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TECHNICAL NOTE

Microsatellite markers for *Juglans cinerea* L. and their utility in other Juglandaceae species

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Abstract Ten polymorphic microsatellite markers were found to amplify in butternut (*Juglans cinerea*; Juglandaceae). These microsatellite loci were found to amplify across most of nine other species and five hybrids examined. Loci were highly polymorphic, with 18 to 32 alleles per locus across species. These nuclear microsatellite markers will be useful in examining genetic diversity within and among populations of butternut, and in distinguishing butternut from interspecific hybrids.

Keywords Butternut · Endangered · Hybridization · Microsatellite markers · Sensitive species

Introduction

Butternut, *Juglans cinerea* L., is a short-lived tree species being extirpated throughout most of its native Eastern North American range by butternut canker (Schultz 2003; Nielsen et al. 2003), a disease caused by the exotic fungus *Sirococcus clavigignenti-juglandacearum* (Renlund 1971). The species was listed as endangered by the Committee on the Status of Endangered Wildlife in Canada in 2003, and in the USA it is currently considered a Regional Forester Sensitive Species.

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Previous research based on allozyme loci reported low levels of genetic diversity across butternut populations in the northern portion of the species range (Morin et al. 2000). Here, we identify a suite of ten nuclear microsatellite markers that amplify in *J. cinerea* and examine the utility of these markers in other Juglandaceae species. Four of these markers have previously been published for *J. nigra* (Woeste et al. 2002; Victory et al. 2006) and *J. regia* (Dangl et al. 2005).

Methods

Primers for this research were derived by further sequencing of a black walnut (*Juglans nigra*) microsatellite library described by Woeste et al. (2002). Leaf samples of the following species and hybrids were obtained: *Carya illinoensis* and *Juglans microcarpa* from L.J. Grauke, USDA ARS National Germplasm Repository for Pecans and Hickories, Somerville, TX; *Juglans ailanthifolia*, *J. cathayensis*, *J. hindsii*, *J. mandschurica*, *J. regia*, and *J.* x *paradox* from Malli Aradhya, USDA ARS National Clonal Germplasm Repository, Davis, CA; *J. nigra* from Mark Coggeshall, University of Missouri Agroforestry Research Center; and *J.* x *quadrangulata*. *J.* x *royal*, and *J. major* from the germplasm collection of the USDA Forest Service Hardwood Tree Improvement and Regeneration Center, West Lafayette, IN. Samples of *J. cinerea* were collected by the authors with the assistance of numerous collaborators.

DNA Extraction, quantification, amplification and genotyping

Genomic DNA was extracted from leaves as described by Victory et al. (2006). All samples were quantified using a

ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). PCR conditions and genotyping was performed as described in Victory et al. (2006) except products were separated using an ABI 3730 sequencer. Two positive and one negative control were run with each PCR to ensure accurate scoring. Failed reactions were repeated for accuracy.

Analysis

GDA (Lewis and Zaykin 2001) software was used to determine mean sample size over all loci (n), mean number of alleles per locus (A), expected (H_E) and observed (H_O) heterozygosity, linkage disequilibrium, and fixation index (f). Linkage disequilibrium was only tested for *J. cinerea*, the only species for which there was a large sample size (n = 422). SAS (v. 9.1; Cary, NC) Proc Princomp and Proc Candisc were used to perform principle components analysis (PCA) and canonical discriminant analysis (CDA), respectively. For both SAS procedures, missing data resulting from non-amplification or from unscorable, multiple peaks were replaced with the grand mean for each locus. Each allele/species combination was considered an independent unit of analysis (i.e., each bi-allelic genotype at each locus generated two data points). To make the dataset more balanced, eight *J. cinerea* genotypes were chosen arbitrarily and included in the PCA and CDA.

Results and discussion

Loci amplified across the majority of species examined (Table 1, 2) and were highly polymorphic, showing 18–32 alleles per locus across species; however, polymorphism within taxa was generally low, ranging from 1.3 for *J.* x *quadrangulata* to 13.0 for *J. cinerea*. This was likely due to limited sample sizes. Linkage disequilibrium was detected in 60% of the pairwise comparisons between loci. Four of the ten loci (WGA 004, WGA 204, WGA 221, and WGA 256) amplified across all species. The loci did not show a high degree of ascertainment bias (Table 2), as the allele numbers for *J. ailanthifolia* and *J. major* were as high or higher as those for *J. nigra*, the species from which the primers were originally derived. Transfer of WGA 004, 204, 221 and 256 to *C. illinoensis* is unusual and may point to either strong conservation of these loci or a relatively recent divergence of the genera (Hale et al. 2005).

Table 1 Primer sequences and label information for microsatellite loci that amplify in *Juglans cinerea*

Locus	Repeat motif	Primer sequence (5′ (3′)[a]	Label[b]	GenBank accession number
WGA 004*	$(GT)_5(GA)_{15}$	F: TGT TGC ATT GAC CCA CTT GT	6–FAM	AY465953
		R: TAA GCC AAC ATG GTA TGC CA		
WGA 033*	$(GA)_{19}(GAGT)_5(GA)_6$	F: TGG TCT GCG AAG ACA CTG TC	6-FAM	DQ307437
		R: GCA TCG TCA TTA CCT GCT CA		
WGA 082*	$(CT)_{20}$	F: TGC CGA CAC TCC TCA CTT C	HEX	AY333956
		R: CGT GAT GTA CGA CGG CTG		
WGA 090*	$(CT)_4T(TC)_{14}$	F: CTT GTA ATC GCC CTC TGC TC	6-FAM	AY352441
		R: TAC CTG CAA CCC GTT ACA CA		
WGA 142	$(CT)_8$	F: CAT ATT CCC GGT GAT TTT GG	6-FAM	DQ307429
		R: TGA CCA CAA ATC GGA GAT GA		
WGA 147	$(GA)_{14}(GT)_8$	F: TGG AAC TTG TTC TGT GCG AG	6-FAM	DQ307430
		R: CCG AGT CCC CTT CAC ATC TA		
WGA 148	$(AG)_{15}$	F: GGT GAA CTC CCA TAG GGG TA	6-FAM	DQ307431
		R: CCA ATG CTA CTT GCA GAA CC		
WGA 204	$(AG)_{15}$	F: GGG TCT CGC CTT CTT TTC TT	6-FAM	DQ307432
		R: CAC AGA GAG AAG CAC GGG TA		
WGA 221	$(CT)_8$	F: CGA CTG CGA AGC CTT TGT AT	6-FAM	DQ307428
		R: TGG GCA TCA CAC CTA CGT TA		
WGA 256	$(CT)_{19}$	F: TGA AGA CAA CAA AAC TGC GC	NED	DQ307435
		R: CCG GCA TTG TTT CTG AAA AT		

[a] F = forward primer; R = reverse primer

[b] Forward primers were modified at the 5′ end with a fluorescent label: HEX (green), 6-FAM (blue), or NED (yellow)

* sequence published previously for other species (WGA 004 in Woeste et al. 2002 and in Dangl et al. 2005; WGA 033 in Woeste et al. 2002; WGA 082 in Woeste et al. 2002 and in Victory et al. 2006; and WGA 090 in Victory et al. 2006)

Table 2 Allele size range (size) and number observed (N_a) for each locus and overall allelic richness (A) for each species

Species (sample size)		WGA 004	WGA 033	WGA 082	WGA 090	WGA 142	WGA 147	WGA 148	WGA 204	WGA 221	WGA 256
Juglans regia L. (8)	Size	228–240	NA	NA	NA	[a]130–218	190–195	253–271	172–178	230–232	227–253
	N_a	5	NA	NA	NA	[a]20	5	7	4	2	7
Juglans hindsii (Jepson) R.E. Smith (8)	Size	245–257	[a]202–258	170–190	126–150	[a]178–191	171–197	251–267	176–186	228–234	207–237
	N_a	4	[a]7	8	2	[a]7	3	3	2	2	4
Juglans major (Torrey) Heller (8)	Size	241–275	249–271	164–198	124–136	[a]163–186	188–196	239–245	172–188	204–238	207–217
	N_a	6	8	12	4	[a]11	5	4	4	6	4
Juglans microcarpa Berlandier (3)	Size	236–246	[a]236–268	166–194	[a]134–165	[a]165–207	195–213	233–247	166–178	224–236	223–231
	N_a	4	[a]9	5	[a]8	[a]12	5	5	4	2	4
Juglans nigra L. (8)	Size	234–246	[a]236–284	156–188	[a]134–172	[a]163–181	187–209	243–247	168–184	216–238	215–237
	N_a	6	[a]12	7	[a]10	[a]8	5	3	4	4	8
Juglans ailanthifolia Carr. (8)	Size	233–249	222–230	NA	157–185	128–158	183–209	230–264	175–191	220–230	207–243
	N_a	6	4	NA	10	6	8	8	7	4	10
Juglans mandschurica Maxim. (8)	Size	239–255	222–230	NA	133–173	128–158	181–209	230–260	169–197	220–228	215–237
	N_a	6	4	NA	7	6	9	7	10	5	10
Juglans cathayensis Dode (2)	Size	239–241	222–228	NA	159–175	142–150	183–213	238–266	179–187	224–234	235–237
	N_a	2	2	NA	3	3	3	4	3	3	2
Juglans cinerea L. (422)	Size	225–273	228–268	150–182	126–144	161–199	173–211	232–282	168–200	221–247	205–241
	N_a	20	6	16	7	10	14	15	16	11	15
Juglans x bixbyi Rehd. (3)	Size	245–275	228	162	132–165	134–158	197	260–264	166–184	226	219–227
	N_a	2	1	1	2	2	1	2	2	1	2
Juglans x intermedia Carr. (7)	Size	228–244	236–286	154–186	142–172	[a]130–205	189–221	235–261	168–178	224–236	215–249
	N_a	6	9	5	6	[a]18	5	7	5	4	6
Juglans x quadrangulata (Carr.) Rehd. (1)	Size	228–243	228	156	144	187–194	185	242	174–182	230	223
	N_a	2	1	1	1	2	1	1	2	1	1
Juglans x paradox Burbank (7)	Size	228–251	202–258	156–194	126–152	[a]130–205	181–197	243–267	174–188	228–236	219–249
	N_a	4	6	6	3	[a]10	3	5	4	4	6
Juglans x royal Burbank (1)	Size	238–245	[a]236–260	156	[a]146–172	165–169	193–209	243–247	168	236–238	221–247
	N_a	2	[a]3	1	[a]3	2	2	2	1	2	2
Carya illinoensis (Wangenh.) K. Koch (8)	Size	235–239	NA	NA	NA	NA	NA	NA	169–185	[a]217–230	220–244
	N_a	8	NA	NA	NA	NA	NA	NA	6	[a]3	10

Species are arranged by Section (i.e., Dioscaryon (*J. regia*), Rhysocaryon (*J. hindsii*, *J. major*, *J. microcarpa*, *J. nigra*), Cardiocaryon (*J. ailanthifolia*, *J. mandschurica*, and *J. cathayensis*) and Trachycaryon (*J. cinerea*)) followed by hybrids and Carya *illinoensis*. NA = no amplification

[a] Multiple peaks (>2) were observed for each individual, which may be explained by a gene duplication event and subsequent independent mutations in the repeat motif (but not the primer sequences) leading to amplification of multiple alleles per individual

Table 3 Mean sample size over all loci (*n*), mean number of alleles per locus (A), expected heterozygosity (H_E), observed heterozygosity (H_O), and an estimate of the fixation index (f). Species are arranged as in Table 2

Species	n	A	H_E	H_O	f
Juglans regia L.	6.57	4.43	0.610	0.401	0.359
Juglans hindsii (Jepson) R.E. Smith	8.00	3.50	0.572	0.563	0.018
Juglans major (Torrey) Heller	8.00	5.89	0.730	0.764	–0.050
Juglans microcarpa Berlandier	3.00	4.14	0.838	0.857	–0.029
Juglans nigra L.	8.00	5.29	0.731	0.750	–0.028
Juglans ailanthifolia Carr.	7.89	7.00	0.821	0.784	0.048
Juglans mandschurica Maxim.	7.10	6.60	0.823	0.677	0.214
Juglans cathayensis Dode	2.00	2.78	0.778	0.778	0.000
Juglans cinerea L.	415.00	13.00	0.723	0.670	0.073
Juglans x bixbyi Rehd.	3.00	1.60	0.353	0.567	–0.889
Juglans x intermedia Carr.	6.78	5.89	0.815	0.619	0.253
Juglans x quadrangulata (Carr.) Rehd.	1.00	1.30	0.300	0.300	–
Juglans x paradox Burbank	7.00	4.67	0.745	0.667	0.113
Juglans x royal Burbank	1.00	1.75	0.750	0.750	–
Carya illinoensis (Wangenh.) K. Koch	8.00	8.00	0.875	0.875	0.000

The usefulness of these microsatellite loci across species is reflected in the number of alleles per locus (Table 2). Both *J. regia* and *J. mandschurica* showed elevated fixation levels compared to other groups (Table 3). In the case of *J. regia*, this may reflect a domestication bottleneck. The hybrids *J.* x *intermedia* and *J.* x *paradox* are complex; they are intersectional hybrids that may contain the genomes of more than two species, and they may be the result of backcrosses or intercrosses (Potter et al. 2002). As a consequence, these taxa may contain genomic incompatibilities that limit recombination and affect inbreeding. Analysis using principle components and plotting of the first two principle component scores, which explained 41% of the variance, showed a clear clustering of the section Cardiocaryon alleles and a discrete position for the alleles of *J. major* and *J. regia* (Fig. 1). *J. cinerea* alleles were located much nearer to *J. nigra* and other members of section Rhysocaryon than to the Asian members of section Cardiocaryon, with which butternut is sometimes lumped (Fig. 1). The taxa could all be significantly separated from one another using canonical discriminant analysis, with the exception of *J. mandschurica* and *J. ailanthifolia* (data not shown).

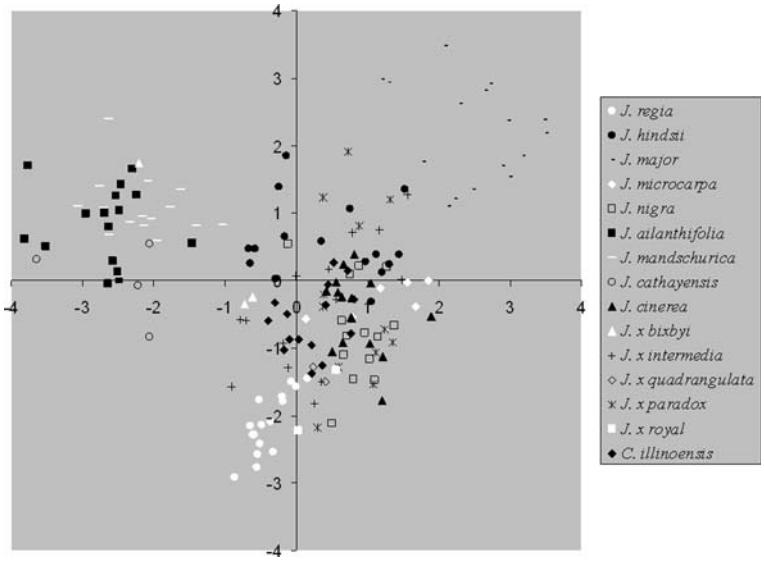

Fig. 1 Scatterplot of individual species/allele units on first two axes of PCA, which account for 41% of the total variance

The interspecific butternut hybrid *J*. x *bixbyi* is vigorous, difficult to distinguish from butternut, produces large numbers of fruit and may be more resistant to butternut canker. Cross-species amplification of these loci may prove useful in distinguishing butternut from hybrids. For example, there was a 13 base pair difference in allele size ranges for WGA 090 between *J*. *ailanthifolia* and *J*. *cinerea* (the two parent species of *J*. x *bixbyi*). Indeed, all *J*. x *bixbyi* individuals examined in this study contained an allele of the *J*. *cinerea* size and an allele of the *J*. *ailanthifolia* size, as expected. However, before a distinct range of allele sizes can be confirmed for a species, additional individuals must be genotyped. Results of PCA and CDA presented here should be considered heuristic and not an indication of phylogeny since allelic states were considered random deviations from a grand mean and not according to any biological model such as stepwise mutation, and the PCR products were not sequenced to verify the relationships among the length variants. The role of homoplasy in determining identity of state is likely in some cases.

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