Ten microsatellite markers identify cultivars of black walnut (*Juglans nigra* L.) in a nut breeding orchard

Peng Zhao[1,2] Keith Woeste[2] Mark Coggeshall[3]

1 College of Forestry, Northwest Agriculture & Forestry University, Yangling, Shaanxi, 712100, China

2 USDA Forest Service, Hardwood Tree Improvement and Regeneration Center (HTIRC), Department of Forestry and Natural Resources, Purdue University, 715 West State Street, West Lafayette, IN 47907, USA

3 Agroforestry Center, University of Missouri, 203 Anheuser-Busch Natural Resources Bldg. Columbia, MO 65211, USA

Abstract

Black walnut (*Juglans nigra* L.) is a large tree native throughout the eastern United States from New England to Texas. It is a valuable hardwood species that also produces a high quality edible nut. The black walnut nut breeding orchard at the University of Missouri contains about 70 accessions used for breeding. Nearly all of these accessions were obtained as named cultivars (clones) propagated by small scale, private, nut growers. To maintain the integrity of the breeding program, it was important to verify the identity of the accessions. We combined morphological, phenological, and genotype data from ten microsatellites to group individual accessions by cultivar (clone). We have found many clones that shared cultivar names did not have identical phenotypes or genotypes, and conversely, some genotypes had been given multiple cultivar names. For example, some accessions of 'Clermont', were found to have an identical genotype with the cultivars Victoria and Hare, which were found to be identical. On the other hand, other accessions of 'Claremont' had a genotype identical to the cultivars Thomas and Thatcher, which were also identical to each other. Our results indicate that multiple errors were committed during the propagation of these important cultivars, and that it may be difficult or even impossible to properly assign a name to a genotype. This set of microsatellite loci clearly provides a powerful tool for the management of black walnut nut breeding populations.

Introduction

Black walnut (*Juglans nigra* L.), is a large tree native throughout the eastern United States from New England to Texas (Fowells 1965) (Fig. 1). Black walnut is prized as a multipurpose species: it provides valuable timber, produces a high quality edible nut, and is attractive to wildlife. The black walnut nut breeding orchard (Fig. 2) at the University of Missouri contains about 70 accessions used for breeding. Phenological and morphological data indicated that some of the cultivars in the breeding orchard were incorrectly labeled, leading to uncertainty concerning their breeding value. We used 10 microsatellite markers to evaluate the genotypes of the accessions in the orchard and compared them to standards.

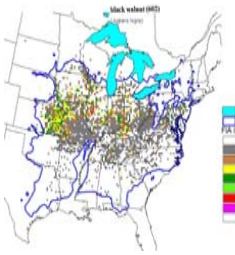

Fig.1 Range of Black walnut (*Juglans nigra* L.) in U.S.

Materials and Methods

Plant material

Genotypes used in this study were obtained from the Black breeding orchard in Missouri University (Table 2)

DNA Extraction

DNA was extracted from leaf samples using a modified version of the methods of Doyle and Doyle (1987), and Robichaud et al. (1997) and stored at – 20°C.

Microsatellite analysis

Primer pairs designed to for this research were derived by further sequencing of a black walnut microsatellite library described by Woeste et al. (2002); Dangel et al. (2005) and Victory et al. (2006) (Table 1, Fig. 4). Polymerase chain reaction (PCR) was conducted in a total volume 10 µL and genotyping was performed as described in Victory et al. (2006) except products were separated using an ABI 373 sequencer. Two positive and one negative control were run with each PCR to ensure accurate scoring. Failed reactions were repeated for accuracy. Used software Genemapper to score genotyping data (Fig. 5) Excel was used to sort all data and to place similar genotypes together (Table 2).

Table1 Primer sequences and label information for microsatellite loci used in this study.

Reverse	Repeat array primer	Length (bp)	Allele size range (bp)	GeneBank Accession Number	Ta	Label	Locus Set	sequence (5'-3')
WGA06	$(AG)_2AA(AG)_{19}AT(AG)_3$	157	134–172	AY333949	53.5	HEX	A	F: CCATGAAACTTCATGCCGTTG R: CATCCCAAGCGAAGGTTG
WGA32	$(TC)_2CG(TC)_{10}$	176	163–217	AY333952	53.5	6-FAM	A	F: CTCGGTAAGCCACACCAATT R: ACGGGCAGTGTATGCATGTA
WGA72	$(AG)_4AA(AG)_4(G)_{12}$	151	135–159	AY333954	53.5	NED	A	F: AAACCACCTAAAACCCTGCA R: ACCCATCCATGATCTTCCAA
WGA76	$(GA)_{12}$	236	224– 254	636615	50	HEX	B	F: AGGGCACTCCTTTATGAGGT R: CAGTCTCATTCCCTTTTTCC
WGA82	$(CT)_{20}$	175	140–234	AY333956	50	6-FAM	B	F: TGCCGACACTCCTCACTTC R: CGTGATGTACGACGGCTG
WGA89	$(TG)_5(GA)_{12}$	215	179–233	AY352440	50	HEX	C	F: ACCCATCTTTCACGTGTGTG R: TGCCTAATTAGCAATTTCCA
WGA90	$(CT)_3T(CT)_{14}$	157	142–178	AY352441	50	6-FAM	C	F: CTTGTAATCGCCCTCTGCTC R: TACCTGCAACCCGTTACACA
WGA24	$(T)_8R_{10}(CT)_{12}N_{10}(CT)_5$	242	222–248	AY333950	50	NED	C	F: TCCCCCTGAAATCTTCTCCT R: TTCTCGTGGTGCTTGTTGAG
WGA27	$(GT)_3TT(GA)_{20}$	242	199–245	AY333951	50	HEX	D	F: AACCCTACAACGCCTTGATG R: TGCTCAGGCTCCACTTCC
WGA69	$(AG)_4N_6(AG)_{17}$	182	164–188	AY333953	50	NED	D	F: TTAGTTAGCAAACCCACCCG R: AGATGCACAGACCAACCCTC

Table 2 Allele sizes at 10 microsatellite loci for cultivars of black walnut (*Juglans nigra*) nut breeding orchard

Cultivar Name; location	WAG06	WAG24	WAG27	WAG32	WAG69	WAG72	WAG76	WAG82	WAG89	WAG90
MO-2190 Victoria	140 158	226 240	225 231	166 188	172 172	145 147	229 243	164 176	208 214	152 152
MO-2195 Hare	140 158	226 240	225 231	166 188	172 172	145 147	229 243	164 176	208 214	152 152
MO-2199 Claremont L	140 158	224 240	225 231	166 188	172 172	145 147	229 243	164 176	208 214	152 152
Clermont_(L),_BC-8	142 160	226 240	226 232	167 167	172 172	146 146	230 244	166 178	209 209	152 152
Hare_AG-1	142 160	226 240	226 232	167 167	172 172	146 146	230 244	166 178	209 209	152 152
Hare_AJ-4	142 160	226 240	226 232	167 167	172 172	146 148	230 244	166 178	209 209	152 152
Hare_BD-10	142 160	226 240	226 232	167 167	172 172	146 148	230 244	166 178	209 209	152 152
Victoria_CE-7	? ?	? ?	226 232	167 167	172 172	146 148	230 244	166 178	209 209	152 152
MO-2183 Thomas	140 142	238 246	211 229	166 180	172 176	147 159	233 237	154 154	188 206	156 172
MO-2191 Thatcher	140 142	238 246	211 229	166 180	172 176	147 159	233 237	154 154	188 206	156 172
MO-2100 Clermont (L)	140 142	238 246	211 229	166 180	172 176	147 159	233 237	154 154	188 206	156 172
Clermont_(L),_BE-5	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Clermont_(W)_BA-9	142 142	238 246	? ?	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Clermont_(W)_BD-3	142 142	238 246	230 234	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thatcher_BC-1	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thatcher_CD-3	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thomas_BD-4	142 142	236 246	? ?	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thomas_AD-2	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thomas_AE-4	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thomas_BB-2	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Thomas (R-8)	142 142	238 246	212 230	167 181	172 176	148 160	234 138	156 156	189 207	? ?
Stambaugh_BB-5	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
Stambaugh_BC-3	142 142	238 246	212 230	167 181	? ?	148 160	234 238	156 156	189 207	156 172
Stambaugh_BF-6	142 144	238 246	212 230	167 181	172 176	148 160	234 238	156 156	? ?	156 172
Rowner_BA-10	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
VV-1_CD-9	142 142	238 246	212 230	167 181	172 176	148 160	234 238	156 156	189 207	156 172
MO-2182 Emma-K	140 154	240 242	217 223	172 186	176 178	145 147	233 235	156 182	184 194	152 158
MO-2185 Emma-K	140 154	240 242	217 223	172 186	176 178	145 147	233 235	156 182	184 194	152 158
Emma_K_AE-2	142 156	? ?	218 224	173 187	176 176	146 146	234 234	158 184	185 195	152 158
Emma_K_AG-5	142 156	240 242	218 224	173 187	? ?	146 146	234 234	158 184	185 195	152 158
Patterson_AH-2	142 156	240 242	218 224	173 187	176 176	146 148	234 234	158 184	185 195	152 158
Patterson_AK-5	142 156	240 242	218 224	173 187	176 178	146 148	234 234	158 184	185 195	152 158
MO-2194 Woody	140 140	230 244	215 225	184 200	168 172	147 147	229 233	170 170	208 208	156 158
davidson	142 142	228 242	214 224	185 201	168 172	148 148	230 234	172 172	207 207	156 158
davidson	? ?	? ?	? ?	185 201	168 172	148 148	230 234	172 172	207 207	156 158
davidson	142 142	228 242	220 232	185 201	168 172	148 148	230 234	170 178	201 209	156 158
Davidson_AA-5	142 142	230 244	216 226	185 185	168 172	148 148	230 234	172 172	209 209	156 156
Davidson_AL-1	142 142	230 244	216 226	185 185	168 172	148 148	230 234	172 172	209 209	156 156
Davidson_BB-7	142 142	230 244	216 226	185 185	168 172	148 148	230 234	172 172	209 209	156 156
Davidson_BD-8	142 142	230 244	216 226	185 185	? ?	148 148	230 234	172 172	209 209	156 158
Woody_BD-12	142 142	230 244	216 226	185 185	? ?	148 148	230 234	172 172	209 209	156 156
MO-2192 sparks 129	140 140	238 244	225 229	180 200	168 176	145 147	233 233	170 170	200 208	156 158
Sparks_129_CC-2	142 142	238 244	? ?	181 181	? ?	146 148	234 234	172 172	201 209	156 156
MO-2198 Christianson(LN)	140 140	x x	229 233	170 180	176 176	145 147	233 235	154 170	200 206	156 158
sparks 177	142 142	236 242	228 232	171 181	176 176	146 148	234 236	156 172	201 205	156 158
sparks 177	? ?	236 242	? ?	171 181	176 176	146 148	234 236	156 172	201 205	156 158
sparks 177	142 156	236 242	228 232	171 181	176 176	146 148	234 236	156 172	201 205	156 158
Hay_BB-9	142 142	? ?	230 234	171 181	? ?	146 146	234 234	156 172	201 207	156 156
Hay_BE-7	142 142	238 244	? ?	171 181	176 176	146 146	234 234	156 172	201 207	156 156
Christianson_(LN)_BC-5	142 142	238 244	230 234	171 181	176 176	146 148	234 234	156 172	201 207	156 156
Christianson_(TT)_A-12	142 142	238 244	230 234	171 181	176 176	146 148	234 234	156 172	201 207	156 156
Thomas_Meyer_AJ-2	142 142	238 244	230 234	171 181	176 176	146 146	234 234	156 172	201 207	156 156
Thomas_Meyer_AE-5	142 142	238 244	230 234	171 181	176 176	146 148	234 234	156 172	201 207	156 156
MO-2196 Surprise	154 156	238 242	215 241	170 216	176 178	145 147	235 235	172 194	200 206	152 160
surprise	154 158	236 240	214 240	171 217	176 178	146 148	236 236	174 196	201 205	152 160
surprise	? ?	236 240	214 214	171 217	176 178	146 148	236 236	174 196	201 205	152 160
surprise	? ?	236 240	? ?	171 217	176 178	148 148	236 236	172 172	207 207	152 160
surprise	? ?	236 240	218 218	171 217	176 178	146 148	234 234	174 196	201 205	152 160
Surprise_AF-1	156 156	238 242	216 240	171 171	? ?	146 146	236 236	174 196	201 207	152 160
Surprise_AH-5	156 156	238 242	216 240	171 171	176 178	146 148	236 236	174 196	201 207	152 160
Surprise_BD-6	156 156	238 242	216 240	171 171	176 176	146 148	236 236	174 196	201 207	152 160
Surprise_BA-5	156 156	238 242	216 242	171 171	? ?	146 146	236 236	174 196	201 207	152 160
Surprise_BE-12	156 156	238 242	? ?	171 171	176 178	146 148	236 236	? ?	201 207	152 160
Surprise (K-03)	156 158	238 242	216 242	179 195	178 180	147 149	232 244	176 196	203 209	x x

Cultivar Name; Marker	WAG06	WAG24	WAG27	WAG32	WAG69	WAG72	WAG76	WAG82	WAG89	WAG90
MO-2180 Brown nugget	140 158	234 240	213 221	182 192	166 168	145 151	227 231	188 204	186 202	158 164
mintle	142 160	232 238	212 220	183 193	166 168	146 152	228 232	? ?	187 203	158 164
mintle	? ?	232 238	? ?	183 193	166 168	146 152	228 232	190 204	187 203	158 164
mintle	? ?	232 238	212 220	183 193	166 168	146 152	228 232	190 204	187 203	158 164
BrNugget_BA-11	142 160	234 240	214 222	183 193	166 166	146 152	228 232	190 204	187 203	158 164
BrNugget_BD-2	142 160	234 240	214 222	183 193	? ?	146 152	228 232	190 204	187 203	158 164
Brown_Nugget_AF-5	142 160	234 240	? ?	183 193	166 166	146 152	228 232	? ?	187 203	158 164
Brown_Nugget_AI-2	142 160	234 240	214 222	183 193	166 166	146 152	228 232	190 204	187 203	158 164
MO-2181 Sauber #1 (W)	140 140	240 248	215 221	162 196	174 174	145 147	231 233	158 176	188 214	150 158
Daniel Niswonger	144 144	238 244	218 222	173 173	174 174	147 149	234 236	186 186	189 215	x x
Daniel_BF-2	142 142	240 248	216 222	163 163	174 174	146 148	232 232	160 178	189 215	150 158
Daniel_BC-9	142 142	240 248	216 222	163 163	? ?	146 146	232 232	160 178	189 215	150 158
Sauber_#1_(W)_AJ-5	142 142	240 248	216 222	163 163	174 174	146 148	232 232	160 178	189 215	150 158
Sauber_#1_(W)_AG-2	142 142	240 248	216 222	163 163	? ?	146 146	232 232	160 178	189 215	150 158
duhois 8415	142 142	238 240	216 222	175 183	172 174	146 148	232 232	176 186	185 201	154 154
duhois 8415	142 142	238 240	216 222	175 183	172 174	146 148	232 232	176 186	185 201	154 154
MO-2101 Eldora	140 148	234 238	227 229	164 178	172 172	147 147	231 241	168 176	200 208	154 162
scrimger	? ?	232 236	? ?	165 179	172 172	148 148	232 242	170 178	201 209	154 162
scrimger	142 150	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 209	154 162
scrimger	? ?	232 236	? ?	165 179	172 176	148 148	232 242	170 178	201 209	154 162
Scrimger_BE-2	142 150	234 238	228 228	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Scrimger_BA-4	142 150	234 238	? ?	165 179	172 172	148 148	232 242	170 178	201 209	154 162
sparrow	? ?	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 207	154 162
sparrow	142 150	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 209	154 162
sparrow	142 150	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 209	154 162
sparrow	142 150	232 236	226 226	165 179	172 172	148 148	232 242	170 178	? ?	154 162
Sparrow_AG-3	142 150	234 238	228 228	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Sparrow_BB-4	142 150	234 238	? ?	165 179	172 172	148 148	232 184	170 178	201 209	154 162
drake	142 150	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 207	154 162
drake	? ?	232 236	226 226	165 179	172 172	148 148	232 242	170 178	201 207	154 162
drake	? ?	232 236	? ?	165 179	172 172	148 148	232 242	170 178	201 207	154 162
Sauber_#2_AK-4	142 150	234 238	228 228	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Sauber_#2_AL-3	142 150	234 238	228 228	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Sauber_#2_BA-8	142 150	? ?	228 228	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Eldora_BF-3	142 150	234 238	230 234	165 179	172 172	148 148	232 242	170 178	201 209	154 162
Eldora_BA-6	? ?	234 238	228 228	165 179	? ?	148 148	232 242	170 178	201 209	154 162
Eldora_(Q-7)	142 150	234 238	228 228	165 179	172 172	148 148	232 242	170 178	201 209	x x
MO-2188 Krause	140 140	240 240	219 221	174 174	172 176	147 155	229 231	182 192	184 184	146 154
Krause_CC-4	142 142	240 240	220 220	175 175	172 176	148 156	230 230	184 194	185 185	146 154
Krause_CE-13	142 142	240 240	220 220	175 175	172 176	148 156	230 230	184 194	185 185	146 154
MO-2187 Cutleaf	140 140	242 242	223 223	186 202	172 172	147 157	231 231	180 184	186 210	152 154
Cutleaf_CC-12	142 142	240 242	224 224	187 187	? ?	148 158	232 232	182 186	187 211	152 154
Krause_CE-13	142 142	240 242	224 224	187 187	? ?	148 158	232 232	182 186	187 211	152 154
MO-2197 Drake_BB-6	140 142	238 244	211 233	180 192	166 176	147 159	231 233	154 168	188 204	152 172
Drake_BB-6	142 142	238 244	212 234	181 181	166 176	148 160	232 232	156 170	189 205	152 172
MO-2193 Elmer Meyer	140 140	238 244	213 233	170 184	176 176	145 149	235 235	170 186	186 200	142 158
Clermont_(Y)_BC-6	142 142	? ?	214 234	171 185	? ?	146 150	236 236	172 188	187 201	142 158
Clermont_(Y)_BF-9	142 142	234 238	216 234	171 185	176 176	146 150	236 236	172 188	187 201	142 158
Clermont-Yates	142 142	238 244	214 234	171 185	176 176	146 150	236 236	172 188	187 201	? ?
Elmer_Myer_BB-13	142 142	238 244	? ?	171 185	176 176	146 150	236 236	172 188	187 201	? ?
Elmer_Myer_BF-12	142 142	234 238	214 234	171 185	176 176	146 150	236 236	172 188	187 201	? ?
MO-2184 Elmer Meyer	142 152	x x	229 237	180 192	172 176	149 159	233 239	204 214	206 206	152 172
Elmer_Meyer_AD-3	144 154	238 246	230 238	181 181	172 176	150 160	234 240	? ?	207 207	152 172
MO-2186 Russell_#3	138 156	238 244	221 227	164 188	172 172	147 147	229 237	170 208	206 208	148 150
Russell_#3_C-13	140 158	238 244	222 228	165 165	168 172	148 148	230 238	172 206	207 207	? ?
Russell_#3_CB-6	142 158	240 244	222 222	183 183	172 176	146 146	230 238	166 178	187 203	156 162
Russell_#3_CA-13	140 140	228 242	222 234	179 179	? ?	148 152	230 230	194 206	191 209	150 158
MO-2189 Harney	132 152	234 248	211 221	182 198	172 174	145 147	229 229	158 174	200 218	150 162
Harney_BF-11	156 156	? ?	? ?	171 171	176 176	146 146	236 236	? ?	201 207	152 160

Results and Discussion

To maintain the integrity of the black walnut nut breeding program, it was important to verify the identity of accessions in the nut breeding orchard. Ten microsatellite markers were used to characterize 22 reference samples provided by Coggeshall. We identified 17 unique genotypes. One-hundred additional samples of cultivars from the University of Missouri nut breeding orchard were also genotyped. All of these accessions were obtained as named cultivars (clones) propagated by small- scale, private, nut growers. The results showed that many clones that shared cultivar names did not share genotypes, and conversely, some genotypes had multiple cultivar names. For example, some accessions of 'Victoria', 'Hare', and 'Clermont', had identical genotypes. Other accessions of 'Clermont' had the same genotype as the cultivars 'Thomas', ' Stambaugh' , ' Rowher ', ' VV ' and 'Thatcher', which were identical to each other (Table 1).

The most likely reasons for these results are mistakes in labeling and mistakes in sample collection. Most black walnut cultivars were first identified as wild trees or chance seedlings by amateur breeders. Over 750 black walnut cultivars have been selected and named since 'Thomas', the first black walnut selected for nut production, was propagated in 1881 (Heiges 1896). Black walnut has great genetic potential for improved nut yield and quality because the extensive native range of the species ensures a large gene pool from which advanced selections can be made. Genetic traits that would lead to advances in a tree improvement program include: lateral bud fruitfulness, late leafing, resistance to anthracnose *Gnomonia leptostyla,* (Fr.) Ces. & Not., precocity and productivity, and improved nut quality. We will combine genotype data with morphological and phenological data to characterize the most important black walnut nut cultivars for breeding .

Fig 2. Breeding Orchard

Fig 3. Black walnut leaves and nuts

Indicates 22 reference samples

Indicates 100 cultivar samples from Missouri

Genotype data did not match reference samples

? ? Indicates no genotype data

x x Indicates possible null allele

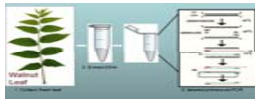

Fig . 4 DNA Extraction and Microsatellite PCR

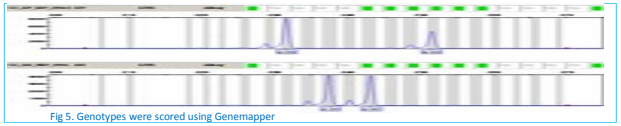

Fig 5. Genotypes were scored using Genemapper

Reference

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