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DNA markers identify hybrids between butternut (*Juglans cinerea* L.) and Japanese walnut (*Juglans ailantifolia* Carr.)

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Abstract Butternut (*Juglans cinerea* L.) is a temperate deciduous hardwood native to the eastern USA and southern Canada valued for its nuts and wood. Butternut's survival is threatened by butternut canker, a disease caused by the exotic fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz. Field observations indicate that trees commonly called buartnut (a hybrid of butternut and its close congener Japanese walnut (*Juglans ailantifolia* × *J. cinerea*)) may be more resistant to butternut canker than is either parental species. Hybrids are difficult to distinguish morphologically from butternuts, and scientists have expressed concern over the possibility of range-wide genetic invasion by Japanese walnut via hybridization with butternut. We used pair-wise combinations of 40 random primers to screen bulked DNA pools of butternut, Japanese walnut, and buartnuts to identify genomic regions unique to Japanese walnut. We ultimately identified one ITS region marker, one chloroplast marker, one mitochondrial marker, and six nuclear markers. The utility of the markers for identifying hybrids was tested and verified using more than 190 genotypes. The markers will be used to identify buartnut hybrids based on the presence of introgressed genomic fragments inherited from Japanese walnut. We confirmed that hybrids have a complex genetic history and present features of the parental species in all possible combinations. These results will assist in the identification and testing of (non-hybrid) butternut for breeding and reintroduction of the species to its former habitats.

Keywords *Juglans* × *bixbyi* · RAPD · SCAR · CAPS · Internal transcribed spacer · *trnT-F* · Hybrid invasion · Butternut canker

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Introduction

Butternut (*Juglans cinerea* L.) also called white walnut, oilnut, or lemonnut is a short-lived, deciduous, cold-tolerant, tree species important for its nuts, wood, and wildlife mast (http://plant-materials.nrcs.usda.gov/). Butternut is native to northeastern and central USA and southern Canada eastward from Ontario (Rink 1990; Farrar 1995), its nuts and wood are of high quality, but its widely scattered distribution, relatively soft wood, medium-sized kernel, and hard shell have prevented it from becoming a commercially important timber or nut species (Williams 1990; Ostry and Pijut 2000). Butternut is threatened by butternut canker, a disease caused by the fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz (Nair et al. 1979). The disease has killed up to 80% of the trees in some states and is threatening its survival (Ostry et al. 1994; Ostry 1998). Japanese walnut (*Juglans ailantifolia* Carr.) is a native to Japan and Sakhalin (Ohwi 1965) and can also serve as a host to *S. clavigignenti-juglandacearum* (Nair et al. 1979; Orchard 1984). It was introduced into North America from Japan about 1870 by a nurseryman in San Jose, CA (Manning 1978; Ostry 1997). A horticultural

selection known as heartnut (*J. ailantifolia* var. *cordiformis*) was widely planted in the eastern USA in the second half of the nineteenth century, and hybrids between butternut and Japanese walnut known as buartnut (technically *Juglans* × *bixbyi*) were described in the early 1900s as amateur horticulturists and nurserymen began to propagate them (Woeste et al. 2009). Japanese walnut and butternut are extremely similar vegetatively, but the nut of most hybrids resembles the (distinctively shaped) butternut, which is why hybrids are commonly confused with butternuts (Woeste et al. 2009; Woeste and Pijut 2009). Biologists have expressed concern over the possibility of genetic invasion of butternut by Japanese walnut because, unlike most *Juglans* hybrids, buartnuts are highly fruitful and vigorous (Ostry and Woeste 2004; Hoban et al. 2009). Because buartnut hybrids apparently are able to cross with other buartnut hybrids, both parental species, and may even self-pollinate, their progeny can present confusing combinations of traits (Ross-Davis and Woeste 2008). Hoban et al. (2009) identified hybrids using a probabilistic approach based on differences in microsatellite allele frequencies in butternut versus Japanese walnut, but additional tools are needed in cases where greater taxonomic certainty is required and for breeding (Michler et al. 2005).

Randomly amplified polymorphic DNA (RAPD) markers are usually dominant. They are simple to use, produce results quickly, and do not require any prior information concerning the DNA template, but they are sensitive to minor changes in reaction conditions during polymerase chain reaction (PCR) amplification, which can result in irreproducible results (Williams et al. 1990). RAPD markers can be made more robust by converting them into sequence-specific, PCR-based markers such as a sequence characterized amplified regions (SCAR; Yang and Korban 1996; Yan et al. 2005; Rahman et al. 2007) or cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel 1993; Farinhó et al. 2007; McCleary et al. 2009).

The internal transcribed spacer (ITS) refers to a region of non-functional RNA situated between multi-copy, structural ribosomal RNAs on a common precursor transcript. The ITS region of the nuclear DNA includes three components: internal transcribed spacer 1, the 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (Baldwin 1992). Sequence diversity among ITS1 alleles has been used to distinguish closely related species and for phylogenetic studies in many angiosperm families and fungi (Baldwin et al. 1995; Potter et al. 2002). Because the ITS region is highly conserved intraspecifically but variable among species, it is often used in taxonomy (Liston et al. 1999).

Chloroplasts are a valuable source of genetic information because their sequence is highly conserved, they undergo little or no recombination, they are uniparentally inherited, primer sites are highly conserved, and the amplification system is robust (Ruf et al. 2007). Chloroplast polymorphisms are powerful genetic tools for identifying matrilineal family groups, studying intraspecific population structure and gene flow from seed versus pollen movement and reconstructing phylogeographic colonization (Marshall et al. 2002). To detect introgression of *J. ailantifolia* into native *J. cinerea* populations, McCleary et al. (2009) developed four chloroplast markers polymorphic between the two species. We focused on the fast-evolving $trnT_{UGU}$-$trnF_{GAA}$ region of the chloroplast, which contains the $trnL_{UAA}$ exon and intron, as well as a pair of spacer regions (Borsch, et al. 2003). The $trnT_{UGU}$-$trnF_{GAA}$ region, like other regions of the chloroplast, has been used extensively in plant systematic to differentiate among taxa (Olmstead and Palmer 1994; Won and Renner 2005). There are only a few species-specific markers for the Juglandaceae (Germain et al. 1993; Potter et al. 2002; Woeste et al. 2009; McCleary et al. 2009). Now that the regeneration of butternut has become a pressing concern, there is a need to develop concomitant molecular methods to identify non-hybrid butternut (Ostry and Woeste 2004). In this work, we identify chloroplast, mitochondrial, and nuclear genomic DNA markers that can be used to distinguish butternut, Japanese walnut, and their hybrids and demonstrate their value in screening germplasm.

Materials and methods

Plant materials

Leaf samples (n=191) were obtained from public and private landowners from across the native range of *J. cinerea* and from the National Clonal Germplasm Repositories in Davis, CA and Corvallis, OR (Table 1). Many of the analyzed genotypes are currently maintained as grafted clones by the USDA Forest Service Hardwood Tree Improvement and Regeneration Center at Purdue University (HTIRC); others were submitted as leaf samples by landowners.

DNA extraction

Leaves of plants maintained by HTIRC were collected, placed in a plastic bag, and stored on ice. Samples contributed by landowners were placed in resealable plastic bags, the air was squeezed out, and they were mailed to HTIRC. DNA was extracted from leaf samples using a modified version of the methods of Doyle and Doyle (1987, 1990) and Robichaud et al. (2006) and stored at −80°C. About 100 mg of fresh leaf tissue and two 1/4 in. cylindrical ceramic beads (Bio 101-Savant, Carlsbad, CA,

Table 1 Genotypes used for identifying and verifying DNA markers and the germplasm source

Sample number	Sample name	Type of accession	Accession number[a]	Species	Sample location[e]	Sample origin, type, 'cultivar'[f]
001	DJUG-0002.01	S	0002.01[b]	*J. ailantifolia*	NCGR, Davis, CA	NCGR
002	DJUG-0003.07	S	0003.07	*J. ailantifolia*	NCGR, Davis, CA	NCGR
003	DJUG-0005.04	S	0005.04	*J. ailantifolia*	NCGR, Davis, CA	NCGR
004	DJUG-0005.12	S	0005.12	*J. ailantifolia*	NCGR, Davis, CA	NCGR
005	DJUG-0006.06	S	0006.06	*J. ailantifolia*	NCGR, Davis, CA	NCGR
006	DJUG-0008.04	S	0008.04	*J. ailantifolia*	NCGR, Davis, CA	NCGR
007	DJUG-0066.02	S	0066.02	*J. ailantifolia*	NCGR, Davis, CA	NCGR
008	DJUG-0067.07	S	0067.07	*J. ailantifolia*	NCGR, Davis, CA	NCGR
009	DJUG-0001.05	S	0001.05	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
010	DJUG-0004.02	S	0004.02	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
011	DJUG-0004.04	S	0004.04	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
012	DJUG-0007.03	S	0007.03	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
013	DJUG-0009.04	S	0009.04	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
014	DJUG-0009.15	S	0009.15	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
015	DJUG-0010.02	S	0010.02	*J. ailantifolia* var. *cordiformis*	NCGR, Davis, CA	Heartnut, NCGR
016	667-1	G	667[c]	*J. ailantifolia* var. *cordiformis*	Marion, IN	Heartnut, 'Pony Boy'
017	1095-1	G	1095	*J. ailantifolia*	Purdue	NCGR-DJUG 0061.01, Japan
018	1096-1	G	1096	*J. ailantifolia*	Purdue	NCGR-DJUG 0061.03, Japan
019	1097-1	G	1097	*J. ailantifolia* var. *cordiformis*	Purdue	DJUG-004.1, Heartnut, UNK
020	1098-1	G	1098	*J .ailantifolia* var. *cordiformis*	Purdue	DJUG-004.2, Heartnut, UNK
021	1099-1	G	1099	*J. ailantifolia* var. *cordiformis*	Purdue	DJUG-007.3, Heartnut, UNK
022	1100-1	G	1100	*J. ailantifolia* var. *cordiformis*	Purdue	DJUG 007.5, Heartnut, UNK
023	1101-1	G	1101	*J. ailantifolia* var. *cordiformis*	Purdue	DJUG 0683, Heartnut, 'Calendar', WA
024	1103-1	G	1103	*J. ailantifolia*	Purdue	UNK
025	'Fodermeir'	G	1010	Buarthian Hybrid	West Point, IN	UNK
026	702 S 32	S	702-17-48[d]	Hybrid	Purdue	New Paris, IN
027	702 S 33	S	702-17-24	Hybrid	Purdue	New Paris, IN
028	704 S 34	S	704-17-41	Hybrid	Purdue	Plymouth, IN
029	704 S 35	S	704-17-15	Hybrid	Purdue	Plymouth, IN
030	704 S 36	S	704-17-16	Hybrid	Purdue	Plymouth, IN
031	705 S 37	G	705-6-64	*J. ailantifolia* × *J. nigra*	Purdue	Plymouth, IN
032	706 S 38	S	706-17-18	Hybrid	Purdue	New Paris, IN
033	706 S 39	S	706-17-38	Hybrid	Purdue	New Paris, IN
034	706 S 40	S	706-17-39	Hybrid	Purdue	New Paris, IN
035	707 S 41	S	707-17-17	Hybrid	Purdue	Brimfield, IN
036	707 S 42	S	707-17-9	Hybrid	Purdue	Brimfield, IN
037	707 S 43	S	707-17-6	Hybrid	Purdue	Brimfield, IN
038	708 S 44	S	708-17-26	Hybrid	Purdue	Steuben Co., IN
039	708 S 45	S	708-17-1	Hybrid	Purdue	Steuben Co., IN
040	708 S 46	S	708-17-29	Hybrid	Purdue	Steuben Co., IN

Table 1 (continued)

Sample number	Sample name	Type of accession	Accession number[a]	Species	Sample location[e]	Sample origin, type, 'cultivar'[f]
041	710 S 47	S	710-13-22	Hybrid	Purdue	Madison, WI
042	710 S 48	S	710-12-6	Hybrid	Purdue	Madison, WI
043	710 S 49	S	710-13-21	Hybrid	Purdue	Madison, WI
044	711 S 50	S	711-13-17	Hybrid	Purdue	Madison, WI
045	711 S 51	S	711-13-16	Hybrid	Purdue	Madison, WI
046	711 S 52	S	711-13-18	Hybrid	Purdue	Madison, WI
047	731 S 53	S	731-13-46	Hybrid	Purdue	Clover Lick, WV
048	731 S 54	S	731-14-44	Hybrid	Purdue	Clover Lick, WV
049	732 S 55	S	732-15-11	Hybrid	Purdue	Loudon, NH
050	732 S 56	S	732-15-13	Hybrid	Purdue	Loudon, NH
051	734 S 57	S	734-13-36	Hybrid	Purdue	Sanford, ME
052	734 S 59	S	734-14-56	Hybrid	Purdue	Sanford, ME
053	735 S 60	S	735-15-18	Hybrid	Purdue	Sanford, ME
054	735 S 61	S	735-16-45	Hybrid	Purdue	Sanford, ME
055	748 S 62	S	748-15-51	Hybrid	Purdue	Chequamegon NF, WI
056	748 S 63	S	748-15-54	Hybrid	Purdue	Chequamegon NF, WI
057	748 S 64	S	748-15-55	Hybrid	Purdue	Chequamegon NF, WI
058	750 S 65	S	750-16-4	Hybrid	Purdue	Ankeny, IA
059	750 S 66	S	750-15-56	Hybrid	Purdue	Ankeny, IA
060	750 S 67	S	750-15-5	Hybrid	Purdue	Ankeny, IA
061	113SLE7-8-12	S	PP 113	Hybrid	Purdue	IA
062	113SLE1-8-13	S	PP 113	Hybrid	Purdue	IA
063	110SB1.51-8-9	C	PP 110	Hybrid	Purdue	IA
064	117SL.5 K71-8-11	C	PP 117	Hybrid	Purdue	IA
065	108sll51-8-14	C	PP 108	Hybrid	Purdue	IA
066	113Su71-8-8	C	PP 113	Hybrid	Purdue	IA
067	Holcomb 36-1	S	1385	Hybrid	East Leroy	MI
068	Manitou 1-1	S	1386	Hybrid	MI	MI
069	Calvert-1	S	1284	Hybrid	Breese	IL
070	7 Mile Road-1	S	1368	Hybrid	Livonia	MI
071	Anderson-1	S	1271	Hybrid	Peoria	IL
072	CCC Camp-1	S	1285	Hybrid	Peoria	IL
073	1075-1	G	1075	Hybrid	Purdue	Athens, OH
074	Detweiller-1	S	LS	Hybrid	Purdue	UNK
075	Detweiller-3	S	LS	Hybrid	Purdue	UNK
076	709 S 93	S	709-12-5	*J. cinerea*	Purdue	Caledonia, MN
077	712 S 94	S	712-13-14	*J. cinerea*	Purdue	WI
078	713 S 95	S	713-13-10	*J. cinerea*	Purdue	Rochester, MN
079	714 S 96	S	714-13-5	*J. cinerea*	Purdue	Rochester, MN
080	715 S 97	S	715-15-5	*J. cinerea*	Purdue	Rochester, MN
081	716 S 98	S	716-16-48	*J. cinerea*	Purdue	Rochester, MN
082	717 S 99	S	717-14-6	*J. cinerea*	Purdue	Whitewater, WI
083	718 S 100	S	718-14-13	*J. cinerea*	Purdue	Whitewater, WI
084	722 S 101	S	722-14-19	*J. cinerea*	Purdue	Nicolet NF, WI
085	723 S 102	S	723-14-12	*J. cinerea*	Purdue	Whitewater, WI
086	724 S 103	S	724-14-27	*J. cinerea*	Purdue	Whitewater, WI
087	725 S 104	S	725-14-29	*J. cinerea*	Purdue	Whitewater, WI
088	726 S 105	S	726-12-60	*J. cinerea*	Purdue	Mazaska Lake, MN

Table 1 (continued)

Sample number	Sample name	Type of accession	Accession number[a]	Species	Sample location[e]	Sample origin, type, 'cultivar'[f]
089	727 S 106	S	727-13-56	*J. cinerea*	Purdue	Rochester, MN
090	728 S 107	S	728-15-6	*J. cinerea*	Purdue	Mark Twain NF, MO
091	730 S 108	S	730-14-38	*J. cinerea*	Purdue	Mark Twain NF, MO
092	733 S 109	S	733-15-33	*J. cinerea*	Purdue	Perch River, NY
093	736 S 110	S	736-14-57	*J. cinerea*	Purdue	Berlin, VT
094	734 S 111	S	736-14-56	*J. cinerea*	Purdue	Sanford, ME
095	738 S 112	S	738-17-52	*J. cinerea*	Purdue	Trade Lake, WI
096	740 S 113	S	740-6-18	*J. cinerea*	Purdue	Whitewater, WI
097	741 S 114	S	741-15-21	*J. cinerea*	Purdue	Whitewater, WI
098	742 S 115	S	742-15-30	*J. cinerea*	Purdue	Stratford, NH
099	743 S 116	S	743-15-34	*J. cinerea*	Purdue	PA
100	744 S 117	S	744-15-37	*J. cinerea*	Purdue	IA
101	746 S 118	S	746-16-20	*J. cinerea*	Purdue	NY
102	747 S 119	S	747-15-48	*J. cinerea*	Purdue	Bark River, MI
103	752-10-5	G	752	*J. cinerea*	Purdue	Whitewater, WI
104	757-4-19	G	757	*J. cinerea*	Purdue	Whitewater, WI
105	766-9-5	G	766	*J. cinerea*	Purdue	Whitewater, WI
106	769-8-27	G	769	*J. cinerea*	Purdue	Whitewater, WI
107	770-8-29	G	770	*J. cinerea*	Purdue	Whitewater, WI
108	773-4-31	G	773	*J. cinerea*	Purdue	Whitewater, WI
109	804-10-25	G	804	*J. cinerea*	Purdue	Freemont, Steuben Co., IN
110	854-11-6	G	854	*J. cinerea*	Purdue	Indiana Dunes, IN
111	859-11-28	G	859	*J. cinerea*	Purdue	Indiana Dunes, IN
112	901-11-8	G	901	*J. cinerea*	Purdue	Chillicothe, OH
113	927-8-17	G	927	*J. cinerea*	Purdue	OS-52, Nicolet NF, WI
114	951-11-23	G	951	*J. cinerea*	Purdue	Heron Rookery, IN Dunes, IN
115	953-9-25	G	953	*J. cinerea*	Purdue	Wayne NF, OH
116	956-9-27	G	956	*J. cinerea*	Purdue	OH
117	964-11-10	G	964	*J. cinerea*	Purdue	IN
118	966-11-11	G	966	*J. cinerea*	Purdue	Rush Co., IN
119	971-8-5	G	971	*J. cinerea*	Purdue	Taylor Co., KY
120	1083-1	G	1083	*J. cinerea*	Purdue	Hoosier NF, IN
121	1073-1	G	1073	*J. cinerea*	Purdue	OH
122	1077-1	G	1077	*J. cinerea*	Purdue	Glouster, OH
123	1090-1	G	1090	*J. cinerea*	Purdue	Hoosier NF, IN
124	1092-1	G	1092	*J. cinerea*	Purdue	Kellogg Creek, Augusta, MI
125	IN-Tnc-1	S	1339	*J. cinerea*	Mill Creek, IN	Mill Creek, IN
126	1089-1	G	1089	*J. cinerea*	Purdue	OH
127	Gmarks4-1	S	1494	*J. cinerea*	Gilmer	WV
128	HNFb22-1	S	HNF B-22	*J. cinerea*	Purdue	Hoosier NF, IN
129	HNFb33-1	S	HNF B-33	*J. cinerea*	Purdue	Hoosier NF, IN
130	Gesek1-1	S	1499	*J. cinerea*	Ritchie	WV
131	Devils Hollow1-1	S	1266	*J. cinerea*	Tuscumbia	AL
132	Cane Creek Island-1	S	1264	*J. cinerea*	Tuscumbia	AL
133	Birchfield-1	S	1448	*J. cinerea*	Christiansburg	VA
134	Steinkraus-1	S	1394	*J. cinerea*	Marcellus	MI
135	Craig-1	S	1372	*J. cinerea*	Livonia	MI

Table 1 (continued)

Sample number	Sample name	Type of accession	Accession number[a]	Species	Sample location[e]	Sample origin, type, 'cultivar'[f]
136	Brake3-1	S	1484	*J. cinerea*	WV	WV
137	Eisman-1	S	1464	*J. cinerea*	Madison	WI
138	Gsmnp-1	S	1442	*J. cinerea*	TN	TN
139	Cane Creek-1	S	1262	*J. cinerea*	Tuscumbia	AL
140	Willis-1	S	1361	*J. cinerea*	Morehead	KY
141	Willis-5	S	LS	*J. cinerea*	Morehead	KY
142	Ernie Taylor	S	1304	*J. cinerea*	Zionsville	IN
143	Ferguson Farms	S	1306	*J. cinerea*	Lawrence	IN
144	Frank Crosby	S	1307	*J. cinerea*	Lawrenceburg	IN
145	Landino-1	S	1364	*J. cinerea*	Turner	ME
148	Farrington	S	1254	*J. cinerea*	West Plains	MO
146	Johnson	S	1418	*J. cinerea*	Waddington	NY
147	'Ayres'	G	B-1	*J. cinerea*	NCGR, Corvallis, OR	MI
149	'Chamberlin'	G	B-3	*J. cinerea*	NCGR, Corvallis, OR	NY
150	'Craxezy'	G	B-19	*J. cinerea*	NCGR, Corvallis, OR	MI
151	'Creighton'	G	B-4	*J. cinerea*	NCGR, Corvallis, OR	IA
152	'Henderson'	G	B-5	*J. cinerea*	NCGR, Corvallis, OR	UNK
153	'Herrick'	G	B-6	*J. cinerea*	NCGR, Corvallis, OR	IA
154	'Johnson'	G	B-7	*J. cinerea*	NCGR, Corvallis, OR	UNK
155	'Kinneyglen'	G	B-8	*J. cinerea*	NCGR, Corvallis, OR	UNK
156	'Van der Poppen'	G	B-16	*J. cinerea*	NCGR, Corvallis, OR	UNK
157	'Weschcke'	G	B-10	*J. cinerea*	NCGR, Corvallis, OR	UNK
158	'Booth'	G	B-2	*J. cinerea*	NCGR, Corvallis, OR	NY
159	'Beckwith'	G	LS	*J. cinerea*	NCGR, Corvallis, OR	OH
160	Cove	S	LS	*J. cinerea*	Tazewell	VA
161	Knapp	S	1432	*J. cinerea*	Titusville	PA
162	Clear Fork	S	LS	*J. cinerea*	Tazewell	VA
163	Littlehouse-2	S	LS	*J. cinerea*	Indiana	PA
164	Rice45-1	S	LS	*J. cinerea*	Rockville	IN
165	SL-5	S	LS	*J. cinerea*	Gatlinburg	TN
166	GB-1	S	LS	*J. cinerea*	Gatlinburg	TN
167	Kumler-1	S	LS	*J. cinerea*	Lafontaine	IN
168	Janus	S	LS	*J. cinerea*	Olyphant	PA
169	Tack-1	S	LS	*J. cinerea*	Warsaw	IN
170	Tack-2	S	LS	*J. cinerea*	Warsaw	IN
171	CCC	S	LS	*J. cinerea*	Petersburg	IL
172	OD-East	S	LS	*J. cinerea*	Petersburg	IL
173	OD-West	S	LS	*J. cinerea*	Petersburg	IL
174	SE tree	S	LS	*J. cinerea*	Springfield	IL
175	Cotter Peach Orchard	S	LS	*J. cinerea*	Springfield	IL
176	Andrew Duncan	S	LS	*J. cinerea*	Hazel Township	PA
177	Lyon-1	S	LS	*J. cinerea*	Brandywine	MD
178	Lyon-2	S	LS	*J. cinerea*	Brandywine	MD
179	Mother Tree	S	LS	*J. cinerea*	Camp Springs	MD
180	Schneider-1	S	LS	*J. cinerea*	Glouster	OH
181	Schneider-2	S	LS	*J. cinerea*	Glouster	OH
182	Stuery	S	LS	*J. cinerea*	McLean	VA
183	Arnold Arboretum	S	LS	*J. cinerea*	Boston	MA

Table 1 (continued)

Sample number	Sample name	Type of accession	Accession number[a]	Species	Sample location[e]	Sample origin, type, 'cultivar'[f]
184	Giam	S	LS	*J. cinerea*	Freehold	NJ
185	Sternberg	S	LS	*J. cinerea*	Purdue	IL
186	Detweiller-4	G	LS	*J. cinerea*	Purdue	IL
187	910-2	G	LS	*J. mandshurica*	Purdue	IN
188	177-20-8	S	LS	*J. nigra*	Purdue	IN
189	177-20-6	S	LS	*J. nigra*	Purdue	IN
190	360-20-23	S	LS	*J. nigra*	Purdue	IN
191	316-17-9	S	LS	*J. nigra*	Purdue	IN

G graft, *C* cutting, *S* seedling, *HITRC* Hardwood Tree Improvement and Regeneration Center, *USDA* United States Department of Agriculture, *NCGR* National Clonal Germplasm Repository, *PP* an accession number from (Ostry and Pijut 2000), *LS* leaf sample submitted by landowner, *HNF* Hoosier National Forest accession number, *UNK* unknown

[a] HITRC accession number

[b] 0000 number indicates USDA-NCGR accession number

[c] Number indicates HITRC accession number of a grafted or rooted clone

[d] Number indicates a seedling accession, each seedling was an individual genotype

[e] Source of the sample

[f] Sample origin

USA) were placed into a 2-mL screw-cap tube, and 1 mL of modified hexadecyltrimethylammonium bromide (CTAB) extraction buffer was added (Lefort and Douglas 1999). The modified buffer contained 2% CTAB, 50 mM Tris–Cl, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 0.4 M LiCl, 2% polyvinylpyrrolidone, 2% SDS. β-Mercaptoethanol (2%) was added immediately before grinding. Samples then were ground in a Fast Prep 120 (Bio 101-Savant, Carlsbad, CA, USA) for three 40-s cycles and cooled on ice between each cycle (Victory et al. 2006; Foroni et al. 2007). After grinding, samples were incubated at 65°C overnight. Chloroform (400 μL) was added to the mixture, and the sample vortexed until a fine emulsion was formed. The phases were separated by centrifugation at 13,000 rpm for 5 min using a tabletop centrifuge. The aqueous phase was retained and subjected to a second chloroform extraction as described. The resulting aqueous supernatant was then extracted with 450 μL phenol/chloroform/isoamyl alcohol (25:24:1), and the phases resolved using centrifugation at 13,000 rpm for 10 min. The aqueous supernatant was then extracted with chloroform two more times. DNA was precipitated by adding 0.9 volumes of ice-cold isopropanol and 0.1 volumes of 3 M sodium acetate (NaAc, pH 6.8). The DNA pellet was washed with 750 μL of 70% ethanol two times, air-dried briefly, and resuspended in 100 μL of TE buffer (10 mM of Tris, 1.0 mM of EDTA, pH 8.0). The concentration of DNA in the samples was quantified by measuring absorbance at 260 nm using a NanoDrop-8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). The quantified DNA was diluted with sterile deionized water to a working stock concentration (10 ng/μL) prior to PCR amplification.

RAPD PCR analysis

A core set of genotypes was evaluated with a species-specific chloroplast CAPS marker (CPS05; McCleary et al. 2009) to ensure the samples' taxonomic assignment based previously on morphology (Ross-Davis et al. 2008b). Trees containing a *J. ailantifolia* chloroplast but producing a butternut-shaped seed were deemed hybrids; sources that originated from forest habitats, contained a *J. cinerea* chloroplast, and showed *J. cinerea* morphology were deemed butternuts. We used RAPD and DNA bulks of three individuals (by species) as an initial primer screen to identify polymorphisms between *J. cinerea* and *J. ailantifolia*. Larger numbers of samples were later examined individually to verify that the markers produced the expected phenotypes in each taxon (Table 1). By using this method, the accidental inclusion of a hybrid in the core *J. cinerea* DNA pool would have inflated type II error (under the conservative H_0 that all non-*J. ailantifolia* samples are actually hybrids) and led to the unnecessary rejection of some markers as not species specific. As long as the markers are interpreted as intended (as evidence of *J. ailantifolia* genomic introgression rather than evidence that a sample "is" or "isn't" a butternut), then the method we used does not require that we have absolute certainty concerning the taxonomic assignment of our butternut samples. Forty RAPD primers (sets A and B, Gene Link,

Hawthorne, NY, USA) were used pair-wise (multiplex RAPD) to increase the number of amplicons per reaction (Elsh and McClelland 1991), and the PCR amplicons from each DNA template pool were compared using agarose gel electrophoresis and documented electronically (Stratagene Eagle Eye, Bio-Rad, Hercules, CA, USA). About 530 primer combinations were screened; each combination produced about ten amplicons. Fifty-nine RAPD amplicons apparently present in *J. ailantifolia* but absent from *J. cinerea* were chosen for further study. PCR was performed using thermal cyclers from MJ Research (Waltham, MA, USA) in a total volume of 20 μL containing 2 μL of 10× Taq DNA polymerase reaction buffer plus $MgCl_2$ (1.5 mM final concentration; Stratagene, La Jolla, CA, USA), 2.5 μL of 200 mM dNTP (0.25 mM), 2 μL of 0.1 mg/mL acetylated bovine serum albumin (BSA; Promega, Madison, WI, USA), 2 μL of 25 mM $MgCl_2$ (2.5 mM; Applied Biosystems, Foster City, CA, USA), 5.0 μL sterilized distilled water, and 4 μL of 50 mM RAPD decamer primer pair. The final reaction included 2 μL sample DNA and 0.5 U Taq polymerase (Promega, Madison, WI, USA or New England Biolabs, Beverly, MA, USA). Thermal cycling conditions were as follows: denaturation 3 min at 92°C; 35 cycles of 1 min at 92°C, 1 min at 35°C, and 2 min at 72°C; and a final extension of 10 min at 72°C at the end of the amplification (Woeste et al. 1996). Each PCR product (20 μL) was mixed with 2 μL of 6× loading dye (40%, *w*/*v* sucrose, 15% ddH_2O, 30% glycerol) and electrophoresed in 2.5% agarose gel made with 1× TAE buffer. Gels were stained with ethidium bromide, and the RAPD fragments were scored visually as the presence or absence of a band. Amplicon size was estimated by comparison with an in-house DNA size standard with prominent bands of 738, 597, 508, 401, 305, 200, and 104 bp or a commercial 100-bp DNA ladder (Promega, Madison, WI, USA). Precise DNA product sizes were determined by sequencing.

ITS and *trnT-F* markers

Samples (n=191) were analyzed using primers that amplified the ITS region of ribosomal nuclear DNA and a chloroplast marker *trnT-F* (Table 2). Amplification conditions for both markers included 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM primers, 1× Taq DNA polymerase reaction buffer (Stratagene, La Jolla, CA, USA), 0.1 mg/mL BSA (Promega, Madison, WI, USA), 0.5 U Taq polymerase (Promega, Madison, WI, USA or New England Biolabs, Beverly, MA, USA), and 10 ng template DNA in a 40-μL total volume. PCR was completed using the following protocol: 3 min at 95°C followed by 35 cycles of 15 s at 93°C, 60 s at 50°C, and 90 s at 72°C and a final extension of 10 min at 72°C. The restriction site maps of the ITS sequences (Stanford et al. 2000) and *trnT-F* sequences (Aradhya et al. 2007) of *J. ailantifolia* and *J. cinerea* were compared using the online tool NEB-cutter V. 2.0 (http://tools.neb.com/NEBcutter2/). ITS amplification products (10 μL) were digested with 1× NEB4 buffer and 1.5 U *Bsi*EI restriction enzyme in 40 μL total volume at the recommended temperature. Digestion conditions for *trnT-F* amplification product (10 μL) included 1× NEB4 buffer and 1.5 U *Mbo*II restriction enzyme in 40 μL total volume at the recommended temperature. Digestion products were separated in 2.0% agarose gels using 1× TAE buffer and visualized with ethidium bromide.

Cloning and sequencing RAPD DNA amplicons

DNA bands polymorphic between *J. ailantifolia* and *J. cinerea* were extracted from agarose gel and the DNA isolated using QlAquick Gel Extraction Kit and QIAEX II Gel Extraction Kit (Qiagen, Germantown, MD, USA). The purified DNA (2 μL, 200 ng/μL) was ligated into a pGEM-T Easy Vector (1 μL, 50 ng/μL; Promega, Madison, WI, USA). The ligation products were used to transform *Escherichia coli* JM 109 cells (Promega Madison, WI, USA). Transformed cells were streaked onto Luria–Bertani agar containing 50 mg/mL ampicillin, bromo-chloro-indolyl-galactopyranoside, and isopropyl β-D-1-thiogalactopyranoside. Plates were incubated overnight at 37°C, and transformed colonies were identified using blue/white selection. Plasmid DNA was extracted with Zyppy™ Plasmid Miniprep Kit (Zymo Research, Orange, CA, USA) and QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA). For sequencing, plasmid DNA templates were diluted to a concentration 300–400 ng/μL; when sequencing directly from genomic DNA templates, we used a DNA concentration of 5–25 ng/μL. Sequencing reactions were performed using a Thermo Sequenase fluorescent-labeled primer cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the BIGDYE terminator method (ABI, Foster City, CA, USA). Sequences were obtained on an ABIprism 377 (Applied Biosystems, Foster City, CA, USA).

Primer design and PCR amplification for SCAR and CAPS markers

Multiple sequences were obtained for each amplicon, and a consensus sequence was derived using Sequencher (Dome, Ann Arbor, MI, USA). Sequence-specific primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) and obtained from IDT (San Diego, CA, USA). The sequence-specific primers (Table 2) were used to amplify genomic templates of *J. ailantifolia*, *J. cinerea*, and *J. ailantifolia* × *J. cinerea* in PCR reactions containing

Table 2 Primers used for PCR amplification of markers for identifying hybrids between butternut and Japanese walnut

Primer name	Primer sequence 5′–3′	T_a (°C)	Type	Enzyme	Digestion reaction			Size (bp)	Result sizes (bp)			NCBI number[d]
					Buffer	BSA	T_m (°C)		*J. ailantifolia*	*J. cinerea*	Hybrid	
22-5F	CTGGAGTTGGAGCATCATCA	55.0	SCAR	NONE[a]	NONE	−	NONE	705	705	~660	*705*, *~660*	GU552448
22-5R	GCCCTTCTTACCATAGTCTGGA											
16R-2F	AAAGCAGCTTCATCGAACAC	58.0	SCAR	NONE	NONE	−	NONE	300	300	NONE	300	GU552447
16R-2R	TTGACTACCCAATGCTCGAGTT											
*trnT-F*F	AGCGGGCTCACATAACAAAA	50.0	CAPS	*Mbo*II	NEB4	+	37	JA 687	471, 380[b]		471, 380[b]	JA:AY293398
trnT-F R	CAACGTTGGTCCAATCAGAA							JC 669		536, 453[b]	or 536, 453	JC:AY293392
3-9F	CCGGCACACAAACTTAATGA	50.0	CAPS	*Ags*I	SEBY	+	60	213	92, 86, 35	86, 84, 35, 8	92, 86, 35	JA:GU552437
3-9R	GGAGAATCAGAGCTGCTTGG										or 86, 84, 35, 8	JC:GU552438
ITS1	TCCGTAGGTGAACCTGCGG	50.0	CAPS	*Bsi*EI	NEB4	−	60	JA 740	305, 240, 195		*430*, *305*, *240*, *195*	JA:FJ043008
ITS4	TCCTCCGCTTATTGATATGC							JC 735		430, 305		JC:AF179572
14R-1F	AGATGTCTCGCTGAGGCTTT	55.0	CAPS	*Bst*UI	NEB4	−	37	JA 395	228, 167[c], 127, 40		*229*, *228*, *167*, *127*, *40*	JA:GU552439
14R-1R	ACCTTGGCGGTTACAAATGA							JC 396		229, 167		JC:GU552440
15R-8F	CCCACCAATAAGCTCCACTG	63.0	CAPS	*Acl*I	NEB4	+	37	429	259, 170	429	*429*, *259*, *170*	JA:GU552441
15R-8R	GGTGTTCGATGCAAGTGAAA											JC:GU552442
39-6F	TCCTTCCAAGGAAAGTCATTT	50.0	CAPS	*Tsp*509I	NEB4	−	37	JA 201	113, 88		*113*, *88*, *57*, *52*	JA:GU552443
39-6R	GTGACGTAGGAGGACCTAGATAAT							JC 197		88, 57, 52		JC:GU552444
40-1F	GGACCCTTACAACGAACTCTTC	55.0	CAPS	*Mfe*I	NEB4	−	60	559, 346	346, 213, 133	559, 346	*559*, *346*, *213*, *133*	JA:GU552445
40-1R	GGAGGAGGTTTGCAGCAGTA											JC:GU552446

Italicized data indicate co-dominant marker phenotype

T_a PCR reaction annealing temperature for tabulated primers (°C), T_m incubation temperature for enzyme digestion (°C), − enzyme digestion cocktail contained no BSA, + enzyme digestion cocktail contained BSA (1 mg/mL)

[a] "NONE" SCAR markers do not require restriction enzyme digestion

[b] In *J. ailantifolia*, fragments of 91, 83, 70, and 63 bp are produced but not listed because they are not routinely visible in agarose gels. The 471-bp product was probably the result of incomplete digestion or mutation in the restriction site between the 380-bp product and the 91-bp product. In *J. cinerea*, fragments of 83, 70, and 63 bp are produced but not listed. The 536-bp product was probably the result of incomplete digestion or mutation in the restriction site between the 453-bp product and the 83-bp product. Hybrids may contain either a *J. ailantifolia* or a *J. cinerea* haplotype chloroplast, although *J. ailantifolia* is much more common (Hoban et al 2009)

[c] The 167-bp product often did not digest completely to yield 127- and 40-bp products, see Fig. 4a

[d] National Center for Biotechnology Information sequence accession number

2 μL of 1× Taq DNA polymerase reaction buffer (Stratagene), 2.0 μL of 200 mM dNTP (0.25 mM), 2 μL of 0.1 mg/mL acetylated BSA, 1 μL each of 10 mM designed primer, and 9.5 μL sterilized distilled water. Final reaction volume was 20 μL, including 2 μL sample DNA (10 ng/μL) and 0.5 U Taq polymerase. SCAR and CAPS markers were optimally amplified using different annealing temperatures (Table 2).

CAPS marker analysis

When newly designed sequence-specific primers amplified fragments from *J. ailantifolia* and *J. cinerea* that were not polymorphic in size, we excised the *J. cinerea* amplicon and sequenced it as described above for *J. ailantifolia*. We identified restriction enzymes targeting sequence polymorphisms between the *J. ailantifolia* and *J. cinerea* PCR products using NEB-cutter. PCR products were diluted and digested at the recommended temperature using the following cocktail (Table 2): 1× reaction buffer, 2.2 U NEB restriction enzyme (New England Biolabs, Ipswich, MA, USA) or SEB restriction enzyme (SibEnzyme US LLC, West Roxbury, MA, USA) and 10 μL PCR product in a total reaction volume of 40 μL. The enzyme-digested PCR products were resolved in 2% to 3% agarose and visualized with ethidium bromide. To evaluate the phenotypes of *J. ailantifolia*, *J. cinerea*, and *J. ailantifolia* × *J. cinerea* for CAPS marker 3–9, enzyme-digested PCR products were resolved in 4% metaphor high-resolution agarose (Cambrex, Rockland, ME, USA) using 1× TAE buffer and visualized with ethidium bromide (Fig. 4).

Results

Randomly amplified polymorphic DNA bulked segregant analysis

We screened about 530 random decamer primer pairs against DNA bulks of *J. cinerea*, *J. ailantifolia*, and *J. ailantifolia* × *J. cinerea*; each combination produced about ten amplicons. About 90% of the primer pairs produced identical products when they amplified DNA bulks of *J. cinerea*, *J. ailantifolia*, and *J. ailantifolia* × *J. cinerea*. RAPD reactions were repeated at least three times to test reproducibility. Candidate amplicons (those apparently present in *J. ailantifolia* bulks but absent from *J. cinerea* bulks) were tested further to validate their species specificity. For example, random primers combination A1 + B4 produced amplicons of about 1,500 and 750 bp that were found in *J. ailantifolia* bulks and absent from *J. cinerea* bulks (Fig. 1). As expected, RAPD amplicons were expressed as dominant markers that also amplified from hybrid bulks.

Use of ITS and *trnT-F* markers to identify hybrids

By using samples from trees that were clearly hybrids based on morphology and chloroplast haplotype (McCleary et al. 2009), we validated that polymorphism in the ITS and *trnT-F* genomic regions could also be used to identify hybrids (Fig. 2; Table 3). Amplification of the ITS region with primers ITS1 and ITS4 (Table 2) produced an amplicon of 740 bp (FJ043008) in *J. ailantifolia* and 735 bp (AF179572) in *J. cinerea*. Subsequent digestion with *Bsi*EI produced distinct patterns corresponding to *J. ailantifolia* and *J. cinerea* genotypes. Pattern 1, associated with all *J. ailantifolia*, was distinguished by amplicons of 305, 240, and 195 bp. Pattern 2, associated with all *J. cinerea* and some *J. ailantifolia* × *J. cinerea*, contained amplicons of 430 and 305 bp, and pattern 3, associated with 20 of 49 hybrids, contained amplicons of 430, 305, 240, and 195 bp (Fig. 2a). Thus, samples producing ITS pattern 1 or 3 revealed evidence of *J. ailantifolia* genome. Amplification of the *trnT-F* regions with primers *trnT-F* F and *trnT-F* R (Table 2; Fig 2b) produced an amplicon of 687 bp in *J. ailantifolia* (AY293398) and 669 bp in *J. cinerea* (AY293392). Amplification of the *trnT-F* region followed by digestion with *Mbo*II produced two haplotypes: Haplotype A showed digestion products of 471, 380, 91, 83, 70, and 63 bp, and haplotype C showed digestion products of 536, 453, 83, 70, and 63 bp (Fig. 2b). Analysis of *trneT-F* sequence data for *J. ailantifolia* and *J. cinerea* using NEB-cutter showed that the largest expected products following *Mbo*II digestion of *trnT-F* were 380 bp for *J. ailantifolia* and 453 bp for *J. cinerea*. The presence of the 471-bp product in *J. ailantifolia* and the 536-bp product in *J. cinerea* samples was superfluous and possibly derived from the unintended amplification of other intergenic spacers that are highly similar to *trnT-F* in *Juglans*. Both the *trnT-F* and ITS molecular markers revealed fixed differences between *J. cinerea* and *J. ailantifolia* based on over 150 samples (Table 3; Fig. 2). All *J. ailantifolia* and most hybrid individuals showed *trnT-F* haplotype A. All *J. cinerea* showed *trnT-F* haplotype C (Table 3).

Cloning and sequencing RAPD DNA amplicons

We cloned and sequenced 32 RAPD amplicons apparently present in *J. ailantifolia* but absent from *J. cinerea* samples. Of these, primers were designed to amplify 24 loci for re-sequencing. The resulting sequence data allowed us to identify two SCAR markers, one mitochondrial marker, and four CAPS markers that were useful for identifying hybrids (Table 2).

Marker 22-5 was identified by sequencing a 711-bp amplicon produced by random primers A1 + B4. Marker 22-5 (GU552448) was a 705-bp PCR product in all *J.*

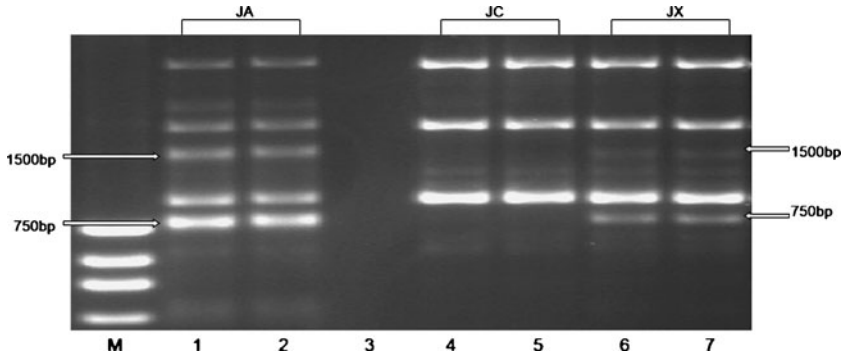


Fig. 1 Agarose gel showing the mobility of amplicons produced from randomly amplified polymorphic DNA primers A1 + B4. *M*=~100 bp DNA size standard. Amplicons specific to *J. ailantifolia* (*JA*; 750 and 1,500 bp) are indicated in *lanes 1* and *2*; *lane 3* was intentionally left blank. *Lanes 4* and *5* amplicons produced by *J. cinerea* (*JC*) samples; *lanes 6* and *7* amplicons produced by hybrid samples, note the presence of the 750- and 1,500-bp products

ailantifolia, 705- and 660-bp products in 29 of 50 *J. ailantifolia* × *J. cinerea*, and a 660-bp product in *J. cinerea* (Table 2; Fig. 3b). Marker 22-5 was co-dominant (Fig. 3b) based on the phenotypes of 24 *J. ailantifolia*, 111 *J. cinerea* individuals, and 50 *J. ailantifolia* × *J. cinerea* (Table 3).

Marker 16R-2 was identified by sequencing a 474-bp amplicon produced by random primers B15 + B10. Marker 16R-2 (GU552447) was a 300-bp PCR product found in all 24 *J. ailantifolia* tested but absent in all *J. cinerea* (Table 2; Fig. 3a). The 300-bp product was found in 21 of 51 *J. ailantifolia* × *J. cinerea* samples tested (Table 3).

Development of the CAPS markers

Marker 14R-1 was identified by sequencing a 471-bp amplicon produced by random primers B15 + B8. Marker

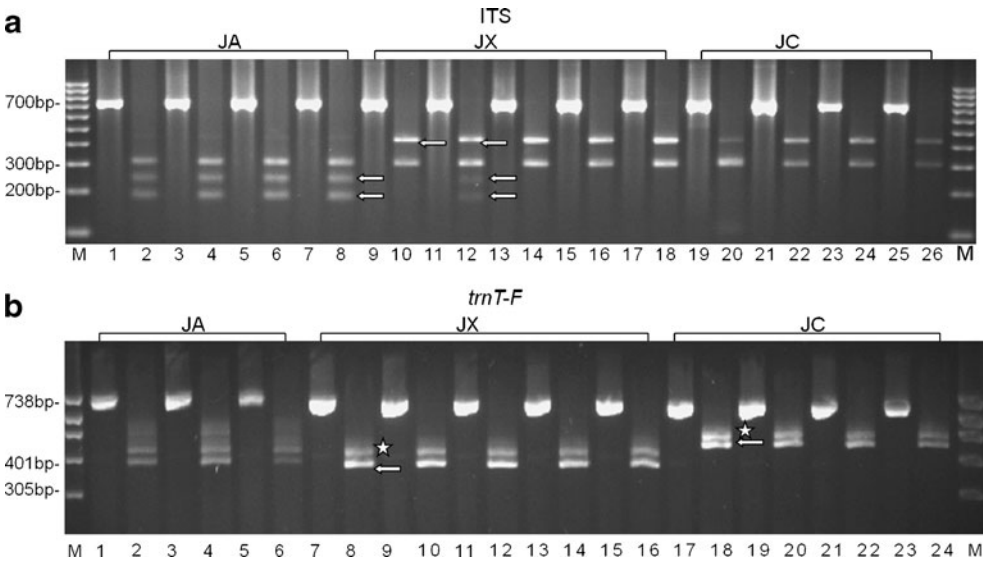


Fig. 2 Phenotypes of ITS and *trnT-F* chloroplast markers visualized using 2% agarose stained with ethidium bromide. For details concerning the primers and digestion conditions, see Table 2. **a** *M*= commercial 100-bp DNA size standard. ITS marker PCR product of *J. ailantifolia* (*JA*) DJUG 0067.07, DJUG 0001.05, DJUG 0004.02, and DJUG 0007.03 uncut (740-bp product in *lanes 1*, *3*, *5*, and *7*) and digested with *Bsi*EI (305-, 240-, and 195-bp products in *lanes 2*, *4*, *6*, and *8*), *arrows* indicate diagnostic product sizes; PCR products of hybrids (*JX*) 702 S 32, 704 S 34, 706 S 38, 707 S 42, and 708 S 45 uncut (740-bp product in *lanes 9*, *11*, *13*, *15*, and *17*) and digested with *Bsi*EI (products in *lanes 10*, *12*, *14*, *16*, and *18*); PCR product of *J. cinerea* (*JC*) 1083-1, 1073-1, 1077-1, and 1089-1 uncut (735-bp product in *lanes 19*, *21*, *23*, and *25*) and digested with enzyme *Bsi*EI (430- and 305-bp products in *lanes 20*, *22*, *24*, and *26*). **b** *M* = in-house DNA size standard. *trnT-F* PCR product of *J. ailantifolia* DJUG 0067.07, DJUG 0001.05, and DJUG 0004.02 uncut (687-bp product in *lanes 1*, *3*, and *5*) and digested with *Mbo*II (471-, 380-, 91-, 83-, 70-, and 63-bp products in *lanes 2*, *4*, and *6*); PCR product of hybrids (*JX*) Holcomb36-1, Manitou1-1, Calvert-1, 7 Mile Road-1, and Anderson-1 uncut (687- or 669-bp product in *lanes 7*, *9*, *11*, *13*, and *15*) and digested with *Mbo*II (products in *lanes 8*, *10*, *12*, *14*, *16*, and *18*); PCR products of JC Devils Hollow-1, Hnf33-1, Cane Creek Island-1, and Brick Field-1 uncut (669-bp product in *lanes 17*, *19*, *21*, and *23*) and digested with *Mbo*II (536-, 453-, 83-, 70-, and 63-bp products in *lanes 18*, *20*, *22*, and *24*). *Arrow* indicates diagnostic product sizes, *asterisk* indicates superfluous digestion products (see footnote, Table 2)

Table 3 Marker phenotypes for germplasm of *J. ailantifolia*, *J. cinerea*, and their hybrids

Sample number[a]	Species[b]	Cytoplasmic markers			Nuclear markers						
		CPS05[e]	*trnT-F*[f]	3-9	ITS	16R-2	22-5	14R-1	15R-8	39-6	40-1
001	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
002	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
003	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
004	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
005	*J. ailantifolia*	A	A	A	A	A	A	ND	A	A	A
006	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
007	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
008	*J. ailantifolia*	A	A	A	A	A	A	A	C	A	A
009	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
010	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
011	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
012	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
013	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
014	*J. ailantifolia*	A	A	A	A	A	A	A	C	A	A
015	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
016	*J. ailantifolia*	A	A	A	A	ND	A	A	A	A	A
017	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
018	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
019	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
020	*J. ailantifolia*	A	ND	A	A	A	A	A	A	A	A
021	*J. ailantifolia*	A	A	A	A	A	A	ND	A	A	A
022	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
023	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
024	*J. ailantifolia*	A	A	A	A	A	A	A	A	A	A
025	Buarthian hybrid[c]	A	A	A	A/C	A	A/C	A/C	A/C	A/C	A/C
026	Hybrid	A	A	A	C	C	C	C	C	A/C	A/C
027	Hybrid	A	A	A	C	A	C	C	C	C	A/C
028	Hybrid	A	A	A	C	A	C	A/C	C	A/C	C
029	Hybrid	A	A	A	C	A	A/C	A/C	C	A/C	ND
030	Hybrid	A	A	A	A/C	A	A/C	A/C	C	C	A/C
031	*J. ailantifolia*× *J. nigra*	A	A	A	A/C	A	A/C	A/C	C	A/C	A/C
032	Hybrid	A	A	A	A/C	A	A/C	C	C	C	A/C
033	Hybrid	A	A	A	A/C	A	A/C	A/C	C	A/C	A/C
034	Hybrid	A	A	A	A/C	A	A/C	A/C	C	A/C	A/C

035	Hybrid	A	A	A	A/C	A	C	A/C	C	C	A/C
036	Hybrid	A	A	A	A/C	C	A/C	A/C	A/C	A/C	A/C
037	Hybrid	A	A	A	A/C	C	A/C	C	C	C	C
038	Hybrid	A	A	A	A/C	C	A/C	ND	C	A/C	A/C
039	Hybrid	A	A	A	A/C	A	C	C	C	C	A/C
040	Hybrid	A	A	A	A/C	C	C	A/C	C	A/C	C
041	Hybrid	ND	A	A	C	C	A/C	A/C	C	C	C
042	Hybrid	A	A	A	C	C	A/C	A/C	C	C	C
043	Hybrid	A	A	A	C	C	C	A/C	C	C	C
044	Hybrid	A	A	A	A/C	C	A/C	A/C	C	A/C	C
045	Hybrid	A	A	A	A/C	C	C	A/C	C	A/C	A/C
046	Hybrid	A	A	A	A/C	C	A/C	C	C	C	A/C
047	Hybrid	A	A	A	C	C	A/C	C	C	A/C	A/C
048	Hybrid	A	A	A	C	A	C	A/C	C	A/C	C
049	Hybrid	C	C	C	A/C	A	A/C	A/C	A/C	C	C
050	Hybrid	A	A	A	A/C	A	C	A/C	C	A/C	A/C
051	Hybrid	A	A	A	C	C	C	A/C	C	C	C
052	Hybrid	A	A	A	C	A	A/C	C	C	C	A/C
053	Hybrid	A	A	A	C	C	C	C	A/C	C	C
054	Hybrid	A	A	A	C	A	A/C	A/C	C	C	C
055	Hybrid	A	A	A	A/C	C	A/C	C	C	A/C	A/C
056	Hybrid	C	C	C	A/C	C	A/C	A/C	C	A/C	A/C
057	Hybrid	A	A	A	A/C	A	A/C	A/C	C	C	C
058	Hybrid	A	A	A	C	C	A/C	A/C	C	A/C	C
059	Hybrid	A	A	A	C	C	ND	A/C	C	ND	A/C
060	Hybrid	A	A	A	C	C	A/C	A/C	C	A/C	A/C
061	Hybrid	A	A	A	C	A	A/C	A/C	A/C	A/C	A/C
062	Hybrid	A	A	A	C	C	A/C	A/C	A/C	A/C	A/C
063	Hybrid	A	A	A	C	C	A/C	A/C	A/C	A/C	A/C
064	Hybrid	A	A	A	C	C	C	A/C	C	A/C	A/C
065	Hybrid	A	A	A	ND	A	C	A/C	A/C	A/C	A/C
066	Hybrid	A	A	A	C	C	A/C	A/C	A/C	A/C	A/C
067	Hybrid	A	A	A	C	C	C	C	A/C	C	A/C
068	Hybrid	A	A	A	C	C	A/C	A/C	C	A/C	A/C
069	Hybrid	A	A	A	C	A	C	C	C	C	C
070	Hybrid	A	A	A	C	C	C	C	C	A/C	C
071	Hybrid	A	A	A	C	C	C	C	C	A/C	A/C
072	Hybrid	A	A	A	C	C	A/C	A/C	A/C	A/C	A/C
073	Hybrid	C	C	C	C	A	C	C	A/C	C	C

Table 3 (continued)

Sample number[a]	Species[b]	Cytoplasmic markers			Nuclear markers						
		CPS05[e]	*trnT-F*[f]	3-9	ITS	16R-2	22-5	14R-1	15R-8	39-6	40-1
074	Hybrid	C	C	C	C	C	C	C	A/C	C	C
075	Hybrid	C	C	C	ND	C	C	C	A/C	A/C	A
076	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
077	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
078	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
079	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
080	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
081	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
082	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
083	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
084	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
085	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
086	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
087	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
088	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
089	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
090	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
091	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
092	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
093	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
094	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
095	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
096	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
097	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
098	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
099	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
100	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
101	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
102	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
103	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
104	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
105	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
106	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
107	*J. cinerea*	C	C	C	C	C	C	C	C	C	C

| | | |

Table 3 (continued)

Sample number[a]	Species[b]	Cytoplasmic markers			Nuclear markers						
		CPS05[e]	*trnT-F*[f]	3-9	ITS	16R-2	22-5	14R-1	15R-8	39-6	40-1
146	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
147	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
149	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
150	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
151	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
152	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
153	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
154	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
155	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
156	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
157	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
158	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
159	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
160	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
161	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
162	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
163	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
164	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
165	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
166	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
167	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
168	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
169	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
170	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
171	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
172	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
173	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
174	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
175	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
176	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
177	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
178	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
179	*J. cinerea*	C	C	C	C	C	C	C	C	C	C
180	*J. cinerea*	C	C	C	C	C	C	C	C	C	C

181	*J. cinerea*	C	C	C	C	C	C	C	C	C	C	C	C	C
182	*J. cinerea*	C	C	C	C	C	C	C	C	C	C	C	C	C
183	*J. cinerea*	C	C	C	C	C	C	C	C	C	C	C	C	C
184	*J. cinerea*	C	C	C	C	C	C	C	C	C	C	C	C	C
185	*J. cinerea*[d]	C	C	C	C	ND	C	C	ND	ND	C	ND	C	C
186	*J. cinerea*[d]	C	C	C	C	C	C	C	C	C	C	C	C	C
187	*J. mandshurica*	A	A	A	A	A	A	A	A	A	A	A	A	A
188	*J. nigra*	C	C	C	A	C	C	C	C	A	A	A	A	A
189	*J. nigra*	C	C	C	A	C	C	C	C	A	A	A	A	A
190	*J. nigra*	C	C	C	A	C	C	C	C	A	A	A	A	A
191	*J. nigra*	C	C	C	A	C	C	C	C	A	A	A	A	A

ND no data, *A/C* co-dominant phenotype, *A J. ailantifolia* phenotype, *C J. cinerea* phenotype

[a] Sample number, see Table 1

[b] Unless otherwise noted, hybrids are assumed to be between *J. ailantifolia* and *J. cinerea* based on morphology

[c] Hybrid believed to be (*J. ailantifolia* × *J. cinerea*) × *J. regia*

[d] DNA extracted from dried leaves

[e] CAPS chloroplast marker (McCleary et al. 2009)

[f] For details concerning this marker and all others, see Table 2

14R-1 (GU552439, GU552440), amplified by primer 14R-1F and 14R-1R, was a 395-bp PCR product in *J. ailantifolia* and 396-bp PCR product in *J. cinerea* (Table 2; Fig. 4a). By analyzing sequence data, we identified a polymorphism in the marker at 268 bp (ctcgAgagc in *J. cinerea* and ctcgCgagc in *J. ailantifolia*; Table 2). Digestion of the 395-bp PCR product from *J. ailantifolia* with *Bstu*UI produced 228-, 167-, 128-, and 40-bp products in *J. ailantifolia*. Digestion of the 396-bp product from 111 *J. cinerea* samples resulted in 229- and 167-bp products, and 32 of 50 *J. ailantifolia* × *J. cinerea* samples produced 229-, 228-, 167-, 127-, and 40-bp products (Table 3; Fig. 4a). Based on these results, we concluded that marker 14R-1 was co-dominant.

Marker 15R-8 was identified by sequencing a 441-bp amplicon produced by random primers B15 + B6. Marker 15R-8 (GU552441 and GU552442), amplified by primer 15R-8F and 15R-8R, was a 429-bp PCR product found in all samples (Table 2; Fig. 4b). Using sequence data, we identified a polymorphism in the marker at 259 bp (aacgGC in *J. cinerea* and aacgTT in *J. ailantifolia*; Table 2). When the 429-bp PCR product was digested using *Acl*I, 170- and 259-bp products were produced by 22 of 24 *J. ailantifolia* samples; all 111 *J. cinerea* samples remained uncut, and 14 of 51 *J. ailantifolia* × *J. cinerea* samples produced bands of 429, 259, and 170 bp (Table 3; Fig. 4b). Based on these results, we concluded that marker 15R-8 was co-dominant. DJUG-0067.07 and DJUG-0009.15 showed a *J. cinerea* phenotype at 15R-8 (Table 3).

Marker 39-6 was identified by sequencing a 426-bp amplicon produced by random primers B20 + A8 (GU552443 and GU552444). Primers 39-6F and 39-6R produced a 201-bp product in *J. ailantifolia* and 197-bp product in *J. cinerea* (Table 3; Fig. 4a). Sequence data revealed a polymorphism at 58 bp (aactAatt in *J. cinerea* and aactGatt in *J. ailantifolia*; Table 2). After digestion of the 201- and 197-bp product with *Tsp*509I, all *J. ailantifolia* samples produced 113- and 88-bp products; all *J. cinerea* samples showed 88-, 57-, and 52-bp products, and 30 of 50 *J. ailantifolia* × *J. cinerea* samples produced 113-, 88-, 57-, and 52-bp products (Table 3; Fig. 4c). We concluded that marker 39-6 was co-dominant based on these results.

Marker 40-1 was identified by sequencing a 371-bp amplicon produced by random primers B20 + A13 (GU552445 and GU552446). Primers 40-1F and 40-1R produced 559- and 346-bp PCR products in all samples (Table 2; Fig. 4a). Sequence data from *J. ailantifolia* and *J. cinerea* revealed a polymorphism at 212 bp (caatCg in *J. cinerea* and caatTg in *J. ailantifolia*; Table 2). After digestion of the 559- and 346-bp products with *Mfe*I, all *J. ailantifolia* samples produced bands at 346, 213, and 113 bp; all *J. cinerea* samples remained uncut (bands at 559 and 346 bp), and 31 of 50 *J. ailantifolia* × *J. cinerea*

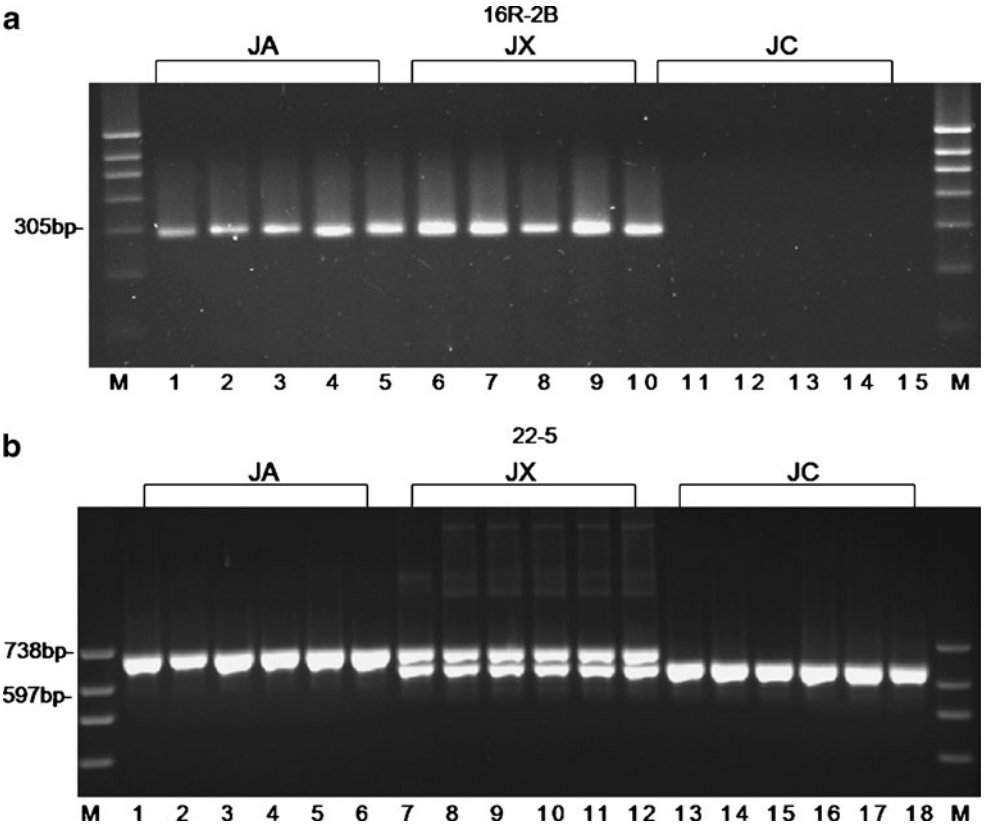


Fig. 3 Phenotypes of SCAR markers 16R-2 and 22-5 for *J. ailantifolia* (*JA*), hybrid (*JX*), and *J. cinerea* (*JC*) samples visualized using 2% agarose stained with ethidium bromide. For details concerning the primers and digestion conditions, see Table 2, *M* = DNA size standard. **a** Marker 16R-2, *lanes 1* through *5*, PCR products from *J. ailantifolia* DJUG 0002.01, DJUG 0005.04, DJUG 0006.06, DJUG 0008.04, and DJUG 0066.02; *lanes 6* through *10*, hybrid 704 S 34, 706 S 38, 734 S 59, 734 S 57, and 708 S 45; *lane 11* through *15*, JC 1083-1, 1073-1, 1089-1, 1090-1, and 1092-1. **b** Marker 22-5, *lanes 1* through *6*, PCR products from *J. ailantifolia* DJUG 0067.07, DJUG 0001.05, DJUG 0004.02, DJUG 0007.03, DJUG 0009.04, and DJUG 0010.02; *lanes 7* through *12*, hybrid Manitou1-1, 706 S 39, 707 S 43, 708 S 44, 710 S 47, and 711 S 52; *lanes 13* through *18*, JC 1083-1, 1073-1, IN-Tnc-1, Gmrks4-1, Hnfb22-1, and Gesek1-1

samples produced four products (559, 346, 213, and 133 bp; Table 3; Fig. 4d). We concluded that marker 40-1 was co-dominant, based on these phenotypes.

Marker 3-9 was identified by sequencing a 262-bp amplicon produced by random primers B13 + B12 (GU552437 and GU552438). Primers 3-9F and 3-9R produced a 213-bp product in all samples (Table 3; Fig. 4e). Sequence data revealed a polymorphism at 41 bp (ttggaattctTgaa in *J. cinerea* and ttgaattctGgaa in *J. ailantifolia*; Table 2). The sequence data were used in a query of the National Center for Biotechnology Information databases, which revealed 92% similarity with a *mitochondrial* sequence from *Beta vulgaris* subsp. *vulgaris* DNA (BA000009 and BA000024). After digestion of the 213-bp product with *Ags*I, all *J. ailantifolia* samples yielded three products (92, 86, and 35 bp); all *J. cinerea* samples yielded three products (86, 84, and 35 bp), and 46 of 51 *J. ailantifolia* × *J. cinerea* yielded 92-, 86-, and 35-bp products (Table 3; Fig. 4e). Marker 3-9 appears to be a dominant, mitochondrial (cytoplasmic) marker based on these phenotypes, which was consistent with results from the chloroplast markers *trnT-F* and CPS05 (McCleary et al. 2009; Table 3).

Of 51 *J. ailantifolia* × *J. cinerea* samples, one showed seven *J. ailantifolia* nuclear markers, five showed six *J. ailantifolia* nuclear markers, nine showed five *J. ailantifolia* nuclear markers, ten showed four, 11 showed three, nine showed two, and six showed one. Thus, all *J. ailantifolia* × *J. cinerea* samples showed both *J. cinerea* and *J. ailantifolia* nuclear marker(s), though nearly all showed *J. ailantifolia* chloroplasts. Of 51 *J. ailantifolia* × *J. cinerea* samples, 46 showed three *J. ailantifolia* cytoplasmic markers, but five hybrid samples (49, 56, 73, 74, and 75; Table 3) did not show the *J. ailantifolia* phenotype for chloroplast markers CPS05 and t*rnT-F* or the mitochondrial marker 3-9. These five samples contained a butternut plastome, but their nuclear genomes showed evidence of hybridization (Table 3).

Marker phenotypes of other *Juglans* species and hybrids

In addition to *J. ailantifolia*, *J. cinerea*, and buartnuts, we analyzed samples of *Juglans nigra* L., *Juglans mandshurica* (Maxim.), a selection reported to be a hybrid commonly called buarthian ((*J. ailantifolia* × *J. cinerea*) × *Juglans regia*) and a hybrid that, based on fruit and nut morphology, appeared to be *J. ailantifolia* × *J. nigra* (Tables 1 and 3). We used all ten markers to evaluate DNA from these samples. For *J. nigra*, we found that markers CPS05, *trnT-F*, and 3-9 and four nuclear markers ITS, 16R-2, 22-5, 14R-1 produced the same phenotype as *J. cinerea*. The phenotype of nuclear markers 15R-8, 39-6, and 40-1 was the same in *J. nigra* as in *J. ailantifolia* (Table 3). For *J. mandshurica*, the phenotypes of all the markers were

identical to *J. ailantifolia*. The putative *J. ailantifolia* × *J. nigra* hybrid showed phenotypes at markers 15R-8, 39-6, and 40-1 that may indicate that it actually contained *J. cinerea* genome. The putative buarthian hybrid contained a plastome consistent with *J. ailantifolia* as the female in the original hybridization.

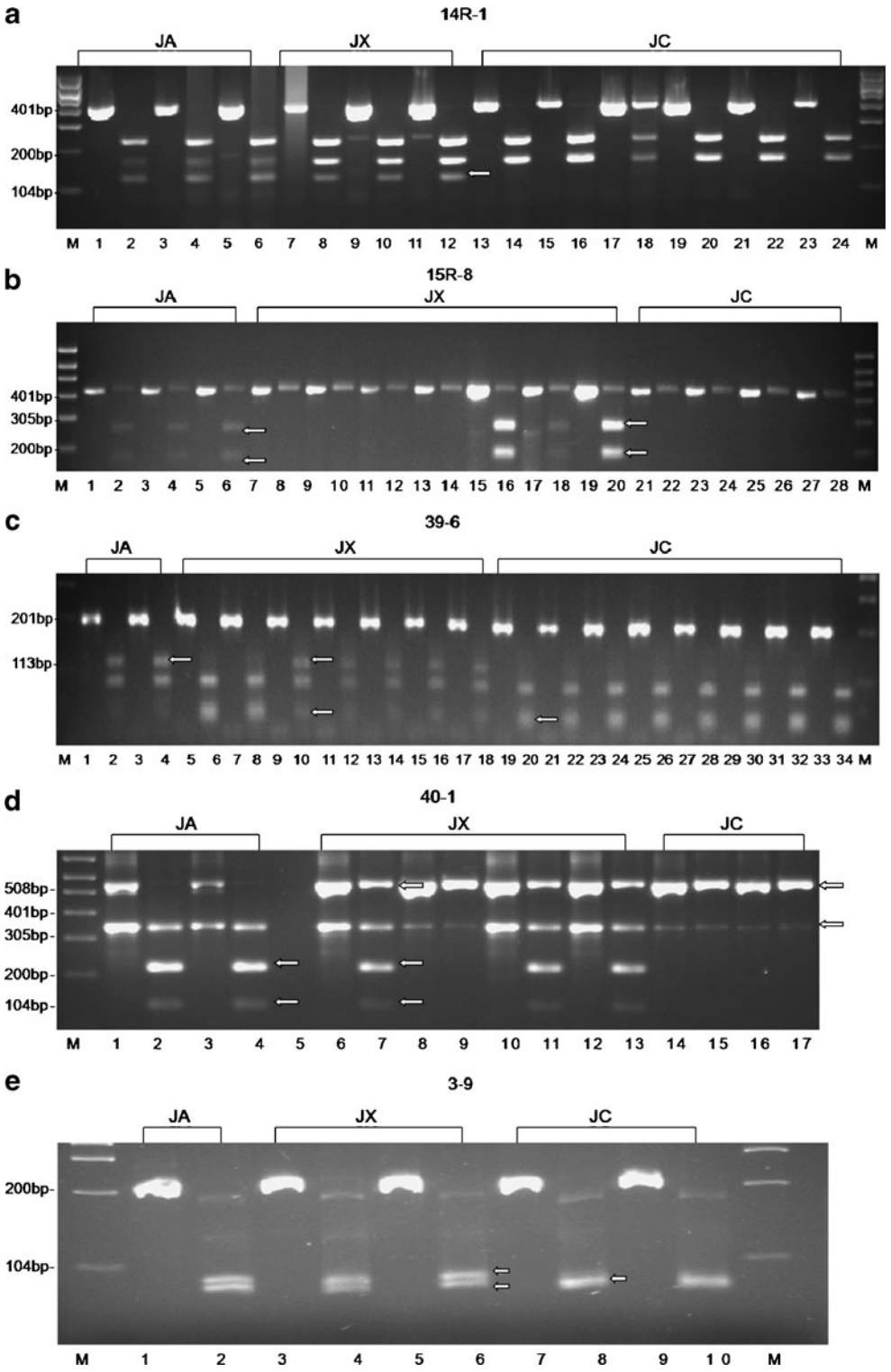
Discussion

The markers described here for identifying buarts and other hybrids were robust, and they provide a new resource management tool. Because the markers are PCR-based, they required only small amounts of DNA that can be obtained through speedy and small-scale extraction procedures. They can be used to analyze the genome of seedlings, which is important because many of the morphological markers that are available for identifying hybrids require the examination of reproductive or mature vegetative tissues (Woeste et al. 2009). The markers are also flexible; a comparison of the sequence data for *J. ailantifolia* and *J. cinerea* at each of the marker loci showed that enzymes other than those listed in Table 2 could be used to produce polymorphic products. The chloroplast marker *trnT-F* produced superfluous products of 471 bp in *J. ailantifolia* and 536 bp in *J. cinerea* after digestion with *Mbo*II (Fig. 2b). These products did not reduce the utility of the marker because the *J. ailantifolia* and *J. cinerea* haplotypes could still be easily discriminated based on more than 150 samples. The utility of the markers was revealed when we tested samples 732-S-55 and 748-S-63 (sample number 049 and 056) that contained *J. cinerea* cytoplasm, based on markers CPS05, *trnT-F*, and 3-9. We found that 748-S-63 was a hybrid based on the phenotype of markers 22-5, ITS, 14R-1, 39-6, and 40-1. Tree 732-S-55 was identified as a hybrid using markers 16R-2, 22-5, ITS, 14R-1, and 15R-8 (Table 3). Additional markers will be needed to detect advanced-generation backcrosses that might be the product of natural hybridizations or controlled crosses as part of a breeding program. We wish to reiterate that the application of the markers described here cannot "prove" that a specimen is a butternut; it can only show or fail to show evidence that it is a hybrid.

Butternut is increasingly threatened by butternut canker disease (Harrison and Hurley 1998; Ostry and Pijut 2000), so proper management of the remaining butternut resource is critical. The presence of an unknown number of *J.* × *bixbyi* and other interspecific hybrid trees (Ostry and Woeste 2004; Ross-Davis and Woeste 2008) complicates conservation and management decisions on several levels. For example, hybrid trees, if retained in a management area, could genetically swamp the remaining butternuts by hybridization or by numerical or fitness advantage (Hufford and Mazer 2003). Although the management plans of some forested lands require the removal of non-native or invasive species, without tools to definitively identify hybrids, managers may be reluctant to remove any tree that could be a butternut, especially if it is healthy or reproductive. Plant breeders will find the markers described here useful because they need to know if trees are hybrids. Trees that appear to have reduced morbidity or increased resistance to *S. clavigignenti-juglandacearum* infection in the field have been identified and re-propagated for use in seed orchards and for breeding (McIlwrick et al. 2000; Ostry and Woeste 2004; Michler et al. 2005). Public and private agencies and landowners that wish to use "pure" butternut, if possible, for reintroduction and afforestation will require seedlings from seed orchards containing only trees that show no evidence of hybridization.

Nuclear markers of hybridization provide several advantages to researchers. Cytoplasmic markers alone cannot identify hybridization events where butternut was the female parent. As hybrids become more common in the landscape relative to butternut, this type of hybridization is more likely (Hoban et al. 2009). Nuclear markers can also make morphological identification more secure and genetic studies more robust. If the taxonomic status of trees with unusual or intermediate phenotypes can be clarified, then the phenotypic variability of *J. cinerea* can be more firmly established and hybrids can be correctly identified for ecological and population genetic studies. Our results showed that the unusual, dark-barked butternuts reported by Ostry and Woeste (2004) presented no evidence of hybridization to *J. ailantifolia*. These trees are important because they may have increased resistance to *S. clavigignenti-juglandacearum* (Ostry and Woeste 2004). Ross-Davis et al. (2008a) hypothesized that hybrids may have misled researchers investigating the range of genetic variation in butternut (Morin et al. 2000).

In the late nineteenth century, nut growers were surprised to discover that some seedlings from heartnut trees did not bear heart-shaped nuts (Bixby 1919). Bixby reasoned that these non-heartnut-producing offspring were in fact (buart) hybrids. Buartnuts were subsequently propagated and cultivated as orchard or farmyard nut trees because of their vigor and fertility; most were likely the offspring of planted *J. ailantifolia* or their hybrid progeny, so they contained *J. ailantifolia* chloroplasts (Hoban et al. 2009). Because there were many more *J. cinerea* trees than *J. ailantifolia* over the past 150 years, pollination of a *J. cinerea* by *J. ailantifolia* has been relatively rare, and when it occurred, the hybrid offspring typically had to compete in a forest environment and were less likely than hybrids (growing in yards and orchards) to reproduce. Thus, hybrids containing a *J. cinerea* plastome are rare, and many are likely offspring of cultivated *J. cinerea* grown in orchards.



J. ailantifolia samples 008 and 014 showed *J. cinerea* phenotypes for nuclear marker 15R-8, indicating that the "C" allele has a frequency in *J. ailantifolia* of about 8% based on 24 *J. ailantifolia* samples. As a consequence, marker 15R-8 is less powerful (higher type II error rate) for identifying hybrids than the other markers. Because we have imperfect knowledge of the range of variation in the *J. ailantifolia* and *J. cinerea* genomes, it remains possible that further investigation will reveal that one or more of these markers can produce erroneous or ambivalent results. Testing

◀**Fig. 4** Phenotypes of CAPS markers 14R-1, 15R-8, 39-6, 40-1, and 3-9 for *J. ailantifolia* (*JA*), hybrid (*JX*), and *J. cinerea* (*JC*) samples. For details concerning the primers and digestion conditions, see Table 2. **a** Phenotypes of marker 14R-1 visualized using 2.5% agarose gel stained with ethidium bromide. PCR products from *J. ailantifolia* DJUG-0002.01, DJUG-0003.07, and DJUG0005 uncut (395-bp product in *lanes 1*, *3*, and *5*) and digested with *Bst*UI (228-, 167-, 127-, and 40-bp products in *lanes 2*, *4*, and *6*), *arrows* indicate diagnostic product size; PCR product of hybrid 731 S 53, 732 S 56, and 734 S 57 uncut (395- or 396-bp product in *lanes 7*, *9*, and *11*) and digested with *Bst*UI (products in *lanes 8*, *10*, *12*); PCR product of JC 1075-1, 1090-1, 1092-1, 1089-1, 966-11-11, and 971-8-5 uncut (396-bp product in *lanes 13*, *15*, *17*, *19*, *21*, and *23*) and digested with *Bst*UI (229- and 167-bp products in *lanes 14*, *16*, *18*, *20*, *22*, and *24*). **b** Phenotypes of marker 15R-8 visualized using 2.5% agarose stained with ethidium bromide. PCR product of *J. ailantifolia* DJUG-0002.01, DJUG-0003.07, and DJUG0005.04 uncut (429-bp product in *lanes 1*, *3*, and *5*) and digested with *Acl*I (259- and 170-bp products in *lanes 2*, *4*, and *6*), *arrows* indicate diagnostic product size; PCR product of hybrid 704 S 35, 706 S 39, 708 S 44, 748 S 62, 113SLE7-8-12, Holcomb36-1, and Manitou1-1 uncut (429-bp product in *lanes 7*, *9*, *11*, *13*, *15*, *17*, and *19*) and digested with *Acl*I (products in *lanes 8*, *10*, *12*, *14*, *16*, *18*, and *20*); PCR products of *J. cinerea* 1092-1, Tnc-1, 1089-1, and Gmarks4-1 uncut (429-bp product in *lanes 21*, *23*, *25*, and *27*) and digested with *Acl*I (429-bp product in *lanes 22*, *24*, *26*, and *28*). **c** Phenotypes of marker 39-6 visualized using 3% agarose gel stained with ethidium bromide. PCR product of *J. ailantifolia* DJUG-0002.01 and DJUG-0003.07 uncut (201-bp product in *lanes 1* and *3*) and digested with *Tsp*509I (113- and 88-bp products in *lanes 2* and *4*), *arrows* indicate diagnostic product sizes; PCR products of hybrids 734 S 57, 735 S 60, 748 S 62, 707-15-6, 113SLE7-8-12, 113SLE1-8-13, and 110SB.51-8-9 uncut (201- or 197-bp product in *lanes 5*, *7*, *9*, *11*, *13*, *15*, and *17*) and digested with *Tsp*509I (products in *lanes 6*, *8*, *10*, *12*, *14*, *16*, and *18*); PCR products of *J. cinerea* 709 S 93, 715 S 97, 716 S 98, 726 S 105, 727 S 106, 728 S 107, 730 S 108, and 738 S 112 uncut (197-bp product in *lanes 19*, *21*, *23*, *25*, *27*, *29*, *31*, and *33*) and digested with enzyme *Tsp*509I (88-, 57-, and 52-bp products in *lanes 20*, *22*, *24*, *26*, *28*, *30*, *32*, and *34*). **d** Marker 40-1 phenotypes visualized using 2.5% agarose stained with ethidium bromide. PCR products of *J. ailantifolia* DJUG-002.01 uncut (559- and 346-bp products in *lanes 1* and *3*) and digested with *Mfe*I (346-, 213-, and 133-bp products in *lanes 2* and *4*), *arrows* indicate diagnostic product sizes; PCR products of hybrids 702 S 32, 704 S 34, 706 S 38, and Holcomb36-1 uncut (559- and 346-bp products in *lanes 6*, *8*, *10*, and *12*) and digested with *Mfe*I (products in *lanes 7*, *9*, *11*, and *13*); JC 1073-1 and Tnc2-1 DNA samples uncut (559- and 346-bp products in *lanes 14* and *16*) and digested with *Mfe*I (559- and 346-bp products in *lanes 15* and *17*). **e** Phenotypes of marker 3-9 visualized using 4% metaphor agarose stained with ethidium bromide. *J. ailantifolia* PCR product uncut (213-bp product in *lane 1*) and digested with *Ags*I (92-, 86-, and 35-bp products in *lane 2*), *arrows* indicate diagnostic product sizes; PCR products of hybrids 706 S 38 and 707 S 41 uncut (213-bp product in *lanes 3* and *5*) and digested with *Ags*I (92-, 86-, and 35-bp products in *lanes 4* and *6*); PCR products of JC 708 S 46 and 711 S 52 uncut (213-bp product in *lanes 7* and *9*) and digested with *Ags*I (86-, 84-, 35-, and 8-bp products in *lanes 8* and *10*)

herbarium specimens of butternut obtained before the introduction of *J. ailantifolia* to the USA would be the best way to characterize and verify the range of morphological and genomic variation of *J. cinerea* without concern that alleles from *J. ailantifolia* had influenced the *J. cinerea* phenotype.

It is believed that butternut hybrids are self-fertile (as are all *Juglans*) and that many hybrid trees are F_2 or the product of backcrosses. The observation that most of the hybrids we examined did not present *J. ailantifolia* marker phenotypes at every locus is additional evidence that they were not F_1 (*J.* × *bixbyi*), but backcrosses, F_2, or other complex hybrids. This result was not surprising given the length of time that *J. ailantifolia* has been in the USA, the frequency with which hybrids are encountered, and the relative rarity of *J. ailantifolia* specimens. Nevertheless, it helps explain why the morphological identification of hybrids is so difficult.

Marker phenotypes in *J. mandshurica*, *J. nigra*, and hybrid samples 024 and 030 revealed that the markers described here will have limited utility for clarifying the pedigree of multi-species hybrids. Markers such as SSRs, which have a large number of alleles per locus, should prove more powerful for this application (Ross-Davis and Woeste 2008). We found that the cytoplasmic marker phenotype of *J. mandshurica* was identical to *J. ailantifolia*, which was not surprising considering their taxonomic and phylogeographic similarity, but the single *J. mandshurica* sample we tested did not contain only *J. ailantifolia* marker phenotypes in its nuclear genome. The four *J. nigra* samples we tested contained a *J. cinerea* cytoplasmic phenotype, consistent with the taxonomic placement of butternut with the other New World *Juglans* rather than the "Asian butternuts" (Aradhya et al. 2007). The marker phenotypes of the putative Buarthian were consistent with its putative pedigree (Table 3).

The markers described here have already been used to identify non-hybrid trees in National Forests of United States, for establishing seed orchards, and to further butternut conservation efforts by the USDA Forest Service and cooperating institutions. It is our goal to use them in conjunction with a canker resistance breeding program.

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