Geographically extensive hybridization between the forest trees American butternut and Japanese walnut

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We investigate the question of naturally occurring interspecific hybrids between two forest trees: the native North American butternut (*Juglans cinerea* L.) and the introduced Japanese walnut (*Juglans ailantifolia* Carrière). Using nuclear and chloroplast DNA markers, we provide evidence for 29 F₁ and 22 advanced generation hybrids in seven locations across the eastern and southern range of the native species. Two locations show extensive admixture (95% *J. ailantifolia* and hybrids) while other locations show limited admixture. Hybridization appears to be asymmetrical with 90.9 per cent of hybrids having *J. ailantifolia* as the maternal parent. This is, to our knowledge, the first genetic data supporting natural hybridization between these species. The long-term outcome of introgression could include loss of native diversity, but could also include transfer of useful traits from the introduced species.

Keywords: conservation; interspecific hybridization; introgression; *Juglans cinerea*

1. INTRODUCTION

Natural hybridization between sympatric forest tree species is common (Craft *et al.* 2002; Lexer *et al.* 2005), but hybridization between introduced species and rare or economically valuable native species has raised concern among conservation geneticists (Hails & Morley 2005; Mallet 2005). Successful hybridization can result in adaptive evolutionary change if circumstances favour hybrid progeny (Lewontin & Birch 1966; Rieseberg 1997). However, successful genetic invasion involving disjunct species seems unlikely, as hybrids are usually assumed to be intrinsically or extrinsically unfit (Mayr 1992; Coyne & Orr 2004). Therefore, the outcome of hybridization is debatable and of theoretical and practical interest.

Many East Asian woody plant taxa can produce fertile hybrid progeny with their North American sister taxa, including species in *Platanus*, *Ulmus*,

Castanea, *Pinus* and *Juglans* (Wen 1999). Cultivated hybrids between varietal forms of Japanese walnut (*Juglans ailantifolia* Carrière) and the native North American tree butternut (*Juglans cinerea* L.) are such vigorous, fruitful trees that forest tree biologists have expressed concern over the possibility of an undetected, range-wide genetic invasion (Ostry & Woeste 2004). Additionally, some varieties of *J. ailantifolia* and some hybrids have shown remarkable tolerance to *Sirococcus clavigignenti-juglandacearum*, a pathogenic fungus causing the disease butternut canker, a major cause of decline in butternut populations (Orchard 1984). The adaptive potential of Japanese walnut genes could have an evolutionary and ecological impact on butternut (specifically for disease dynamics) if the hybrids produce successful descendants, but it is not known whether hybrids persist in natural settings.

To investigate the possibility of genetic invasion, we developed a set of nuclear microsatellite markers (Hoban *et al.* 2008) and a set of chloroplast markers (McCleary *et al.* 2009) to reliably identify the parent species and hybrids. As no definitive silvicultural characters distinguish hybrids from *J. cinerea* (Ross-Davis *et al.* 2008), identification using DNA markers is essential. We qualified both types of DNA markers on reference sets (*J. ailantifolia* *n*=69 and *J. cinerea* *n*=71) to assess accuracy of species identification, and then genotyped putative butternuts from seven populations in eastern North America (*n*=187). We address the question: ‘do interspecific hybrids occur and reproduce in natural populations?’

2. MATERIAL AND METHODS

(a) Species

Juglans ailantifolia, native to the mountain forests of Japan, was introduced to North America ca 1870, was widely planted and soon naturalized in woodlots, abandoned fields and roadsides (Neilson 1930). *Juglans cinerea*, native to eastern North American riparian and bottomland forest ecosystems, has suffered severe decline throughout the twentieth century and is currently a species of concern for both state and federal threatened species lists (Schultz 2003).

(b) Study populations

The *J. cinerea* reference set consisted of 71 individuals: 11 butternut cultivars from the USDA National Germplasm Repository (Corvallis, OR, USA) and 60 individuals from a naturally regenerated, protected population in Kentucky. The *J. ailantifolia* reference set comprised 69 individuals from germplasm repositories, arboreta and botanic gardens (see table S1 in the electronic supplementary material). Between 2005 and 2008, we sampled 187 individuals from seven additional populations of *J. cinerea* where hybridization is suspected (see table S2 in the electronic supplementary material). Samples comprised approximately 500 g of twigs or leaves. DNA was extracted using a modified cetyltrimethylammonium bromide protocol (modifications in the electronic supplementary material).

(c) Genotyping

We genotyped with three different chloroplast cleaved amplified polymorphic sequence (CAPS) markers to detect species-specific chloroplast haplotypes (McCleary *et al.* 2009). CAPS products were visualized using electrophoresis in 1.5 per cent agarose gels. We also genotyped with eight nuclear microsatellite markers: Jcin_B114; Jcin_B121; Jcin_B147; Jcin_B159; Jcin_B249; Jcin_B262; Jcin_B264 (Hoban *et al.* 2008); and WGA82 (Robichaud *et al.* 2006). Fragment length polymorphisms were visualized on an ABI 3730 XL DNA analyser and scored using GENEMAPPER (Applied Biosystems, Inc., Foster City, CA, USA). Matching genotypes were identified using identity analysis in CERVUS (Kalinowski *et al.* 2007). Exact and near (mismatch=1) matches were removed prior to genetic analysis, as duplicate genotypes bias the allele frequencies used for clustering algorithms. We removed 18 individuals from our reference sets and five from our natural populations. After removing duplicate genotypes, we calculated allele frequencies, *F*_{ST} and *R*_{ST} for each species

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Figure 1. Posterior probability distribution and the most likely genotypic class assignments of hybrid individuals. Dark green, F_1 ; light green, F_2 ; light yellow, $Ja\ BC_1$; light blue, $Jc\ BC_1$; dark blue, Jc ; dark yellow, Ja .

reference set using GENALEX (Peakall & Smouse 2006) and I_n statistics using INFOCALC (Rosenberg et al. 2003; see table S3 in the electronic supplementary material).

(d) Genetic analysis

We used the Bayesian approach implemented by STRUCTURE (Pritchard et al. 2000) to identify admixed individuals. We tested values of K from 2 to 10 and used the method of Evanno et al. (2005) to determine the number of genetic clusters (K) for which the data were most likely (see table S4 in the electronic supplementary material). For the most likely K ($K=2$), we report estimated membership in each cluster for every individual, and 95% posterior probability intervals (see table S5 in the electronic supplementary material). We complement this analysis with the Bayesian approach in NEWHYBRIDS (Anderson & Thompson 2002) to assign individuals to one of six genotypic classes (see table S5 in the electronic supplementary material). We ran the analysis using the z and s options to incorporate knowledge about our reference sets. We also examined the posterior assignment of individuals in our reference sets.

(e) Hybrid assignment

The presence of the *J. aillantifolia* chloroplast in a native *J. cinerea* population is evidence of hybridization in that population. For the nuclear genome, we classify individuals with less than 0.90 probability of membership in a STRUCTURE species cluster as probably admixed. We also assign each individual to the most likely (probability > 0.50) NEWHYBRIDS genotypic class. Individuals for which no single class had more than or equal to 0.50 probability are not assigned to a class.

3. RESULTS

All of the individuals in our reference sets had the chloroplast haplotype of the expected species. STRUCTURE analysis for the reference sets showed that all individuals in our reference groups had more than 0.90 probability of belonging to the species expected (either *J. cinerea* or *J. aillantifolia*), with one exception suggesting admixture, the 'Johnson' butternut cultivar (accession CJUG 10.001 from the USDA National Germplasm Repository; see table S1 in the electronic supplementary material).

In the seven study sites, using STRUCTURE and our cut-off value (0.90), we report 60 admixed individuals, and 12 *J. aillantifolia*. Using NEWHYBRIDS, we report 55 probable hybrids and 10 *J. aillantifolia*. The most probable hybrid classes with our cut-off value (0.50) were as follows: 29 F_1 s; 14 F_2 s; eight backcrosses

to *J. cinerea*; and four not assigned (figure 1; table S5 in the electronic supplementary material). No individual had more than 0.34 probability of backcross to *J. aillantifolia*. Of those assigned, 25 individuals were assigned to a hybrid class with more than or equal to 0.90 probability, 13 were assigned with more than or equal to 0.80 probability, and 13 were assigned with probabilities between 0.80 and 0.50 (figure 1). Of the 55 putative hybrids, 50 (90.9%) contained the *J. aillantifolia* chloroplast, and the remaining five contained the *J. cinerea* chloroplast (see table S5 in the electronic supplementary material). All seven populations showed evidence of at least one probable hybrid (figure 2) and non-admixed native *J. cinerea* occurred at proportions from 0.05 to 0.92.

4. DISCUSSION

Our data indicate that introgression occurs across a large portion of the native species range. Although hybridization was highest in fragmented semi-rural landscapes (sites CT and NC, USA), we also found small numbers of probable hybrids in four other locations, including three national forests. Extensive introgression could alter the gene pool and evolutionary potential of the native species, with potentially cascading ecosystem consequences. Our data suggest that caution should be taken in choosing individuals for restoration efforts.

Most (90.9%) of the probable hybrids contained the *J. aillantifolia* chloroplast. As the chloroplast is maternally inherited in *Juglans* (Zhang et al. 2003), this result indicates that most maternal parents of hybrids are *J. aillantifolia*. One possible explanation is the higher number of *J. cinerea* and thus higher amount of *J. cinerea* pollen in the pollen pool, so most paternal parents will be *J. cinerea*, similar to observations in *Populus* (Lexer et al. 2005). Alternatively, the explanation may be partial one-way intrinsic incompatibility, which is common in angiosperms (Tiffin et al. 2001).

Figure 2. Native range of butternut and admixture in each collection site. Histograms represent the number of individuals assigned to each genetic class by NEWHYBRIDS. The four individuals with less than or equal to 0.50 probability in any class are not shown. Dark blue, Jc; dark yellow, Ja; dark green, F₁; light green, F₂; light blue, Jc BC₁; light yellow, Ja BC₁.

Interestingly, all eight probable backcross individuals are to *J. cinerea*. This also may be due to high representation of *J. cinerea* pollen in the pollen pool. While eight is an exceedingly small sample size, we can speculate that a long-term consequence of repeated backcrossing to *J. cinerea* could be chloroplast capture, in which the *J. ailantifolia* chloroplast is retained mostly in a *J. cinerea* nuclear background. However, interaction between hybrids and butternut canker may preserve large blocks of the *J. ailantifolia* genome, if *J. ailantifolia* tolerance has a genetic basis. The extent of *J. ailantifolia* introgression over time may depend on the strength of selection against *J. cinerea* and asymmetry in survival of backcross individuals.

We note that our Bayesian probabilities are based on a small number of moderately to highly differentiated markers, so assignments are rough estimates. The four unclassifiable individuals and the 13 individuals with less than or equal to 0.80 probability in a single hybrid class reflect the limits of our ability to distinguish genotypic classes with the current marker sets. Genotyping with additional well-differentiated markers will improve the resolution of this analysis and the number of F₁ versus advanced generation hybrids may change. Nevertheless, the presence of the *J. ailantifolia* chloroplast and many highly probable hybrids in multiple populations allows us to strongly suggest that hybrids persist and interbreed with the native species in natural settings, in contrast to expectations for disjunct species.

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