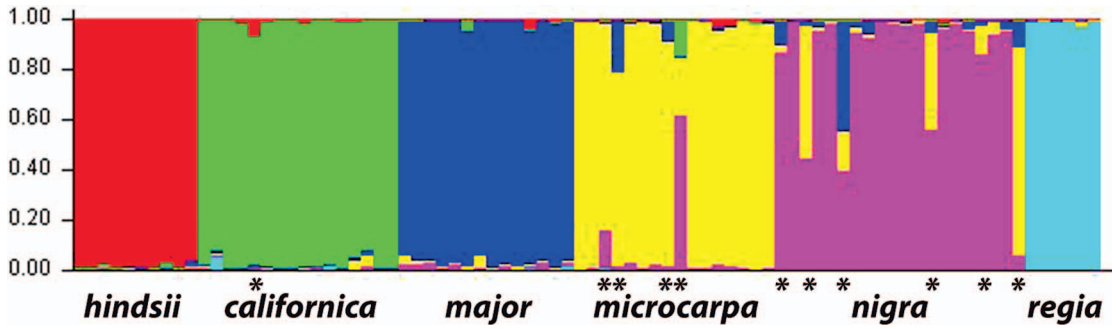

FIG. 2. Sample result of STRUCTURE analysis (K = 6) of the 82 species standards included in this study. Individuals marked with asterisks were excluded from final analyses (see Table S2).

(Dangl et al. 2015). Genotypes were assigned using the software package STRand (Toonen and Hughes 2001; http://www.vgl.ucdavis.edu/STRand).

Data Analyses

In order to assess levels of genetic diversity in *J. hindsii* compared to other walnut species, basic statistics (including numbers of alleles and observed and expected heterozygosities for each locus and each species) were calculated using GenAlEx (Peakall and Smouse 2012). To investigate the number of genetic clusters among the samples based on our microsatellite marker data, Principal Coordinate Analyses (PCoA) of the data were conducted using GenAlEx and Bayesian clustering analyses were implemented in STRUCTURE (Pritchard et al. 2000). For all Bayesian clustering analyses, the admixture ancestry model, in which each individual can to be assigned to more than one cluster, was used, allowing identification of hybrids. Further details about these analyses are provided in the Results section.

RESULTS

Eight of the 10 primer pairs produced amplification bands in the all or nearly all of the 238 individuals (Table 1). Specifically, two (WGA 089, WGA 069) succeeded in all individuals, three (WGA 276, WGA 331, and WGA 332) each failed in one individual, one (WGA 376) failed in two individuals, one (WGA 118) failed in five individuals, and one (WGA 009) failed in 15 individuals. For those eight primers, the individuals for which no products were obtained were treated as missing data for all analyses. Two primer pairs failed in a substantially larger number of individuals. Primer pair WGA 001 failed in 150 individuals (63.0%), including nine species standards for *J. hindsii* and 10 for *J. californica* as well as 131 of the field collections, while primer pair WGA 321 failed in 136 individuals (59.5%), including all 10 species standards for *J. hindsii*, one for *J. californica*, and 125 of the field collections. For GenAlEx analyses, all of these cases were coded as missing data. For the 10 microsatellite loci across the 238 individuals included in the final analyses, the numbers of observed and effective alleles, observed heterozygosity, and expected heterozygosity ranged from 11 to 28, 1.9 to 11.1, 0.25 to 0.70, and 0.47 to 0.91, respectively (Table 1).

Because the large number of failed amplifications with limited taxonomic distribution suggested the possibility of presence of a null allele in the two native California species at the microsatellite loci amplified by primer pairs WGA 001 and WGA 321, an approach that allows for this possibility was used for those loci in the Bayesian clustering analyses. Specifically, presence of a possible recessive allele, i.e., a change in sequence such that no PCR amplification product is generated, was indicated for each of the two loci, and individuals for which amplification failed were coded as homozygotes for that allele, following the instructions in the STRUCTURE manual (Pritchard et al. 2000).

The results of PCoA were generally consistent with those from Bayesian clustering analyses; only the latter will be presented here. Bayesian clustering analyses were conducted in three phases. The goal of the first phase was to ensure the reliability of our standards for *J. hindsii* and each of the other potential parental lineages. Thus, only the genotypes of the 82 individuals initially designated as species standards were included. The number of genetic clusters (K) was set at 6, corresponding to the six species, with the genetic admixture ancestry model in effect, and no *a priori* information about the expected assignment of each individual was provided. The clustering procedure assigns each individual to one or more of the six clusters. We calculated the average assignment coefficient (q) of each individual to each of the six species across 25 STRUCTURE runs. Based on the findings and recommendations of previous studies (Vähä and Primmer 2006; Lepais et al. 2009), we set the threshold for designating an individual as a genetically pure member of a species at $q \geq 0.90$. Using this criterion, our results showed that 11 of the species standards (one individual of *J. californica*, four of *J. microcarpa*, and six of *J. nigra*) were hybrids with one or more other species (Fig. 2, Table S2). These samples were excluded from further

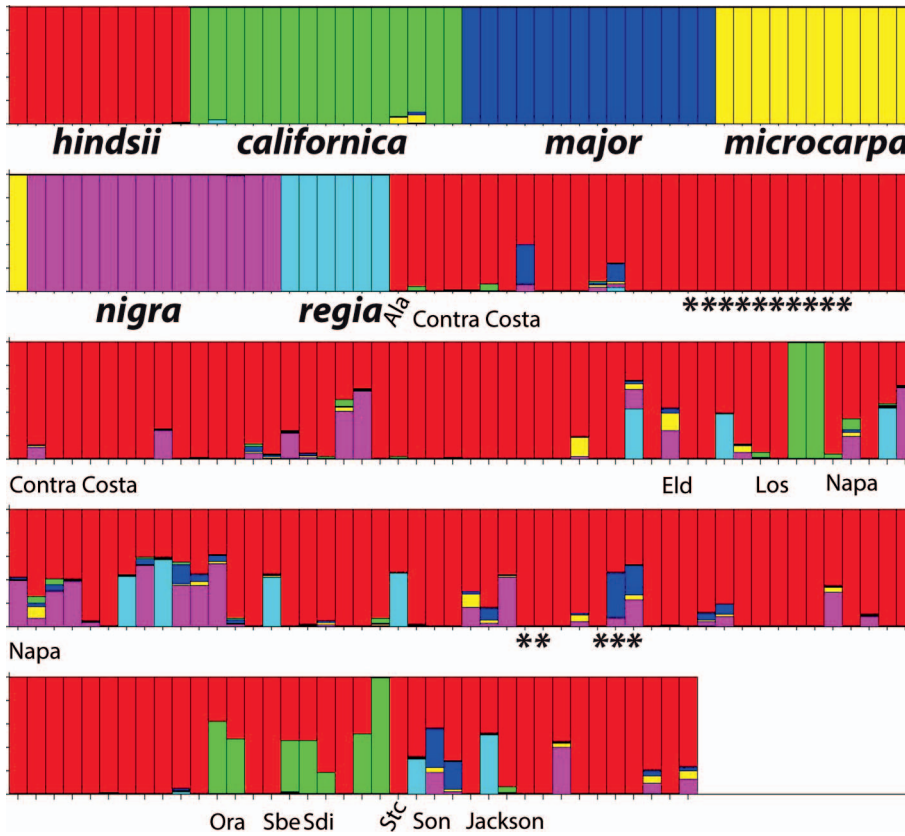

FIG. 3. Sample result of STRUCTURE analysis (K = 6) for the 71 final species standards (Table S2) plus the 167 field-sampled trees, presented in the same order as in Table S1, included in this study. County name abbreviations: Ala = Alameda; Eld = El Dorado; Los = Los Angeles; Ora = Orange; Sbe = San Bernardino; Sdi = San Diego; Stc = Santa Clara; Son = Sonoma. Individuals marked with asterisks were collected from putative original populations of *J. hindsii* in Contra Costa and Napa Counites (see Table S1).

analyses; thus the final number of species standards was 71, comprised of 10 *J. hindsii*, 15 *J. californica*, 14 *J. major*, 12 *J. microcarpa*, 14 *J. nigra*, and six *J. regia*.

The goal of the second phase of the clustering analyses was to check the species assignments of the 167 field-sampled trees (158 collected as pure *J. hindsii*, seven as Paradox hybrids, and two as pure *J. californica*). Thus, in this phase, the genotypes of the 167 field collections were added to the 71 species standards. The number of clusters was set at 6, each of the 71 species standards was flagged as belonging to its respective cluster, but no *a priori* information about the expected assignment of any of the 167 field collections was provided. We calculated the average assignment of each of the 167 trees to each of the six species across 25 STRUCTURE runs (example in Fig. 3), and the results were used to designate each individual as either a pure member of one of the species or a hybrid with two or more species in its ancestry.

Genealogical histories were inferred based on the relative percentages of assignment to each species, with inferences of hybridization limited to the three previous generations. Individuals that were assigned at $q < 0.90$ to *J. hindsii* were considered putative hybrids, while $q = 0.10$ was in most cases set as the minimum assignment value of an individual to a species required for that species to be considered an ancestor of that individual. In eight cases, individuals assigned to *J. hindsii* at $q < 0.90$ but did not assign to any other species at $q \geq 0.10$. For each of those individuals, the species with the next-highest value of q was designated as a potential great-grandparent of the individual, but with a question mark to due to our lack of confidence in these inferences (Tables 2, S1).

Of the 158 trees identified in the field as *J. hindsii* based on morphology, 113 (71.5%) were found to be pure members of that species based on their microsatellite marker genotypes, while the seven trees identified as Paradox hybrids and two identified as *J. californica* based on morphology were confirmed as such (Table 2). The remaining 45 putative *J. hindsii* were found to be F1 (*J. hindsii* × *J. californica*, *J. hindsii* × *J. major*, or *J. hindsii* × *J.*

TABLE 2. SUMMARY OF GENEALOGICAL HISTORIES OF THE 167 FIELD-SAMPLED TREES INCLUDED IN THIS STUDY, BASED ON THE RESULTS OF STRUCTURE ANALYSES. Species name abbreviations: cal = *J. californica*; hin = *J. hindsii*; maj = *J. major*; mic = *J. microcarpa*; nig = *J. nigra*; reg = *J. regia*. County name abbreviations: Ala = Alameda; Eld = El Dorado; Los = Los Angeles; Ora = Orange; Sbe = San Bernardino; Sdi = San Diego; Stc = Santa Clara; Son = Sonoma.

Inferred ID	Total	County										
		Ala	Con	Eld	Los	Nap	Ora	Sbe	Sdi	Stc	Son	Jac
hin	113	1	54	2	2	41	1	1	1	1		9
hin × (hin × (hin × maj?))	2					2						
hin × (hin × (hin × mic?))	1					1						
hin × (hin × (hin × nig?))	5		1	1		2						1
hin × (hin × (hin × nig))	1		1									
hin × (hin × cal)	1								1			
hin × (hin × maj)	3		2								1	
hin × (hin × mic)	2		1			1						
hin × (hin × nig)	7		2			4						1
hin × (hin × reg)	1										1	
hin × (hin × (nig × mic))	1					1						
hin × (maj × nig)	2					2						
hin × (nig × mic)	1			1								
hin × (maj × (maj × nig))	1										1	
hin × cal	5						2	1	2			
hin × maj	1					1						
hin × nig	9		2			6						1
Paradox	7			1		5						1
(hin × (hin × nig)) × reg	1		1									
cal	3				2				1			
Total	167	1	64	5	4	66	3	2	5	1	3	13

nigra) or more complex hybrids. Among the trees collected from the putative original locations for the species, all 10 from the Las Trampas site in Contra Costa County and three from the Circle Oaks Drive site in Napa County were identified as pure *J. hindsii,* while two from the latter site were identified as hybrids involving *J. major* and *J nigra* (Fig. 3, Table S1). Of 12 trees from southern California identified as *J. hindsii* at the time of collection based on morphology, five were confirmed as such, five were found to be F1 hybrids between *J. hindsii* and *J. californica*, one was determined as *J. hindsii* × (*J. hindsii* × *J. californica*), and one was assigned completely to *J. californica* by our analyses.

In the third phase of Bayesian clustering analysis, only the 113 individuals identified as pure *J. hindsii* in the second phase were included in order to test for evidence of genetic structuring within the species. For all four values of K tested (2, 3, 4, and 5), contributions from all gene pools were detected in all individuals, albeit to varying degrees of assignment (Fig. 4), suggesting no significant genetic structuring within the species based on the samples included here.

Analyses of average heterozygosity, F-statistics, and polymorphism across the 10 loci for each species, with putative hybrids excluded, revealed that *J. hindsii* had the lowest genetic diversity (as measured by numbers of effective alleles, information index, and observed and expected heterozygosity) of the six species sampled (Table 3).

DISCUSSION

Bayesian cluster analyses with the program STRUCTURE revealed that at least 71.5% of the putatively wild *J. hindsii* represent genetically pure members of that species; the proportion could be as high as 76.6% since the contributions of other species as great-grandparents were considered questionable in eight cases. The remaining trees show evidence of past hybridizations with one or more of the other North American black walnut species. Only one sample (HB 1299) showed evidence of introgression of *J. regia* into *J. hindsii* beyond first-generation Paradox hybrids. This was a tree from Luther Burbank's home in Sonoma County whose ancestry was inferred as *J. hindsii* × (*J. hindsii* × *J. regia*) and may represent the result of an intentional cross. The tree was noted as a potential hybrid at the time of collection. These results suggest that, as expected, widespread planting of *J. regia* is not a significant threat to genetic purity of *J. hindsii*.

Our sampling was not designed to test any specific hypotheses about the precise geographic origin of *J. hindsii* as a species or the histories of establishment of extant populations (whether natural or anthropogenic) and, given the very limited number of individuals present at the putative original sites for the species (CNDDB 2017), it would be difficult, if

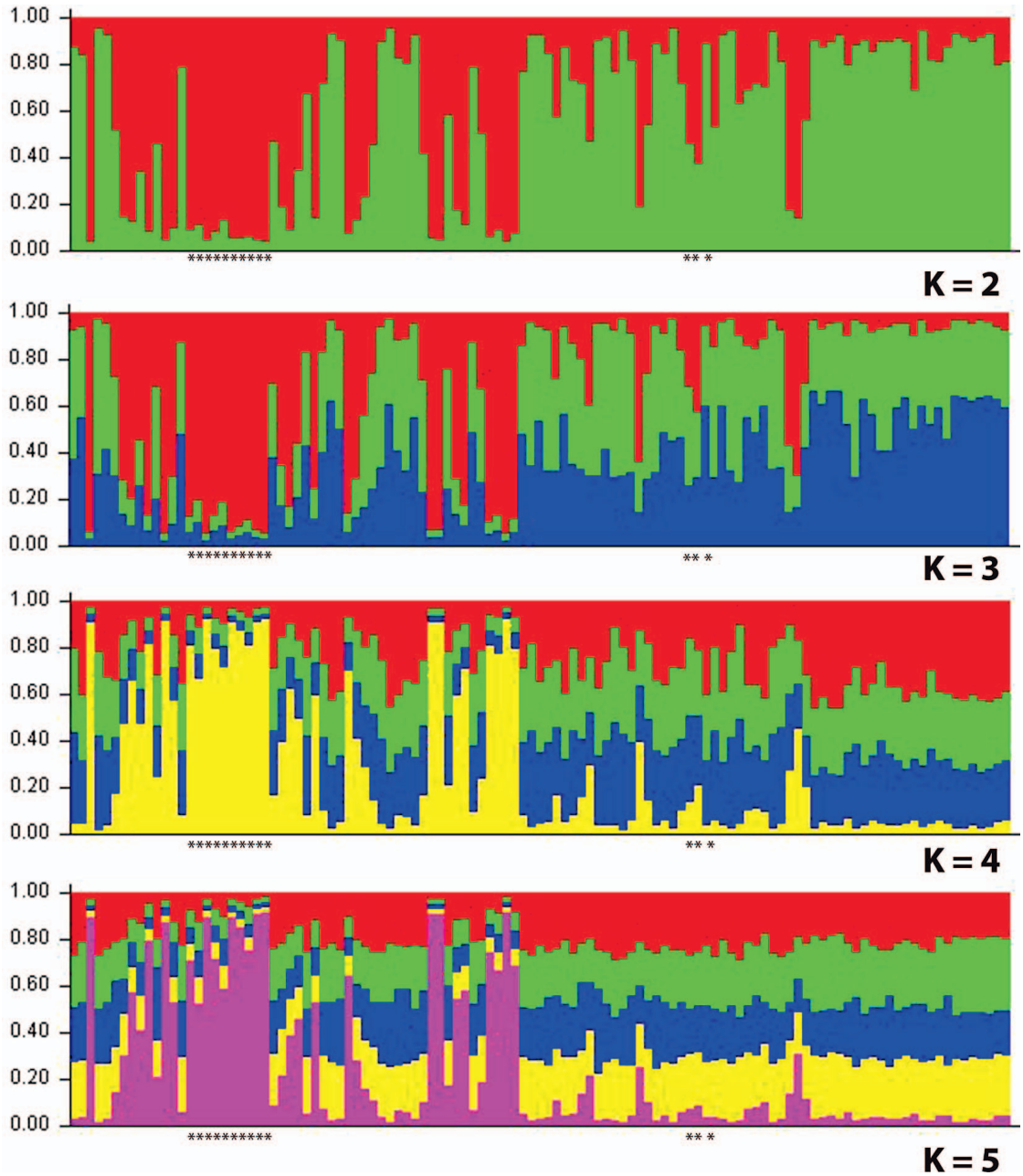

FIG. 4. Sample results of STRUCTURE analyses (K = 2, 3, 4, and 5) for the 113 field-sampled trees included in this study that were inferred to be pure *J. hindsii*. Individuals marked with asterisks were collected from putative original populations of *J. hindsii* in Contra Costa and Napa Counties (see Table S1).

not impossible, to conduct a study that would rigorously test any such hypotheses. Not surprisingly, the results of our STRUCTURE analyses including only individuals identified as pure *J. hindsii* (Fig. 4) suggest that, on one hand, the greatest genetic diversity was captured within the two most extensively sampled counties, Contra Costa and Napa, while, on the other hand, the full range of diversity was not captured among the very limited number of trees collected at the two putative original sites (Fig. 4). Nonetheless, we found no evidence of geographically based genetic structuring within *J. hindsii* across the areas sampled here. Of the 14 trees sampled from southern California, the six that were identified as pure *J. hindsii* are almost certainly the result of human introductions, as there is no

TABLE 3. HETEROZYGOSITY, F-STATISTICS, AND POLYMORPHISM BY SPECIES. Putative hybrids based on prior STRUCTURE analyses (Tables 2 and S1) were excluded from this analysis. Values reported are means and standard errors (SE) across 10 microsatellite loci (Table 3). n = sample size (in some cases less than number of individuals sampled due to missing data at some loci), N_a = number of alleles; N_e = number of effective alleles; I = information index, H_o = observed heterozygosity, H_e = expected heterozygosity, uH_e = unbiased expected heterozygosity, and F = fixation index.

Species	n	N_a	N_e	I	H_o	H_e	uH_e	F
hindsii								
Mean	94.100	3.700	1.784	0.628	0.281	0.342	0.344	0.159
SE	15.518	0.716	0.232	0.160	0.073	0.086	0.086	0.035
californica								
Mean	16.200	5.700	3.254	1.269	0.562	0.612	0.630	0.101
SE	1.200	0.790	0.451	0.180	0.093	0.076	0.078	0.078
major								
Mean	13.800	8.900	5.650	1.869	0.707	0.801	0.831	0.120
SE	0.200	1.100	0.632	0.124	0.053	0.024	0.025	0.060
microcarpa								
Mean	11.800	7.300	5.090	1.602	0.782	0.698	0.729	-0.125
SE	0.133	1.096	0.967	0.220	0.096	0.084	0.087	0.048
nigra								
Mean	13.600	7.500	4.608	1.676	0.736	0.755	0.784	0.019
SE	0.221	0.563	0.444	0.099	0.038	0.035	0.036	0.039
regia								
Mean	6.000	3.700	2.803	1.111	0.750	0.617	0.673	-0.231
SE	0.000	0.300	0.258	0.083	0.057	0.034	0.037	0.087

historical evidence to suggest that the species is native in those areas, while three trees were in fact *J. californica* and five were hybrids between the two species.

Our finding of lower genetic diversity in *J. hindsii* compared to other species (Table 3), similar to results reported by Fjellstrom and Parfitt (1994) based on RFLPs, is consistent with a past genetic bottleneck as would be expected if extant populations were derived from a relatively small number of ancestral populations. Nonetheless, the widespread occurrence of genetically pure *J. hindsii* suggests that the reduced genetic diversity has not, to date, impeded the persistence and spread of this species and that the CNPS (2017) rare plant rank of 1B.1 (rare, threatened, or endangered in California and elsewhere; seriously threatened in California) is not warranted.

On the other hand, *J. californica*, which showed higher levels of genetic diversity among our samples consisting primarily of germplasm repository accessions, is ranked 4.2 (watch list: plants of limited distribution; moderately threatened in California) by CNPS (2017), but may in fact be more seriously threatened due to extensive urbanization in southern California (Holstein 1984). A thorough study of the conservation status of *J. californica* is needed.

In addition to their implications for conservation and taxonomy, our results confirm the utility of microsatellite loci originally developed from *J. nigra* (Woeste et al. 2002) in other walnut species, as previously shown by Dangl et al. (2005) in *J. regia*; they also demonstrate the importance of careful inspection and interpretation of data when transporting molecular markers across species. Two of the 12 loci we tried to use initially produced multi-locus genotypes across several species and were excluded from final analyses. Among the remaining 10 loci, two primer pairs each failed in more than 50% of the individuals of *J. hindsii* and *J. californica*. Because of the restricted and concentrated taxonomic distribution of these failed reactions, we are confident in our interpretation that they are due to the presence of a null allele in the two native California species at the loci amplified by primer pairs WGA 001 and WGA 321.

In summary, our results show that genetically pure representatives of *J. hindsii* are common throughout the areas in California and southern Oregon sampled here. The limited, but appreciable, levels of hybridization and introgression from other native North American *Juglans* species probably resulted from occasional past introductions of those species as street trees or rootstocks. There is no evidence, however, of significant introgression from the widely introduced *J. regia*. Taken together, our results indicate that individual *J. hindsii* trees should not be considered a rare or imperiled species as currently treated. This study therefore effectively settles a long-held conservation concern or point of confusion about northern California black walnut trees and their conservation status.

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