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Morphological and molecular methods to identify butternut (*Juglans cinerea*) and butternut hybrids: relevance to butternut conservation†

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Summary Butternut (*Juglans cinerea* L.) is a native, cold-tolerant, hard-mast species formerly valued for its nuts and wood, which is now endangered. The most immediate threat to butternut restoration is the spread of butternut canker disease, caused by the exotic fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz. Other threats include the hybridization of butternut with the exotic Japanese walnut (*Juglans ailantifolia* Carr.) and poor regeneration. The hybrids, known as buartnuts, are vegetatively vigorous, highly fecund, more resistant than butternut to butternut canker disease and difficult to identify. We review the vegetative and reproductive morphological traits that distinguish butternut from hybrids and identify those that can be used by field biologists to separate the taxa. No single trait was sufficient to separate butternut from hybrids, but pith color, lenticel size, shape and abundance, and the presence or absence of a notch in the upper margin of leaf scars, can be used in combination with other traits to identify butternuts and exclude most hybrids. In at least one butternut population, reduced symptoms of butternut canker disease were significantly associated with a dark barked phenotype. We also describe two randomly amplified polymorphic DNA (RAPD) markers that differentiate butternuts from hybrids based on DNA polymorphism. Together, these results should assist in the identification and testing of non-hybrid butternut for breeding and reintroduction of the species to its former habitats.

Keywords: conservation genetics, Japanese walnut, Juglans ailantifolia, Juglans × bixbyi, morphology, RAPD.

Introduction

Butternut, *Juglans cinerea* L., is a relatively short-lived tree species native to eastern North America, from New Brunswick, south to Georgia and west to Minnesota and Arkansas (Rink 1990). A rapid decline in butternut population size has been attributed to an epiphytotic of the exotic fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz, first described as a new species in 1979 (Nair et al. 1979). This fungus causes branch and stem cankers that grow and coalesce, ultimately girdling and killing the tree. Although butternut is susceptible to damage from other diseases and pests (Rink 1990), butternut canker is the greatest threat to the survival of the species (Loo et al. 2007). Restoration of butternut will require a concerted effort to identify germplasm for both ex situ and in situ conservation and the identification of apparently disease-resistant phenotypes (McIlwrick et al. 2000, Ostry et al. 2003, Michler et al. 2005). Ostry and Woeste (2004) have described an uncommon dark-barked phenotype that is common among healthy trees. Healthy dark-barked trees often grow adjacent to diseased trees of the same size and age.

Butternut freely hybridizes with at least two exotic species, Persian or English walnut (*Juglans regia* L., the hybrid known as *Juglans* × *quadrangulata* (Carr.) Rehd. (pro sp.)) and Japanese walnut (*Juglans ailantifolia* Carr., the hybrid known as *Juglans* × *bixbyi* Rehd.), the progeny of such crosses commonly being called butter-japs or buartnuts. The second hybrid is far more common. Most hybrid trees are of unknown parentage, however, and may be the product of a backcross, an intercross of F1s, or the result of introgression with the same or additional species. For this reason we refer to butternut hybrids generically as buartnuts or just hybrids. Hybrids are often difficult to distinguish from butternuts. Bixby (1919) stated "[c]ertain Japan walnuts [are] so near like butternuts as to be readily mistaken for them… [A]s far as the appearance of the nuts was concerned, the butternut could not be well separated from certain Japan walnuts." The prevalence of hybrids in the landscape has been little appreciated by dendrologists, silviculturists and arboretum curators. Taxonomic treatments (Dode 1909, Manning 1978) rarely mention hybrids. Floristic and dendrological species descriptions are often brief, based on an unknown number of samples of unidentified provenance, and not written with the objective of separating hybrids from native species. Nevertheless, comparisons of vegetative and reproductive morphological traits of Japanese walnut

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(Ohwi 1965) and butternut in which the species differ (e.g., lenticel size and shape, pith color, terminal bud shape (Trelease 1896), catkin length (Rehder 1940), nut shape and length (Dode 1909), leaf scar shape and fruit stalk length (Blackburn 1952), number of fruits per cluster (Steyermark 1963) and number of flowers per cluster (Manning 1978)) can be used to identify tentatively trees with intermediate phenotypes as hybrids.

Molecular methods for distinguishing butternuts from hybrids cannot be used for field identification, but they may provide more definitive identifications than methods based on vegetative morphology, and they have value for breeding and germplasm operations (Ostry and Woeste 2004). Differences in protein mobility or DNA sequence among members of the Juglandaceae have become a mainstay of *Juglans* phylogenetics and conservation genetics (Germain et al. 1993, Fjellstrom and Parfitt 1995, Stanford et al. 2000, Orel et al. 2003, Aradhya et al. 2007, Ross-Davis and Woeste 2007). Although most of these studies included both butternut and Japanese walnut genotypes, only Germain et al. (1993) identified species-specific (allozyme) markers for these taxa.

Now that the regeneration of butternut has become a pressing concern, there is a need to develop methods to identify non-hybrid butternut. Our objective was to quantify morphological and molecular differences within and among butternut, Japanese walnut and their hybrids. Although no single vegetative or reproductive morphological trait examined distinguished between butternut and hybrid genotypes, a combination of several morphological traits can be used to separate the taxa. In addition, two RAPD markers differentiated butternuts from hybrids.

Materials and methods

Plant material and morphological measurements

We measured morphological traits on three arbitrarily selected dormant branches of butternut ramets, hybrid and *J. ailantifolia* genotypes drawn from a range of geographical sources (Table 1). Ramets were primarily maintained at the Hardwood Tree Improvement and Regeneration Center (HTIRC), and in Rosemount, MN by the St. Paul office of the Northern Research Station of the USDA Forest Service. Additional samples were obtained from the Daniel Boone National Forest, Clark County, KY; Hoosier National Forest, Lawrence County, IN; Mammoth Cave National Park, Edmonson County, KY; Havelock, Ontario, Canada; and the Cincinnati Park Board, Cincinnati, OH. Assignment to species was based on morphology; individuals with phenotypes intermediate for at least one morphological trait were deemed hybrids.

Traits measured included: pith width (PW) and branch width (BW) of the previous-year's growth at two locations that divided the branch into thirds; the length (VBL) and width (VBW) of axillary vegetative buds; the length (CBL) and width (CBW) of catkin buds; the length (TBL) and width (TBW) of terminal buds; the width of the lower lobe of a leaf scar and the length of the same leaf scar; the length of the notch protruding downward from the upper margin of leaf scars (NOTCH); and the length and abundance of lenticels (LENTL and LEND). Although morphological traits can be strongly affected by environment, they can be taxonomically informative (Green 1969, Odell and Vander Kloet 1991), especially in detecting hybrids, when combined with other traits that remove some of the effects of allometry (Woeste et al. 1998). Therefore, the following ratios were calculated: PW/BW; VBL/VBW; CBL/CBW; TBL/TBW; and the ratio of the width of the lower lobe of the leaf scar to the total length of the leaf scar (SCAR). To reduce environmental effects on trait development, samples from HTIRC were taken from 4-year-old grafted trees grown at the Martell Forest on the property of the Department of Forestry and Natural Resources (FNR), Purdue University, West Lafayette, IN (Table 1). To remove the effect of unbalanced numbers of observations, data for catkin length

Table 1. Genotypes used to evaluate morphological and molecular differences among *Juglans ailantifolia*, *Juglans cinerea* and their hybrids, and the germplasm source.

Genotype	Source[1]	Species	Trait[2]
DJUG0002	NCGR	*J. ailantifolia*	D
DJUG0003	NCGR	*J. ailantifolia*	D
DJUG0004	NCGR	*J. ailantifolia*	D
DJUG0004.1	NCGR	*J. ailantifolia*	DV
DJUG0004.2	NCGR	*J. ailantifolia*	DV
DJUG0005	NCGR	*J. ailantifolia*	D
DJUG0006	NCGR	*J. ailantifolia*	D
DJUG0007	NCGR	*J. ailantifolia*	D
DJUG0007.3	NCGR	*J. ailantifolia*	DV
DJUG0007.5	NCGR	*J. ailantifolia*	DV
DJUG0008	NCGR	*J. ailantifolia*	D
DJUG0009	NCGR	*J. ailantifolia*	D
DJUG0061.1	NCGR	*J. ailantifolia*	V
DJUG0061.3	NCGR	*J. ailantifolia*	V
DJUG0067	NCGR	*J. ailantifolia*	D
667	HTIRC	*J. ailantifolia*	V
683	HTIRC	*J. ailantifolia*	DV
60-2	RSMT	*J. cinerea*	D
60-3	RSMT	*J. cinerea*	DV
226	RSMT	*J. cinerea*	D
234	RSMT	*J. cinerea*	D
243	RSMT	*J. cinerea*	D
685	HTIRC	*J. cinerea*	DF
717	HTIRC	*J. cinerea*	VFL
718	HTIRC	*J. cinerea*	VFL
719	HTIRC	*J. cinerea*	DFL
723	HTIRC	*J. cinerea*	VF
724	HTIRC	*J. cinerea*	VL
725	HTIRC	*J. cinerea*	VFL
739	HTIRC	*J. cinerea*	VFL
740	HTIRC	*J. cinerea*	VL
741	HTIRC	*J. cinerea*	VFL
744-1	HTIRC	*J. cinerea*	D
746-1	HTIRC	*J. cinerea*	D
747	HTIRC	*J. cinerea*	D
747-1	HTIRC	*J. cinerea*	D

Continued overleaf.

Table 1 Cont'd. Genotypes used to evaluate morphological and molecular differences among *Juglans ailantifolia*, *Juglans cinerea* and their hybrids, and the germplasm source.

Genotype	Source[1]	Species	Trait[2]
766	HTIRC	*J. cinerea*	DF
770	HTIRC	*J. cinerea*	F
804	HTIRC	*J. cinerea*	F
854	HTIRC	*J. cinerea*	F
858	HTIRC	*J. cinerea*	F
951	HTIRC	*J. cinerea*	F
953	HTIRC	*J. cinerea*	F
956	HTIRC	*J. cinerea*	F
971	HTIRC	*J. cinerea*	F
8047	WI	*J. cinerea*	D
9289	WI	*J. cinerea*	D
8098	WI	*J. cinerea*	D
DB6	DBNF	*J. cinerea*	D
DB16	DBNF	*J. cinerea*	D
DB30	DBNF	*J. cinerea*	D
H1	HNF	*J. cinerea*	D
H28	HNF	*J. cinerea*	D
MACA82	MCNP	*J. cinerea*	D
DJUG0066	NCGR	*J. ailantifolia*	D
Ontario3	Ontario	*J. cinerea*	D
Ontario4	Ontario	*J. cinerea*	D
Slocum128	WI	*J. cinerea*	D
AO-9	RSMT	hybrid	V
CPB1	CPB	hybrid	V
CPB2	CPB	hybrid	V
CPB3	CPB	hybrid	V
Hort3	HTIRC	hybrid	F
OS-7	RSMT	hybrid	V
OS-45	RSMT	hybrid	V
OS-46	RSMT	hybrid	V
OS-128	RSMT	hybrid	V
OS-165	RSMT	hybrid	V
638	HTIRC	hybrid	D
656	HTIRC	hybrid	D
696	HTIRC	hybrid	LV
701	HTIRC	hybrid	FLV
702	HTIRC	hybrid	FV
704	HTIRC	hybrid	LV
780	HTIRC	hybrid	FLV
781	HTIRC	hybrid	LV
802	HTIRC	hybrid	LV
803	HTIRC	hybrid	FLV
805	HTIRC	Hybrid	F
807	HTIRC	hybrid	LV
810	HTIRC	hybrid	F
855	HTIRC	Hybrid	D

[1] Abbreviations: NCGR = National Clonal Germplasm Repository, Davis, CA; RSMT = Rosemount, MN; HTIRC = Hardwood Tree Improvement and Regeneration Center; WI genotypes were from an autochthonous stand in Rock Co., WI; DBNF = Daniel Boone National Forest; HNF = Hoosier National Forest; MCNP = Mammoth Cave National Park; Ontario = Havelock, Ontario, Canada; and CPB = Cincinnati Park Board.

[2] Abbreviations: D = genotype used for DNA marker development; V = genotype used to compare vegetative morphology; F = genotype used to compare floral morphology; and L = genotype used to compare lenticel size and density.

were first averaged across all branches of all ramets for each clone, and then the genotypic means were used to calculate the mean, standard deviation (SD) and range for each taxon. Lenticel density was determined by counting lenticels on three branches from eight hybrid and butternut genotypes (Table 1). Samples of Japanese walnut were not examined for lenticel density or catkin length. The number of lenticels in three 1-cm-circumferential bands was divided by the diameter of the branch at the point where the lenticels were counted, to adjust the count data to a common branch surface area. Details concerning the germplasm are available from the corresponding author.

Statistical analysis

The effects of species and genotype on the measured variables were evaluated by analysis of variance (ANOVA). Species was considered a fixed effect and genotypes were considered random effects and nested within species. Hybrids were considered a "species" in the analysis. Least squares means were estimated by restricted maximum likelihood; pairwise differences were adjusted for multiple comparisons by Tukey's method. The correlations between bark color and disease symptoms and between bark depth and disease symptoms were tested by Mantel-Haenszel Chi-Square. Statistical analysis of the association between the dark-barked phenotype and butternut canker resistance was based on a single large population of butternuts growing in Rock County, WI, near the town of Whitewater. The phenotypes of 301 butternut trees were characterized for bark color, disease rating and other traits by two of the authors (KW and MO). Trees were rated in 2003 for canker abundance and size as: "severe," when they harbored large bole cankers that were oozing and coalescing and > 50% of the canopy branches were dead or dying; "many," when they harbored numerous small cankers and 50–75% of the canopy was healthy; or "few," when at least one active canker was observed and < 25% of the canopy was affected. At the same time, trees were rated for bark color as "light," when the bark was ash gray or silver; "dark," when it was similar in color to the bark of black walnut; or "moderate," when the bark was an intermediate color. Bark fissure was rated as: "shallow," when it was of a depth typical of butternut; "deep," when it was of a depth typical of black walnut (*Juglans nigra* L.); or "moderate," when it was of intermediate depth (Baskauf 2007).

RAPD markers to identify butternuts and hybrids

We used bulked DNA pools of each species to identify randomly amplified polymorphic DNAs (RAPDs) producing amplification products specific to butternut and *J. ailantifolia* (Michelmore et al. 1991; Table 1) The bulked DNA pools were screened based on primers from Gene Link (Hawthorne, NY), and two promising primers, A15 (5′-TTCCGAACCC-3′) and B12 (5′-CCTTGACGCA-3′) were chosen for further study. These RAPD primers were then screened against the individuals comprising the bulked DNA pools and an additional test set of individuals (Table 1) to confirm that amplification of the markers was consistent with the species being tested. The PCR analysis was performed as described by Woeste et al. (1996)

except that gels were documented with a Bio-Rad system (Hercules, CA) running Quantity One software Version 4.6.3.

Results

Vegetative morphology

Members of the *Juglans* genus have a distinctive chambered pith that is often used to separate *Juglans* members from species of other genera. The pith of butternut occupies a slightly smaller area of the branch in longitudinal section than the pith of the hybrids we measured. The ratio of pith width to branch width at the same point was 0.26 for butternuts and 0.32 for hybrids (Table 2), but butternut did not differ from Japanese walnut for this trait. The shape of the axillary (vegetative) buds and terminal buds differentiated the butternuts and hybrids we measured. The vegetative buds of butternut were significantly longer and thinner (VBL/VBW) than those of Japanese walnut and hybrids (Table 2); hybrids did not differ significantly from Japanese walnut in this trait. We did not obtain sufficient terminal bud samples of Japanese walnut to make comparisons; however, the terminal buds of butternut were significantly longer and thinner than those of the hybrids we measured (Table 2). The shape of the dormant catkin buds of butternut and hybrids did not differ significantly, although the catkin buds of hybrids tended to be slightly longer and narrower than those of butternut.

We measured the width of the lower lobe of leaf scars and the total length of leaf scars and compared the ratio of width to length for the three taxa. Although there was a clear trend, with butternut exhibiting less deeply lobed scars than Japanese walnut and hybrids exhibiting intermediate values, variability was high and differences among the taxa were not significant (Table 2). Taxonomists and dendrologists have frequently noted that the top edge of the leaf scars of butternuts are straight, and that this trait separates them from related species (Werthner 1935, Rehder 1940), although at least one author (Manning 1978) noted that the leaf scars of butternut are occasionally

Table 2. Morphological comparison of Japanese walnut (*Juglans ailantifolia*; *Ja*), hybrids (*Jx*) and butternut (*Juglans cinerea*; *Jc*).

Trait	Species	LSM[1]	Test of species effects			Differences of LSM[2]			
			df	*F*	*P* > *F*	Comparison	df	*t*	*P*(adj.)
Pith width/branch width	*Ja*	0.287 (0.022)	2	3.53	0.045	*Ja* vs. *Jc*	25	0.083	0.69
	Jx	0.328 (0.016)				*Ja* vs. *Jx*	25	–1.49	0.31
	Jc	0.269 (0.019)				*Jc* vs. *Jx*	25	–2.61	0.038
Vegetative bud length/width	*Ja*	1.11 (0.065)	2	17.9	0.001	*Ja* vs. *Jc*	63	–4.43	0.0001
	Jx	1.45 (0.042)				*Ja* vs. *Jx*	63	–0.71	0.76
	Jc	1.16 (0.031)				*Jc* vs. *Jx*	63	5.56	0.0001
Terminal bud length/width	*Jx*	1.45 (0.05)	1	17.2	0.0002	*Jc* vs. *Jx*	34	4.14	0.0006
	Jc	1.74 (0.05)							
Catkin bud length/width	*Jx*	1.92 (0.11)	1	1.96	0.175	*Jc* vs. *Jx*	23	–1.40	0.175
	Jc	1.66 (0.15)							
Leaf scar width/length	*Ja*	0.45 (0.03)	2	5.96	0.004	*Ja* vs. *Jc*	61	–3.45	0.0029
	Jx	0.54 (0.02)				*Ja* vs. *Jx*	61	–2.15	0.089
	Jc	0.60 (0.03)				*Jc* vs. *Jx*	61	1.77	0.187
Leaf scar notch length	*Ja*	1.01 (0.27)	2	7.58	0.001	*Ja* vs. *Jc*	66	2.53	0.036
	Jx	1.12 (0.15)				*Ja* vs. *Jx*	66	–0.34	0.939
	Jc	0.15 (0.20)				*Jc* vs. *Jx*	66	–3.81	0.0009
Lenticel length	*Ja*	2.59 (0.19)	2	25.0	0.0001	*Ja* vs. *Jc*	68	7.04	0.0001
	Jx	1.67 (0.11)				*Ja* vs. *Jx*	68	4.15	0.0003
	Jc	0.87 (0.15)				*Jc* vs. *Jx*	68	–4.18	0.0002
Lenticel density[3]	*Jx*	2.54 (0.547)	1	8.37	0.0118	*Jc* vs. *Jx*	14	2.89	0.0118
	Jc	4.78 (0.547)							
Catkin length (cm)	*Jx*	17.1 (1.77)	1	15.3	0.0018	*Jc* vs. *Jx*	13	–3.92	0.0018
	Jc	9.01 (1.08)							

[1] Least squares means and (standard deviation).
[2] Pairwise comparisons adjusted by Tukey's method.
[3] Number of lenticels per 3.14 cm^2 branch surface.

weakly notched. We also observed that some butternut leaf scars had small notches, but there was a clear and significant difference in the length of the notch between butternut, Japanese walnut and their hybrids (Table 2). Of 18 (putative) hybrid genotypes examined, notches were absent in all three observed scars per genotype only for the hybrid genotype AO-9; notches were absent in one or two leaf scars in two other hybrid genotypes (OS46 and 802, respectively; Table 1). In two of the nine butternut genotypes measured, small notches were present in all three scars (717 and 739); a notch was found in only one of the three examined scars of HTIRC 740. Thus, most hybrids have leaf scars with notches, and, although some butternuts have leaf scars with notches, the notches in the leaf scars of butternut were significantly shorter and smaller than those of the hybrids and Japanese walnuts we examined.

Lenticel size can be strongly influenced by environment (Mooney and Emboden 1968), but in certain cases this trait can be a reliable descriptor of a species (Aldasoro et al. 1998). We found that lenticels can be used to help separate butternut from Japanese walnut and hybrids (Table 2). In butternut, lenticels were small, abundant and primarily round or rarely elongated longitudinally and often elongated slightly in the transverse direction (across the branch). Lenticels of hybrids were larger than those found on butternut (1.6 versus 0.87 mm); lenticels of hybrids were sometimes uniformly distributed, as in butternut, but were most often patchy, and often elongated longitudinally. Lenticels of Japanese walnut were often elongated (2.6 mm) and uneven in distribution. Lenticel density was significantly greater ($P < 0.0001$) in butternut than in hybrids after normalization for branch diameter. Mean branch diameter was almost identical for butternuts and hybrids (8.9 and 8.8 mm, respectively), and the mean (unadjusted) number of lenticels per 1-cm-circumferential band of bark (± SD) was 40.7 ± 13.9 for butternut and 24.7 ± 15.6 for hybrids.

Juglans catkins, which are borne on the previous year's growth, were shorter in butternuts than in hybrids. At peak pollen shed, butternut catkin length ranged from 5 to 12 cm, with a mean ± SD of 9.0 ± 2.3 cm, significantly shorter than hybrid catkins (P (adjusted) = 0.0018) that were 13–26 cm in length, with a mean ± SD of 16.7 ± 4.8 cm (Table 2).

RAPD markers to identify butternut and hybrids

Analysis of the amplification products of the RAPD primers A15 and B12 showed that DNA bulking could reveal amplicons specific to butternut and Japanese walnut. An amplicon of about 600 bp was produced with primer A15; it was present in all butternuts tested and absent from all Japanese walnuts. The reverse was true for an amplicon of about 650 bp (Figure 1). When RAPD A15 was used to screen hybrids, we observed that amplicons from both parents were present (Figure 1). A similar result was obtained for marker B12, which produced an amplicon of about 490 bp in Japanese walnut and about 550 bp in butternut. In hybrids, marker B12 expressed both the Japanese walnut and the butternut amplicon sizes (Figure 1). The RAPDs are generally understood to be dominant markers, so the results we observed probably indicate that the amplicons of A15 and B12 are not alleles but distinct genetic regions that amplify in one species but not in the other, and that amplicons from both parents are produced in hybrids.

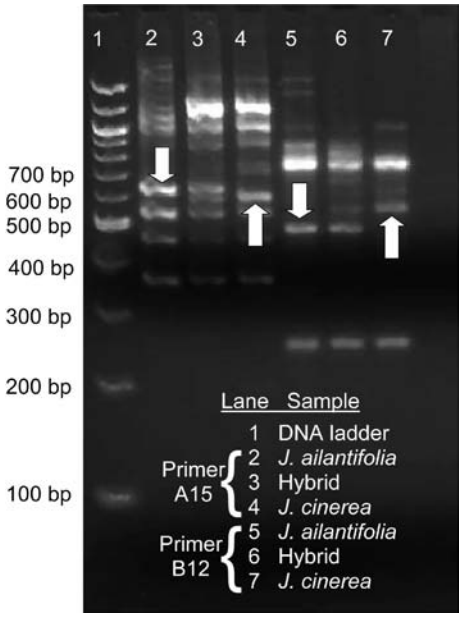

Figure 1. Agarose gel showing the mobility of amplicons produced from the randomly amplified polymorphic DNA (RAPD) primers A15 and B12. Amplicons specific to Japanese walnut (*Juglans ailantifolia*) are indicated by arrows pointing down; amplicons specific to butternut (*J. cinerea*) are indicated by arrows pointing up; both bands are amplified in hybrids. Results shown were typical for all genotypes tested (see Table 1).

Butternut canker disease susceptibility

The bark of butternut is described as light gray (Whittemore and Stone 1997) or grayish brown with smooth ridges (Gleason 1958). We have observed that butternut bark on the oldest parts of a tree may vary widely in appearance among trees. In the even-aged population we examined, the trend associating increasingly dark bark color and fewer butternut canker disease symptoms was highly significant (Mantel-Haenszel Chi-Square = 97.7, $P = 0.0001$). The association between bark depth and disease symptoms did not differ significantly from zero.

Discussion

We found that samples of butternut, Japanese walnut and their hybrids differed significantly for several vegetative and reproductive traits previously described only qualitatively, and that the morphology of hybrids was usually intermediate between the parental species. When characterizing the morphology of a species, it is impossible to know if the full range of variation for the species has been observed for a particular trait. None of the scientists we consulted recognized regional variation or

ecotypes in the morphology of butternut, but it is likely that variation of this type is present given the large natural range of the species.

We determined that the ratio of pith width to branch width was similar in butternut and Japanese walnut, with the pith occupying about 25% of the branch in longitudinal section, whereas in hybrids the pith occupied nearly 33% of the stem. The difference between butternut and hybrids was statistically significant for this trait, whereas butternut did not differ from Japanese walnut. Other studies have shown that hybrids sometimes express traits outside the range of the parental species (Rosenthal et al. 2002). The axillary vegetative buds and terminal buds of butternut were significantly more elongated and more conical than those of Japanese walnut and hybrids, which in the samples we examined had roughly rectangular terminal buds, broader and flatter at the tip than those of butternut. The shape of catkin buds did not differ between butternut and hybrids. Furthermore, there were no differences between butternut and hybrids in the shape of the leaf scar; intraspecific variability for this trait was high (Table 2). However, the presence of a descending V-shaped notch in the center of the upper margin of the leaf scar of many hybrids, although not unequivocal proof of a hybrid, was strongly indicative. We observed notches in some leaf scars of some butternut genotypes, but these notches were significantly shorter than those of hybrids and, in general, the butternut leaf scars we examined nearly always had a straight upper margin. The lenticels of butternuts were more abundant and smaller than those of hybrids. Butternut lenticels were most often evenly distributed, small and round, but if elongated, they were usually transversely oriented (across the branch). Lenticels of hybrids were larger than those of butternuts, most often elongated rather than round, less densely scattered than in butternut, and frequently patchy in distribution. Although the measured and observed differences in lenticel size and density may seem too small to be of practical significance, when branches are compared side-by-side, the differences were striking. The catkins of butternut were significantly shorter than those of hybrids; although this trait is observable only during a short period in the spring it may be a useful guide for trees grown ex situ that can be routinely evaluated.

We employed a DNA bulking method to identify RAPD markers that distinguish (presumed) butternut and hybrid genotypes, and used RAPD primers A15 and B12 to amplify taxon-specific bands. We predict that by cloning and sequencing these amplicons we will be able to develop markers with less complex amplification products that are more amenable to large-scale screens of germplasm and of seed sources. The development of a larger number of taxon-specific markers targeted at both the nuclear and plastid genomes should assist in identifying hybrids even when they are part of a hybrid swarm. Butternut is described as a short-lived tree (Rink 1990), and there are few (if any) trees alive today that predate the introduction of Japanese walnut to North America. Because of the history of hybridization and limited access to samples with a clear and certain identity, there will always be doubt as to the full range of molecular and morphological variability of butternut proper.

The identification of disease-resistant butternuts

We determined a statistically significant association between bark color and canker resistance in a population apparently segregating for both traits. The association between bark color and resistance to butternut canker is important because the traits may be genetically related or linked, and if this is borne out by further research it could simplify breeding of resistant selections. Over the past decade, dark-barked trees have been identified in numerous locations around the Midwestern USA, usually as isolated individuals. Research on heritability of disease resistance and the dark-barked phenotype is needed to confirm that the association between bark color and disease resistance in the Wisconsin butternut population was not the result of local linkage disequilibrium. We have observed that not all dark-barked trees appear highly resistant and not all apparently resistant trees have dark bark.

Prospects for butternut

Hybrids have attractive qualities as horticultural selections (Bixby 1919), but their silvicultural and ecological properties have not been described, and they are not acceptable substitutes for butternut in settings that require the retention of native species (Lake States Working Group, FSC 2005). Hybrids can colonize natural areas, although not aggressively (data not shown), but even limited colonization may present a threat where butternut regeneration is poor or non-existent. The simplest means to forestall hybrid invasion may be a concerted effort to improve the availability of butternut seed and to verify the identity of seed sources used for afforestation and reintroduction. The future availability of pure butternut seedlings from tested, resistant parents may help reduce the use of hybrids as seed sources for afforestation.

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