CULTIVAR IDENTIFICATION AND GENETIC RELATEDNESS AMONG 25 BLACK WALNUT (*JUGLANS NIGRA*) CLONES BASED ON MICROSATELLITE MARKERS

Kejia Pang, Keith Woeste, and Charles Michler[1]

Abstract.—A set of eight microsatellite markers was used to genotype 25 black walnut (*Juglans nigra* L.) clones within the Purdue University germplasm repository. The identities of 212 ramets were verified using the same eight microsatellite markers. Some trees were mislabeled and corrected as to clone using analysis of microsatellite markers. A genetic dendrogram was constructed to show the degree of genetic relatedness between clones. Two additional dendrograms, one based on crown architecture traits and the other on tree size and form traits, were also built and compared with the genetic dendrogram. The genetic dendrogram showed that these eight molecular markers had the ability to distinguish genetically related clones from less related ones. Crown architecture traits and tree size and form traits were able to group genetically related clones together, but less accurately than the genetic matrix.

INTRODUCTION

Black walnut is a highly valuable timber species that is planted and grown widely in the eastern United States. A genetic improvement program for black walnut at Purdue University was initiated in 1967, and a clone bank containing black walnut timber genotypes was established (Beineke 1983, 1989). This clone bank contained genotypes from various parts of Indiana and other states.

Accurate and fast cultivar identification is particularly important for vegetatively propagated species to improve the efficiency of breeding and to protect property rights (Nicese et al. 1998). Numerous molecular techniques have been developed to verify cultivar identity, conduct parentage analysis, and evaluate genetic correlation among walnut cultivars (Dangl et al. 2005). These techniques include isozymes (Arulsekar et al. 1985; Rink et al. 1989, 1994; Solar et al. 1994); restriction fragment length polymorphism (Fjellstrom and Parfitt 1994); randomly amplified polymorphic DNA (Nicese et al. 1998); and microsatellite (simple sequence repeats [SSR]) markers (Woeste et al. 2002). Microsatellite markers developed by Woeste et al. (2002) have been widely used in genetic studies of walnut (*Juglans* spp.) (Dangl et al. 2005, Parks et al. 2014, Zhao et al. 2013). Molecular techniques genetically characterize commercial plant species more accurately than traditional methods that rely on the evaluation of phenological and morphological traits that are usually time consuming and subject to large errors because of environmental factors (Nicese et al. 1998, Weising et al. 1994).

The goals of this study were to (1) use microsatellite markers to verify the clonal identities of 212 black walnut grafted ramets believed to represent 25 clones; (2) examine the genetic relatedness of 25 black walnut clones; and (3) determine the level of correspondence between genotypic and phenotypic dendrograms of 25 black walnut clonal selections.

[1] Ph.D. candidate (KP), Purdue University, Department of Forestry and Natural Resources, West Lafayette, IN. Research Geneticist (KW) and Research Horticulturist (CM, deceased), U.S. Forest Service, Northern Research Station. KP is corresponding author: to contact, email at pangkejia@gmail.com. Current address: University of Missouri, Center for Agroforestry, 203 Natural Resources Bldg., Columbia, MO.

MATERIALS AND METHODS

Plant Material

We genotyped samples of 25 grafted clones of black walnut that have been growing in a plantation in West Point, IN (Table 1) since 2002. The clones were either mass selected from wild populations, from the Purdue University Black Walnut Improvement Program, or from a commercial company. Five to 10 grafted ramets were randomly sampled from each clone, for 212 trees in total. Some trees were excluded from the crown architecture and tree size analyses because of wind damage that occurred in summer 2009, 2010, and 2011.

Table 1.—Origin of the 25 black walnut (*Juglans nigra*) clones investigated in this study and documented paternal relationship

Clone	Mother	Origin[a]
C55	Unknown	Darlington, IN
C130	Unknown	wild
C715	C130	West Lafayette, IN
C720	Fayette-1	West Lafayette, IN
C702	BW95	West Lafayette, IN
C703	BW249	South Raub, IN
C707	BW95	South Raub, IN
C710	C55	South Raub, IN
C714	C55	South Raub, IN
C705	BW205[b]	West Lafayette, IN
C701	BW41	West Lafayette, IN
C717	BW36	West Lafayette, IN
C718	C55	wild
C730	C55	wild
C700	Unknown	wild
C708	Unknown	wild
C709	Unknown	West Lafayette, IN
C712	Unknown	wild
C713	Unknown	wild
C716	Unknown	wild
C719	Unknown	wild
C726	Unknown	wild
C728	Unknown	wild
C729	Unknown	wild
C777	Unknown	wild

[a] The places where the selections were found, usually from the progeny test of a previous elite black walnut selection; some, however, were found from the wild populations (wild).
[b] The grandmother of BW205 was BW97.

Study Area

The local annual average temperature in West Point, IN (40.4°N, 87°W) is 11.1 °C, and the annual average precipitation is 92.2 cm (U.S. Climate Data 2012). The soil type is well-drained Elston loam (NRCS 2016); site index is approximately 29 m on a 50 year basis (Zellers et al. 2012). All black walnut trees were planted at a spacing of 4.57 m × 6.10 m and were intensively managed.

Genomic DNA Extraction and Polymerase Chain Reaction Amplification

Fresh leaf samples were collected from each tree, put in plastic bags, and placed in a cooler in the field. All leaves were stored at 4 °C in a refrigerator before the DNA was extracted and quantified as described in Zhao and Woeste (2011). For polymerase chain reaction (PCR) amplification, 12 pairs of primers (Integrated DNA Technologies®, Table 2) from Woeste et al. (2002) were selected to fingerprint these 25 black walnut clones. Genomic DNA was diluted to 50-100 ng·μL^{-1}. PCRs were performed in a volume of 15 μL in 96-well plates, containing 1.5 uL of 2 mM deoxynucleotide solution (GeneMate), 1.5 μL of 1 mg·mL^{-1} bovine serum albumin, 1.5 μL of 1× *Taq* buffer, 1.5 μL of 1 mM magnesium chloride, 1.5 μL of 10 μM reverse primer, 0.3 μL of 10 μM forward primer, 1.5 μL of 10 μM M13 tag with 5' 6-Carboxyfluorescein or 6-Hexachlorofluorescein fluorescent (Schuelke 2000), 0.5 μL of 5 units·μL^{-1} *Taq* polymerase, 1 μL of genomic DNA, and 4 μL of nanopure water. The thermal cycle procedure for all primers was 3 minutes at 94 °C, 35 cycles of 45 seconds at 94 °C, 1 minute at 55 °C, and 45 seconds at 72 °C, and 1 cycle of 5 minutes at 72 °C at the end. Size multiplexing, which combines different markers with nonoverlapping size ranges (Dangl et al. 2005), was used with color multiplexing (5' 6-carboxyfluorescein and 6-hexachlorofluorescein). PCR products were diluted using nanopure water to 1/20 of its original concentration. One μL of diluted PCR product with 14 μL of formamide: rox (67:3) was added. Next, the DNA was denatured by heating at 95 °C for 5 minutes, snap cooled, and submitted to Purdue Genomic Center for analysis with an ABI 3700 sequencer (Applied BioSystems, Foster City, CA).

Table 2.—Characteristics of 12 microsatellite markers used to genotype 25 black walnut (*Juglans nigra*) clones

	Microsatellite Loci	Primer sequence (5′ - 3′)	Allele size range
1	WAG 06	F: CCATGAAACTTCATGCGTTG	134–172
		R: CATCCCAAGCGAAGGTTG	
2	WAG 32	F: CTCGGTAAGCCACACCAATT	163–217
		R: ACGGGCAGTGTATGCATGTA	
3	WAG 72	F: AAACCACCTAAAACCCTGCA	135–159
		R: ACCCATCCATGATCTTCCAA	
4	WAG 27	F: AACCCTACAACGCCTTGATG	199–245
		R: TGCTCAGGCTCCACTTCC	
5	WAG 69	F: TTAGTTAGCAAACCCACCCG	164–188
		R: AGATGCACAGACCAACCCTC	
6	WAG 82	F: TGCCGACACTCCTCACTTC	140–234
		R: CGTGATGTACGACGGCTG	
7	WAG 76	F: AGGGCACTCCCTTATGAGGT	228–254
		R: CAGTCTCATTCCCTTTTTCC	
8	WAG 90	F: CTTGTAATCGCCCTCTGCTC	142–178
		R: TACCTGCAACCCGTTACACA	
9	WAG 24	F: TCCCCCTGAAATCTTCTCCT	222–248
		R: TTCTCGTGGTGCTTGTTGAG	
10	WAG 86	F: ATGCCTCATCTCCATTCTGG	208–250
		R: TGAGTGGCAATCACAAGGAA	
11	WAG 89	F: ACCCATCTTTCACGTGTGTG	179–233
		R: TGCCTAATTAGCAATTTCCA	
12	WAG 97	F: GGAGAGGAAAGGAATCCAAA	149–189
		R: TTGAACAAAAGGCCGTTTTC	

Note: Sequence of M13 tag: AGTAAAACGACGGCCAGT; F: forward; R: reverse.

Data Analysis

Allele peaks were marked using GeneMapper v3.7.1 (Applied BioSystems, Foster City, CA). Reference samples from genotype C55 were used as positive controls in each plate. Errors with two base pairs' difference in allele size were allowed when binning and labeling the allele peaks. Allele frequency, probability of identity, probability of exclusion, and pair-wise genetic distance were calculated using the software GenAlEx (Peakall and Smouse 2006, 2012). Allele frequency calculation followed the method in Hartl and Clark (1997), using the equation for codominant data. Two types of probability of identity were calculated: (1) the unbiased probability of identity (P_I), i.e., the probability that two unrelated, randomly sampled trees would have identical genotypes, estimated following Paetkau et al. (1998); and (2) the probability of identity of full siblings (P_{Isib}), i.e., the probability that two randomly selected full siblings would have identical genotypes, calculated using the formula in Waits et al. (2001). The probability of exclusion of these loci for paternity analysis (P_1), i.e., to exclude a putative parent when the genotype of the mother is known, calculated following Jamieson and Taylor (1997) equations 1a and 4; the possibility of excluding a putative parent when the genotype of another parent is unavailable for test (P_2), estimated following Jamieson and Taylor (1997) equations 2a and 4; the probability of excluding a pair of putative parents when the genotypes of both parents were unknown (P_3), calculated using equations 3a and 4 (Jamieson and Taylor 1997).

A pair-wise genetic distance (D_s, a form of Euclidean distance) matrix was generated following equation 1 in Smouse and Peakall (1999). Then a cluster dendrogram based on D_s was prepared by NTSYSpc 2.0 (Rohlf 1997) using the unweighted pair-group method, which uses arithmetic averages (Sokal and Michener 1958). Two phenotypic dendrograms were constructed based on Euclidean distance using PROC CLUSTER and PROC TREE in SAS 9.3 (SAS Institute., Cary, NC), also following the unweighted pair-group algorithm. One phenotypic dendrogram was based on crown architecture traits; a second was based on tree size and form traits. All phenotypic data were collected between 2009 and 2012 (Pang 2014). The crown architecture traits were clonal means of each tree's average branch angle, branch frequency, branch diameter, and branch basal area per meter of stem; the tree size and form traits included clonal mean of d.b.h. (diameter at breast height, 1.37 m), tree height, crown radii, the ratio of tree height to d.b.h. (height ratio), and stem straightness (Tables 3 and 4). Mantel tests (Mantel 1967) were performed following Smouse and Long (1992) to evaluate the correspondence between the genetic matrix and each of the two phenotypic matrices using GenAlEx (Peakall and Smouse 2006, 2012).

Table 3.—Clone means of four crown architecture traits of 25 black walnut (*Juglans nigra*) clones (standard deviation)

Clone	Branch angle[a]	Branch diameter[b]	Branch frequency[c]	Branch basal area[d]
	degrees	*cm*	*per meter*	*cm^2*
C130	69.7(5.1)	2.8(0.3)	13.0(1.3)	79.0(10.8)
C55	65.3(3.7)	2.5(0.2)	14.6(2.5)	57.2(11.6)
C700	70.3(5.4)	2.8(0.3)	13.1(1.8)	55.1(7.2)
C701	65.5(2.4)	2.8(0.1)	11.9(1.8)	61.5(7.6)
C702	55.3(2.9)	2.6(0.2)	16.9(1.7)	63.3(7.1)
C703	70.3(2.7)	2.8(0.3)	16.2(1.8)	79.6(12.3)
C705	66.0(2.3)	2.9(0.2)	13.8(1.3)	70.9(15.4)
C707	61.9(3.8)	2.7(0.3)	15.2(1.7)	62.9(15.2)
C708	65.7(6.6)	2.9(0.4)	18.4(1.1)	71.9(10.7)
C709	65.7(3.7)	2.4(0.3)	13.8(0.6)	54.3(9.9)
C710	57.5(3.6)	2.8(0.2)	13.7(1.5)	75.2(9.6)
C712	68.4(1.8)	2.9(0.2)	13.1(1.6)	76.5(12.1)
C713	53.2(2.4)	2.9(0.2)	14.6(1)	88.9(11.3)
C714	66.6(4.2)	3.1(0.2)	15.5(1.2)	74.0(17.3)
C715	57.0(4.9)	2.8(0.3)	16.5(1.9)	81.9(12.6)
C716	67.7(7.7)	2.8(0.2)	15.3(1.4)	65.5(11.7)
C717	66.4(6.9)	2.7(0.1)	13.1(1)	80.8(16.1)
C718	70.3(3.3)	3.2(0.1)	13.3(1.3)	69.1(11.3)
C719	67.5(0.7)	2.7(0)	12.2(3)	66.7(22.9)
C720	60.8(3.5)	3.0(0.3)	12.6(1.5)	66.2(9.7)
C726	70.0(3.3)	2.9(0.2)	8.9(1.1)	74.3(8)
C728[e]	67.7	2.9	14.5	70.9
C729[e]	79.6	2.4	12.6	55.2
C730	61.7(4.4)	2.2(0.2)	14.4(1.2)	66.2(7.3)
C777[e]	66.4	2.8	16	94.6

[a] The average of the insertion angle of all living branches
[b] The average diameter of all living branches
[c] The number of branches per meter of stem
[d] The accumulated branch basal area per meter of stem
[e] Clones that had only one tree left because of wind damage, making standard deviation unavailable

Table 4.—Clone means for tree size and form traits for 25 black walnut (*Juglans nigra*) clones (standard deviation)

Clone	d.b.h. 2009	d.b.h. 2010	d.b.h. 2011	Tree height 2009	Tree height 2010	Tree height 2011	Crown radius 2009	Crown radius 2010	Crown radius 2011	Height: d.b.h. 2009	Height: d.b.h. 2010	Height: d.b.h. 2011	Straightness[a] 2008
	cm												
C130	13.1(1.2)	15.2(1)	16.7(0.9)	717(51)	887(41)	1023(33)	288(26)	286(20)	237(27)	55.1(3)	58.6(2.3)	63(4.8)	3.2(0.9)
C55	12.3(0.8)	14.5(1)	16.1(1)	850(62)	1009(52)	1113(56)	280(15)	266(17)	245(27)	69(5.1)	69.8(4.1)	69.3(3)	4.2(0.7)
C700	10.9(0.8)	13(0.7)	14.8(0.9)	807(41)	902(86)	975(120)	273(16)	271(34)	220(54)	74.6(6.2)	69.7(7)	66.1(8)	3.2(0.5)
C701	10.5(0.7)	12.8(0.5)	14.6(0.5)	784(43)	909(24)	1046(41)	304(24)	273(27)	199(25)	74.6(6)	71.2(1.8)	71.7(2.1)	3.5(0.5)
C702	12.8(0.7)	14.8(0.6)	16.4(0.5)	815(52)	986(41)	1085(43)	291(21)	248(27)	251(25)	63.9(4.4)	66.6(2.8)	66.2(1.9)	3.9(0.7)
C703	12.1(1.1)	14.5(0.9)	16.3(0.9)	755(52)	936(30)	1055(31)	284(17)	263(26)	264(26)	62.6(2.9)	64.7(4.2)	64.8(2.6)	3(0.6)
C705	11.3(0.9)	13.5(1)	15.2(1.2)	832(117)	963(53)	1061(69)	300(19)	308(25)	254(23)	73.6(7.2)	71.6(2.9)	69.1(4.1)	2.9(0.4)
C707	12.2(1.2)	14.2(1)	15.4(1.2)	831(54)	952(78)	1059(51)	289(29)	249(33)	258(30)	68.7(6.8)	67.2(6.8)	68.9(4.5)	3.1(0.5)
C708	12.4(0.8)	14.9(0.9)	16.3(0.9)	838(49)	1002(31)	1106(35)	291(25)	269(23)	253(18)	67.7(5.6)	67.4(3.9)	68.1(5.3)	2.9(0.7)
C709	11.3(0.6)	13.2(0.5)	14.9(0.5)	797(23)	921(9)	1031(29)	274(22)	250(15)	247(16)	70.9(3.1)	69.7(2.6)	69.1(2)	2.4(0.9)
C710	12.6(0.5)	14.7(0.4)	16.2(0.4)	880(33)	1020(48)	1134(25)	291(21)	279(24)	282(30)	70.1(1.7)	69.4(3.5)	69.8(1.8)	4.3(0.8)
C712	13(0.9)	15.3(1)	17.1(1.1)	827(39)	1000(73)	1156(41)	302(24)	269(17)	252(30)	63.8(3.9)	65.3(3.8)	67.9(4.3)	4(0.8)
C713	13.1(1.1)	15.4(1)	17(1.1)	811(50)	972(60)	1105(51)	306(15)	284(30)	247(31)	62(3.2)	63.3(3.6)	65.2(3.3)	3.7(0.5)
C714	14.1(1.1)	16.4(0.9)	18.1(0.8)	938(48)	1098(40)	1233(51)	304(20)	295(25)	281(19)	66.6(3.9)	67.1(2.4)	68.2(2.5)	4.4(0.5)
C715	14.1(1.2)	16.2(1.1)	17.6(1.1)	774(59)	911(60)	1021(54)	308(18)	285(24)	274(27)	55.1(2.9)	56.4(2.4)	58.4(3.7)	4.5(0.9)
C716	11.9(0.7)	14.2(0.9)	15.7(0.9)	859(35)	955(68)	1101(73)	297(29)	267(28)	269(30)	72(2.2)	67.4(3.3)	70.2(1.6)	4.5(0.8)
C717	11.9(1)	13.8(0.6)	15.2(0.2)	754(48)	780(56)	953(86)	300(20)	288(25)	250(29)	63.3(3.9)	56.5(6)	62.6(6.2)	2.7(0.6)
C718	13(0.3)	15.3(0.2)	16.9(0.3)	851(32)	1032(29)	1130(19)	275(17)	258(21)	249(33)	65.6(2.4)	67.3(2)	66.7(1.6)	3.8(1.1)
C719	10.4(1)	12.9(0.8)	14.4(0.5)	693(13)	747(37)	920(35)	304(4)	265(35)	254(13)	67.3(7.7)	58.2(6.5)	64.2(4.9)	3(0)
C720	11.5(1.5)	13.9(1.3)	15.5(1.4)	772(45)	942(60)	1082(25)	284(24)	294(15)	267(29)	67.8(7.7)	67.8(3.8)	67.6(2.9)	3.2(1.2)
C726	11.2(0.5)	13.2(0.5)	14.7(0.5)	797(75)	886(24)	1016(39)	298(14)	279(21)	262(23)	71.3(5.5)	67.1(2.1)	69.1(1.9)	2.8(0.7)
C728[b]	12.2	14.2	15.8	777	867	1035	299	264	248	63.7	60.9	65.7	3
C729[b]	8.5	9.9	11.1	584	729	771	259	252	240	68.6	73.6	69.8	1
C730	11.7(0.7)	13.9(0.9)	15.3(1)	739(42)	893(16)	1047(27)	283(23)	258(13)	229(12)	63.3(4.5)	64.5(4.1)	68.5(4.4)	4.2(0.8)
C777[b]	12.4	14.9	16.8	789	915	/	312	322	315	63.4	61.6	/	3

[a]1 to 5 with 5 as the straightest

[b]Clones that had only one tree left because of wind damage, making standard deviation unavailable

RESULTS AND DISCUSSION

Allele Frequency, Probability of Identity, and Probability of Exclusion

Although the 12 markers we used to characterize the clones in our study were published SSRs with successful applications (Robichaud et al. 2006, Woeste et al. 2002), the visualization of allele peaks at loci WAG06, WAG24, WAG69, and WAG90 was not consistent or clear, so these loci were dropped. Among them, WAG06, WAG24, and WAG90 often presented multiple (more than three) allele peaks; thus, it was difficult to make allele calls. We omitted WAG69 because of the likely presence of a null allele. In all, then, only eight markers yielded clear results with regard to allele sizes (Table 5).

Based on the allele profile (size and frequency) produced by the eight SSRs for the 25 black walnut clones (Tables 5 and 6), WAG32, WAG27, WAG86, WAG89, and WAG97 were more polymorphic than WAG72 and WAG76, with only eight and five alleles observed for the latter two loci, respectively. Most markers had strong power of exclusion, which means they could precisely identify relatives and determine paternity/parentage reliably. The exceptions,

Table 5.—Allele sizes (in base pairs) at eight microsatellite loci for 25 black walnut (*Juglans nigra*) clones

Clone	WAG32		WAG86		WAG72		WAG82[a]		WAG27		WAG89		WAG76		WAG97	
C130	183	185	222	222	146	146	172	182	217	227	199	209	230	238	171	189
C55	169	191	222	240	146	146	182	190	225	229	197	197	232	236	155	161
C700	181	181	220	232	144	144	168	178	223	227	209	211	232	236	163	169
C701	179	189	216	226	146	146	194	196	221	227	185	219	230	236	155	161
C702	189	191	228	240	146	146	162	190	225	229	191	197	232	236	159	161
C703	171	171	222	236	144	146	184	196	221	225	189	197	236	236	155	173
C705	181	187	216	238	146	148	178	182	213	213	201	213	232	232	157	161
C707	181	181	222	224	146	148	190	196	221	233	209	211	232	236	155	169
C708	187	195	232	238	146	146	178	180	221	221	211	217	232	238	159	161
C709[a]	177	199	212	232	144	144	–	–	219	221	189	209	232	234	163	171
C710	181	191	214	240	146	152	170	184	221	229	197	209	230	236	161	173
C712	179	183	214	222	146	146	176	194	241	241	195	207	230	236	161	161
C713	183	193	216	224	144	144	194	200	211	219	191	209	236	236	157	159
C714	169	173	234	240	146	146	176	182	221	229	187	197	232	232	155	161
C715	181	183	222	230	146	146	182	194	211	217	199	209	232	238	161	189
C716	169	169	222	222	146	146	182	206	223	225	197	201	230	236	155	173
C717	169	181	222	230	146	158	178	200	221	221	187	187	230	236	167	173
C718	171	191	220	222	146	146	168	182	223	229	197	207	232	234	155	163
C719[a]	183	214	222	250	146	156	–	–	211	241	187	199	234	234	163	167
C720	175	207	214	238	146	146	166	180	211	223	201	215	230	232	153	155
C726[a]	179	189	214	216	146	154	–	–	219	219	199	211	230	236	159	167
C728	181	181	216	228	146	146	178	194	213	213	197	213	232	232	161	173
C729	183	197	214	222	144	150	168	188	225	225	205	205	232	236	161	169
C730	169	187	216	222	146	152	182	190	221	229	197	209	232	236	155	175
C777	181	212	220	236	144	152	162	178	211	225	169	191	232	232	133	149

[a] C709, C719, and C726 were not successfully amplified by primer WAG82. As a result, their allele sizes were missing.

Table 6.— Frequencies of observed alleles at eight microsatellite loci based on 25 black walnut (*Juglans nigra*) clones (number of alleles observed for each locus)

Locus	Allele size (base pair)	Frequency	Locus	Allele size (base pair)	Frequency
WAG32	169	0.120	WAG89	169	0.020
(19)	171	0.060	(17)	185	0.020
	173	0.020		187	0.080
	175	0.020		189	0.040
	177	0.020		191	0.060
	179	0.060		195	0.020
	181	0.220		197	0.200
	183	0.120		199	0.080
	185	0.020		201	0.060
	187	0.060		205	0.040
	189	0.060		207	0.040
	191	0.080		209	0.160
	193	0.020		211	0.080
	195	0.020		213	0.040
	197	0.020		215	0.020
	199	0.020		217	0.020
	207	0.020		219	0.020
	212	0.020	WAG76	230	0.160
	214	0.020	(5)	232	0.380
WAG86	212	0.020		234	0.080
(15)	214	0.100		236	0.320
	216	0.120		238	0.060
	220	0.060	WAG97	133	0.020
	222	0.280	(14)	149	0.020
	224	0.040		153	0.020
	226	0.020		155	0.180
	228	0.040		157	0.040
	230	0.040		159	0.080
	232	0.060		161	0.240
	234	0.020		163	0.080
	236	0.040		167	0.060
	238	0.060		169	0.060
	240	0.080		171	0.040
	250	0.020		173	0.100
WAG72	144	0.180		175	0.020
(8)	146	0.640		189	0.040
	148	0.040	WAG82	162	0.045
	150	0.020	(16)	166	0.023
	152	0.060		168	0.068
	154	0.020		170	0.023
	156	0.020		172	0.023
	158	0.020		176	0.045
WAG27	211	0.100		178	0.136
(11)	213	0.080		180	0.045
	217	0.040		182	0.182
	219	0.080		184	0.045
	221	0.220		188	0.023
	223	0.080		190	0.091
	225	0.140		194	0.114
	227	0.060		196	0.068
	229	0.120		200	0.045
	233	0.020		206	0.023
	241	0.060			

Table 7.—Discrimination power of the black walnut (*Juglans nigra*) microsatellites used in this study

Locus	P_I[a]	P_{ISibs}[b]	P_1[c]	P_2[d]	P_3[e]
WAG32	0.0181	0.3057	0.7986	0.6651	0.9381
WAG86	0.0262	0.3206	0.7552	0.6056	0.9147
WAG72	0.2340	0.5329	0.3441	0.1743	0.5347
WAG82	0.0163	0.3016	0.8098	0.6804	0.9428
WAG27	0.0260	0.3169	0.7585	0.6091	0.9114
WAG89	0.0179	0.3049	0.8003	0.6673	0.9384
WAG76	0.1275	0.4231	0.4764	0.3036	0.6583
WAG97	0.0274	0.3200	0.7522	0.6019	0.9094
Combined	0.0000	0.0002	1.0000	0.9987	1.0000

[a]The probability that two unrelated, randomly sampled trees would have identical genotypes

[b]The probability that two randomly selected full siblings would have identical genotypes

[c]The possibility of excluding a putative parent when the genotype of mother is known

[d]The possibility of excluding a putative parent when the genotype of another parent is unavailable for test

[e]The probability of excluding a pair of putative parents when the genotypes of both parents were unknown

WAG72 and WAG76, were less powerful than the other markers (Table 7) because they were less polymorphic. Although usable markers were reduced from 12 to 8, combined together, these eight markers had a probability of exclusion (P_I) that was less than 2.9×10^{-12} and a P_{Isibs} of 0.0002 (Table 7). These data indicate that the SSRs provided high power of discrimination for cultivar identification, paternity determination, parentage analysis, and relatedness. The likelihood that individuals would share genotypes simply by chance was essentially zero.

Several trees were found to be wrongly labeled based on their allele sizes at the eight loci. Sample BD111 was labeled as C55, and BM111 was marked as C703; however, both of their genotypes matched clone C707. AS115 was labeled as C728, but its genotype did not match any other trees of C728 or any other clones. AY138 and AZ130 were both recorded as C716, but their genotypes were different from other trees of C716. The allele sizes indicated that AY138 and AZ130 shared the same genotype, so they were temporarily named C777, a clone that was not on the original list. Ramets of C704 were found to be identical to those of C715. These results were in accordance with the phenological observations recorded for each clone from 2009 and 2010 (Pang 2014). As a result, there were still 25 genotypes in total, and we corrected the mislabeled trees before conducting further analysis.

Table 8.—Pair-wise genetic distance among 25 black walnut (Juglans nigra) clones based on the allele information at eight microsatellite loci, calculated by GenAlEx 6.41

130[a]	55	700	701	702	703	705	707	708	709	710	712	713	714	715	716	717	718	719	720	726	728	729	730	777	
0																									130
13	0																								55
18	18	0																							700
13	12	17	0																						701
15	5	17	11	0																					702
16	11	16	12	13	0																				703
17	14	15	15	14	19	0																			705
14	12	10	12	13	12	13	0																		707
15	14	16	12	12	16	12	13	0																	708
18	19	12	18	18	16	18	16	16	0																709
14	9	14	11	9	11	15	11	13	16	0															710
13	13	20	10	13	16	17	16	15	21	12	0														712
18	18	13	15	15	14	18	15	19	14	15	17	0													713
15	6	18	12	9	15	12	13	11	17	11	14	20	0												714
6	11	15	12	12	16	12	11	12	17	12	11	15	11	0											715
11	7	19	13	13	11	17	14	17	21	13	14	19	12	13	0										716
15	15	16	13	16	13	17	12	13	18	11	16	17	13	14	12	0									717
12	7	14	13	10	12	14	13	14	16	12	14	19	9	11	10	16	0								718
14	17	19	17	17	18	19	17	18	13	17	15	18	17	13	17	15	13	0							719
14	13	17	12	13	16	13	14	12	18	13	14	18	12	12	12	16	11	16	0						720
16	17	18	12	14	17	18	16	16	14	14	15	14	18	16	17	16	17	13	15	0					726
18	13	15	14	12	18	6	13	14	20	13	16	20	12	11	17	16	14	20	15	19	0				728
17	14	14	17	14	14	17	15	18	17	15	15	15	17	15	16	18	16	18	17	18	19	0			729
12	6	15	11	10	11	12	9	12	15	9	15	14	8	11	9	12	9	16	13	15	14	15	0		730
19	16	11	18	13	16	13	14	16	15	15	20	15	15	14	19	17	15	18	15	19	14	14	14	0	777

[a]Letter "C" was omitted from all clone names to save space

Genetic Relatedness between 25 Black Walnut Clones

A genetic dendrogram can summarize microsatellite data and reveal genetic relationships among tested cultivars (Dangl et al. 2001). Three pairs of clones were grouped into three clades: C702 and C55 had the smallest genetic distance (five); there were two pairs with a genetic distance of six, C130/C715 and C705/C728 (Table 8, Fig. 1). The proximity of C130 and C715 was not surprising because C130 was believed to be the female parent of C715. C55 and C702 shared one allele at all eight loci (Table 5), indicating that C55 may be the sire of C702, because C702's mother was believed to be BW95, a clone not included in this study. C705 and C728 also shared one allele at each locus, indicating close consanguinity, almost certainly as parent-offspring, but possibly full siblings. Breeding records indicate that C55 was the mother of C710, C714, C718, and C730. All these relationships were supported by the close genetic distance between them (Table 8) and the dendrogram based on genetic distance (Fig. 1). The genetic dendrogram and relatedness matrix also disclosed that C702 is closely related to C710, C714, C716, C718, and C730, almost certainly as a full sibling. According to previous breeding records, C702 and C707 are maternal half-siblings from BW95, but this was not reflected in the dendrogram as were the half siblings of C55 mentioned earlier. C702 and C707 may be half-siblings, but the SSR genotype profiles failed to show it because they inherited, by random segregation, different alleles from their mother at many of the eight SSR loci. At least one of the two clones may also have been incorrectly assigned as an offspring of BW95. The genotype of BW95 will need to be compared with both C702 and C707 to obtain more information.

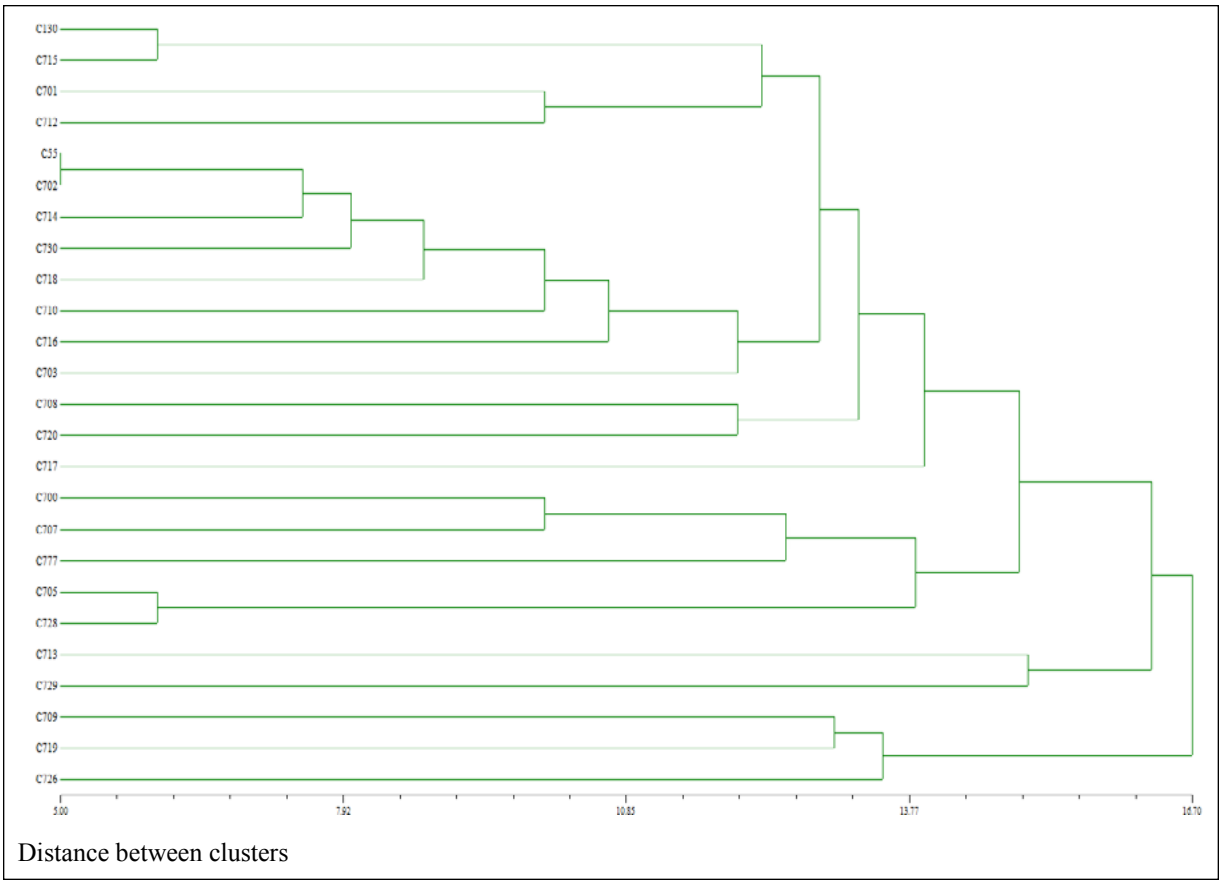

Figure 1.—Dendrogram generated using cluster analysis based on the proportion of shared alleles among 25 black walnut (*Juglans nigra*) genotypes by NTSYSpc 2.0.

The Concordance and Discordance between Genetic Relatedness and Phenotypic Relatedness

The crown architecture dendrogram (Fig. 2) revealed some phenotypic similarities between clones. C705 and C728 were closely grouped based on cluster analysis of crown architecture traits, just as they were in the genetic dendrogram (Fig. 1). C702 and C707 were half-siblings and were grouped in one clade at a distance of 0.52. Half-siblings C718, C710, and C714 were close to each other; C718 and C714 were more closely clustered. C55, as the presumed female parent of C730, and possible male parent of C702, was located near C730 and C702 in the crown architecture dendrogram as well. C716 was near C714, C718, and C710, as it was in the genetic dendrogram. The positions of these clones in the dendrogram make sense given their known genetic relationship. Not all clones were located within the dendrograms where they were expected, however; for example, C715, believed to be an offspring of C130, was far from C130 based on the branch attributes that comprised the crown architecture (Fig. 2). This disparity between genetic relatedness and crown architecture similarity was probably because C715 had larger branch frequency than C130 (16.5 ± 1.9 versus 13 ± 1.3, respectively), and smaller branch angle than its supposed female parent (57 ± 4.9 versus 69.7 ± 5.1) (Table 3).

The tree size and form dendrogram (Fig. 3) clustered clones with similar size and form together. C716 was located close to C710, C718, and C55, as it was in the genetics dendrogram. Again, C728 and C705, which are most likely a parent-offspring pair or full siblings, were not far apart in the dendrogram. Although C715 and C130 were unlike each other in branch characteristics,

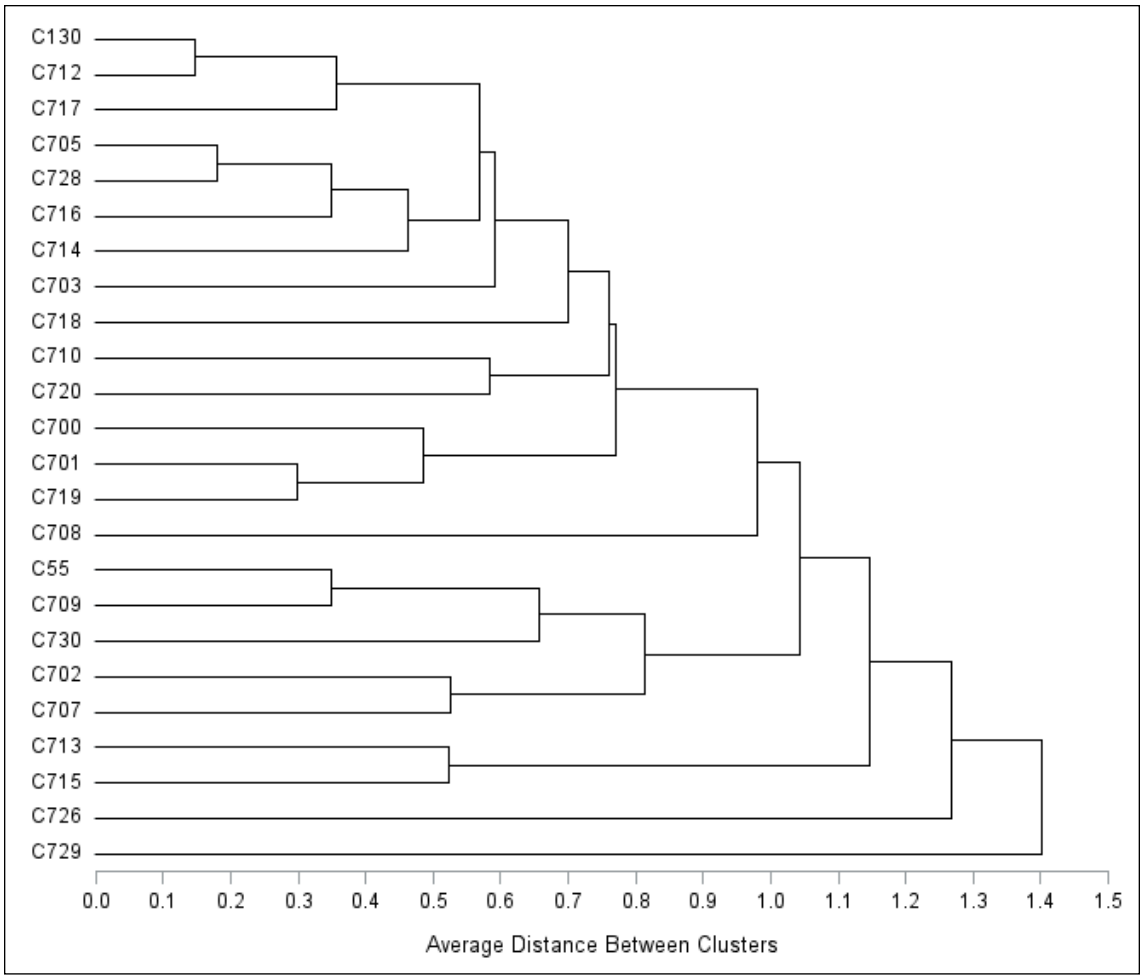

Figure 2.—Dendrogram generated using cluster analysis based on crown architecture traits of 25 black walnut (*Juglans nigra*) genotypes.

as shown in the crown architecture dendrogram, they were grouped in one cluster in this dendrogram, indicating that C715 possibly inherited size and form from its female parent, but not branch structure. C702 and its presumed half-sibling C707 clustered closely. C55 was close to its presumed offspring C718, C710, and its possible offspring C702. Among all the presumed offspring of C55, C714 was furthest from C55. In fact, C55 was closer to the unrelated C130 than it was to C714. It was probably because C714 was much larger in d.b.h., tree height, and crown radius than C55, its siblings, and C130 (Table 4). The most phenotypically divergent clone based on tree size and form traits was C729, which was also most divergent in terms of crown architecture. C729 was not closely related genetically to any other clone (Fig. 1).

Overall, the genetic dendrogram showed that the eight microsatellites we used had the ability to distinguish genetically related clones from unrelated ones, which was reflected by the high probabilities of exclusion (>0.9987, Table 7). Each of the two phenotypic dendrograms grouped some genetically related clones together, but less accurately than the genetic distance dendrogram. Phenotypic similarity as reflected in architecture, tree size, and form traits, was not accidental but a consequence of genetic relatedness. Phenotypes imperfectly reflect genotypic relatedness in part because growth is a deviation-amplifying process (Stage 1987) because of the accumulated effects of local environments over time and genotype × environment interactions over time. Variability in important traits increases as trees grow. In this way, even clones, which are genetically identical, can begin to look different over time. The accumulated effects of local

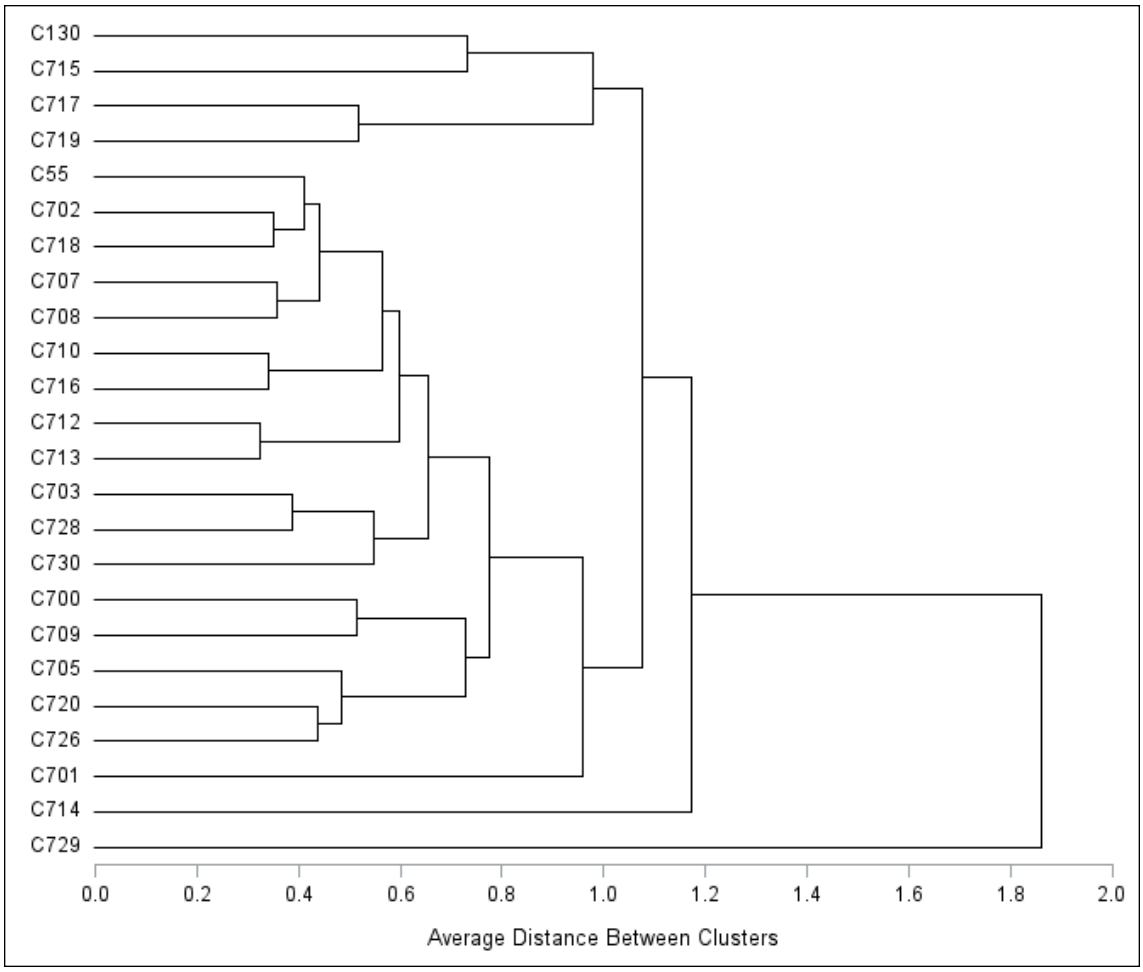

Figure 3.—Dendrogram generated using cluster analysis based on tree size and form traits of 25 black walnut (*Juglans nigra*) genotypes.

environmental differences on a tree's growth can make it difficult to untangle which genes control the growth and form of trees and to what extent. Some confusing associations, such as C55 being closer to C130 than its presumed offspring C714 in the size and form dendrogram, and the unexpected placement of C715 far away from its maternal parent C130 in the crown architecture dendrogram, may in part be the result of such deviation-amplifying processes. The lack of correspondence between the phenotypic and genotypic dendrograms can also be attributed to the relatively small sample size of trees and the high level of genetic relatedness among these trees. If there were more clones like C729 that are more distinct from the rest of the clones, we would have a better perspective. The discordance between genetic relatedness and phenotypic relatedness, however, implies that desirable crown architecture can be obtained from genetically unrelated parents within the breeding program, which is positive because it reduces the necessity for inbreeding. The dendrograms of crown traits and tree size and form traits are useful for understanding how trees can be grouped by architecture and form, which may ultimately be useful in understanding whether certain tree characteristics are physiologically more desirable for efficient growth in plantations.

The attempt to associate phenotypic and genetic similarity is the basis for considerable research in modern quantitative genetics. The correspondence between the genetic distance measures and the phenotypic matrices can be either concordant (Atchley et al. 1982, Villani et al. 1992), or discordant (Atchley et al. 1988, Hampl et al. 2001, Woeste et al. 1998). In our case, the Mantel

correlation between the matrix of genetic relatedness and the matrix of similarity in crown architecture was low (0.105), as was the correlation between the genetic relatedness matrix and tree form and size matrix of similarity (0.141). Complex traits usually have a complex genetic basis, and when a large number of genes are involved in a trait, it often has a wide variance that is strongly affected by environment. The phenotypes entered into the matrix were complex—a mathematical combination of several distinct phenotypes, each with its own error of estimation, reducing its connection to common underlying genetic causes. Stated another way, in general, the more complex the phenotype, the more genes involved in its expression, and the lower its heritability, which was shown in this case by the low correspondence between the genotypic and phenotypic matrices. The process of reducing multidimensional reality to a single number facilitates comparison, but a lot of information is lost in the process. Having a more detailed genotype can increase the connection between genotype and phenotype, but even if the number of DNA markers were increased to thousands and a full quantitative trait loci (QTL) analysis performed, such an analysis is often able to explain only a small percentage of the total phenotypic variance for quantitative traits such as yield or plant height. Despite their drawbacks, our results have value because they represent the first systematic attempt to measure and categorize phenotypic variability for important growth and form traits in black walnut. By adding more microsatellite markers and more black walnut clones from the genetic improvement program, more accurate and more comprehensive pedigree information will be available to black walnut breeders in the future.

ACKNOWLEDGMENTS

Funding for this project, which was part of a larger project (CAFS 08.01), was provided by National Science Foundation Industry/University Cooperative Research Center for Advanced Forestry Systems (0736399). We thank Nick LaBonte and Paola Pollegioni for their help in data analysis, and John Cannaday, Kayla Leach, Jim McKenna, Brian Beheler, Hector Matute, Jason Cook, and Lijie Zhao for their assistance in tree measurements. We extend our appreciation to ArborAmerica, Inc., for granting access to its black walnut plantation. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

LITERATURE CITED

Arulsekar, S.; Parfitt, D.E.; McGranahan, G.H. 1985. **Isozyme gene markers in *Juglans* species inheritance of GPI and AAT in *J. regia* and *J. hindsii*.** Journal of Heredity. 76: 103-106.

Atchley, W.R.; Newman, S.; Cowley, D.E. 1988. **Genetic divergence in mandible form in relation to molecular divergence in inbred mouse strains.** Genetics. 120: 239-253.

Atchley, W.R.; Rutledge, J.J.; Cowley, D.E. 1982. **A multivariate statistical analysis of direct and correlated response to selection in the rat.** Evolution. 36: 677-698.

Beineke, W.F. 1983. **The genetic improvement of black walnut for timber production.** In: Janick, J., ed. Plant breeding reviews. Hoboken, NJ: John Wiley & Sons: 236-266.

Beineke, W.F. 1989. **Twenty years of black walnut genetic improvement at Purdue University.** Northern Journal of Applied Forestry. 6: 68-71.

Dangl, G.S.; Mendum, M.L.; Prins, B.H.; Walker, M.A.; Meredith, C.P., et al. 2001. **Simple sequence repeat analysis of a clonally propagated species: a tool for managing a grape germplasm collection.** Genome. 44: 432-438.

Dangl, G.S.; Woeste, K.; Aradhya, M.K.; Koehmstedt, A.; Simon, C. [et al.]. 2005. **Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut.** Journal of the American Society for Horticultural Science. 130: 348-354.

Fjellstrom, R.G.; Parfitt, D.E. 1994. **Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms.** Genome. 37: 690-700.

Hampl, V.; Vaňáčová, Š.; Kulda, J.; Flegr, J. 2001. **Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains.** BMC Evolutionary Biology. 1: 11.

Hartl, D.L.; Clark, A.G. 1997. **A primer of population genetics, 3rd ed.** Sunderland, MA: Sinauer Associates. 221 p.

Jamieson, A.; Taylor, S.C.S. 1997. **Comparisons of three probability formulae for parentage exclusion.** Animal Genetics. 28: 397-400.

Mantel, N. 1967. **The detection of disease clustering and a generalized regression approach.** Cancer Research. 27: 209-220.

Natural Resources Conservation Service. 2016. **Web soil survey.** Washington, DC: U.S. Department of Agriculture, Natural Resources Conservation Service. http://websoilsurvey.sc.egov.usda.gov/

Nicese, F.P.; Hormaza, J.I.; McGranahan, G.H. 1998. **Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers.** Euphytica. 101: 199-206.

Paetkau, D.; Waits, L.P.; Clarkson, P.L.; Craighead, L.; Vyse, E. [et al.]. 1998. **Variation in genetic diversity across the range of North American brown bears.** Conservation Biology. 12(2): 418-429.

Pang, K. 2014. **Crown ideotypes for genetically improved black walnut (*Juglans nigra* L.) clones under an intensive management regime in Indiana, USA.** West Lafayette, IN: Purdue University. Ph.D. dissertation.

Parks, A.; Jenkins, M.; Ostry, M.; Zhao, P.; Woeste, K. 2014. **Biotic and abiotic factors affecting the genetic structure and diversity of butternut in the southern Appalachian Mountains, USA.** Tree Genetics & Genomes. 10: 541-554.

Peakall, R.; Smouse, P.E. 2006. **GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research.** Molecular Ecology Notes. 6: 288-295.

Peakall, R.; Smouse, P.E. 2012. **GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update.** Bioinformatics. 28: 2537-2539.

Rink, G.; Carroll, E.R.; Kung, F.H. 1989. **Estimation of *Juglans nigra* L. mating system parameters.** Forest Science. 35: 623-627.

Rink, G.; Zhang, G.; Jinghua, Z.; Kung, F.H.; Carroll, E.R. 1994. **Mating parameters in *Juglans nigra* L. seed orchard similar to natural population estimates.** Silvae Genetica. 43: 261-262.

Robichaud, R.L.; Glaubitz, J.C.; Rhodes, O.E. Jr.; Woeste, K. 2006. **A robust set of black walnut microsatellites for parentage and clonal identification.** New Forest. 32: 179-196.

Rohlf, F.J. 1997. **NTS RohlfYS-pc version 2.0.** East Setauket, NY: Exeter Software. http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html.

Schuelke, M. 2000. **An economic method for the fluorescent labeling of PCR fragments.** Nature Biotechnology. 18: 233-234.

Smouse, P.E.; Long, J.C. 1992. **Matrix correlation analysis in anthropology and genetics.** American Journal of Physical Anthropology. 35(S15): 187-213.

Smouse, P.E.; Peakall, R. 1999. **Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure.** Heredity. 82: 561-573.

Sokal, R.R.; Michener, C.D. 1958. **A statistical method for evaluating systematic relationships.** University of Kansas Science Bulletin. 38: 1409-1438.

Solar, A.; Smole, J.; Štampar, F.; Viršček-Marn, M. 1994. **Characterization of isozyme variation in walnut (*Juglans regia* L.).** In: Schmidt, H.; Kellerhals, M., eds. Progress in temperate fruit breeding, developments in plant breeding. Wädenswil/Einsiedeln, Switzerland: Springer: 313-320.

Stage, A.R. 1987. **Progress in yield estimation: a history and prescription.** In: Chappell, H.N.; Maguire, D.A., eds. Predicting forest growth and yield: current issues, future prospects. Seattle, WA: University of Washington, College of Forest Resources, Institute of Forest Resources: 61-77.

U.S. Climate Data. 2012. http://www.usclimatedata.com/.

Villani, F.; Pigliucci, M.; Lauteri, M.; Cherubini, M.; Sun, O. 1992. **Congruence between genetic, morphometric, and physiological data on differentiation of Turkish chestnut (*Castanea sativa*).** Genome. 35: 251-256.

Waits, L.P.; Luikart, G.; Taberlet, P. 2001. **Estimating the probability of identity among genotypes in natural populations: cautions and guidelines.** Molecular Ecology. 10(1): 249-256.

Weising, K.; Nybom, H.; Pfenninger, M.; Wolff, K.; Meyer, W. 1994. **DNA Fingerprinting in plants and fungi.** Boca Raton, FL: CRC Press. 336 p.

Woeste, K.; Burns, R.; Rhodes, O.; Michler, C. 2002. **Thirty polymorphic nuclear microsatellite loci from black walnut.** Journal of Heredity. 93: 58-60.

Woeste, K.; McGranahan, G.; Bernatzky, R. 1998. **Low correlation between genomic and morphological introgression estimates in a walnut backcross.** Journal of the American Society for Horticultural Science. 123: 258-263.

Zellers, C.E.; Saunders, M.R.; Morrissey, R.C.; Shields, J.M.; Bailey, B.G. [et al.]. 2012. **Development of allometric leaf area models for intensively managed black walnut (*Juglans nigra* L.).** Annals of Forest Science. 69: 907-913.

Zhao, P.; Woeste, K.E. 2011. **DNA markers identify hybrids between butternut (*Juglans cinerea* L.) and Japanese walnut (*Juglans ailantifolia* Carr.).** Tree Genetics & Genomes. 7: 511-533.

Zhao, P.; Zhang, S.; Woeste, K. 2013. **Genotypic data changes family rank for growth and quality traits in a black walnut (*Juglans nigra* L.) progeny test.** New Forests. 44: 357-368.