

NOTE / NOTE

Asymmetrical natural hybridization between *Populus deltoides* and *P. balsamifera* (Salicaceae)¹

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Abstract: Natural hybridization has long been recognized as a means for gene flow between species and has important evolutionary consequences. Although hybridization is generally considered to be symmetrical, with both hybridizing species being equally likely to be the male or female parent, several studies have demonstrated the presence of asymmetrical hybridization and introgression from one species to the other. We investigated the direction of natural hybridization between two sympatric forest tree species in North America (*Populus deltoides* Bartr. ex Marsh. and *Populus balsamifera* L.) using species-specific single nucleotide polymorphism (SNP) markers in both the nuclear and chloroplast genomes. All natural hybrid individuals, identified from morphological traits, had nuclear alleles corresponding to both parental species, while the chloroplast genotypes showed similarity to *P. deltoides*, indicating asymmetrical hybridization with *P. deltoides* as the maternal and *P. balsamifera* as the paternal donor species. This observed asymmetrical hybridization may be attributable to cytonuclear interactions.

Key words: *Populus*, natural hybridization, asymmetry, SNP markers.

Résumé : On sait depuis longtemps que l'hybridation naturelle contribue au flux de gènes entre les espèces, avec d'importantes conséquences évolutives. Bien qu'on considère généralement l'hybridation comme symétrique, les deux espèces impliquées contribuant également comme parent mâle et femelle, plusieurs études ont démontré la présence d'hybridation asymétrique et d'introgression, d'une espèce à l'autre. Les auteurs ont examiné la direction de l'hybridation naturelle entre deux essences forestières sympatriques de l'Amérique du Nord (*Populus deltoides* Bartr. ex Marsh. et *Populus balsamifera* L.), en utilisant des marqueurs de polymorphisme à simple nucléotide (SNP), spécifiques à l'espèce, à la fois dans les génomes nucléique et chloroplastique. Tous les individus hybrides, identifiés sur la base des caractères morphologiques, possèdent des allèles nucléiques correspondant aux deux parents, alors que les génotypes chloroplastiques montrent une similarité avec le *P. deltoides*, ce qui indique une hybridation asymétrique où le *P. deltoides* agit comme parent femelle et le *P. balsamifera* comme parent paternel. On pourrait attribuer cette hybridation asymétrique observée, à des interaction cytonucléiques.

Mots-clés : *Populus*, hybridation naturelle, asymétrie, marqueurs SNP.

Introduction

Natural hybridization among species influences the evolution of biological diversity in a variety of ways including an increase in intraspecific genetic diversity (Anderson 1948), altering genetic adaptations (Anderson 1948; Stebbins 1950), the origin of new ecotypes or species (Grant 1981; Whitham et al. 1994), and the reinforcement or breakdown

of reproductive barriers (Ellstrand and Elam 1993; Rieseberg and Gerber 1995; Levin et al. 1996). Introgression, or the transfer of genetic material from one species to another, has been documented in a wide variety of plant and animal taxa (Arnold 1992; Rieseberg and Wendel 1993) and may serve as a source of genetic variation for adaptive evolution (Lewontin and Birch 1966; Grant and Grant 1994, 1996; Wang et al. 1997). Hybridization between species has traditionally been examined for its role in finalizing the speciation process through reinforcement of reproductive barriers (Dobzhansky 1940; Mayr 1963; Howard 1993). The evolution of barriers to interspecies gene flow plays significant roles in the process of speciation. Therefore, understanding the patterns of reproductive isolation between species is crucial for gaining insights into the mechanisms that control interspecific gene exchange or natural hybridization.

Interspecies hybridization is one of the common features among many members of the genus *Populus* L., a group of widely distributed forest trees in the northern hemisphere

Received 9 May 2007. Published on the NRC Research Press Web site at canjbot.nrc.ca on 8 January 2008.

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¹This note is one of a selection of papers published in the Special Issue on Poplar Research in Canada.

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(Barnes 1961; Brayshaw 1965; Eckenwalder 1982, 1996; Barnes and Pregitzer 1985; Stettler et al. 1996; Whitham et al. 1996). In North America, several species of the genus *Populus*, particularly species of the sections *Tacamahaca* Spach. and *Aigeiros* Duby, are broadly sympatric (Eckenwalder 1984a, 1984b), and known to hybridize extensively (Brayshaw 1965; Ronald et al. 1973a, 1973b; Eckenwalder 1984a, 1984b; Rood et al. 1986; Greenaway et al. 1991; Floate 2004).

Through multivariate analysis of leaf morphology of *Populus* species, Rood et al. (1986) showed continuous variation of characters and suggested the hybridization between *Populus deltoides* Bartr. ex Marsh., *Populus balsamifera* L., and *Populus angustifolia* James. These observations were further confirmed with gas chromatography – mass spectrometry data (Greenaway et al. 1991). Comprehensive analysis of leaf morphological characters of *P. balsamifera*, *P. angustifolia*, and *P. deltoides* in the hybrid zones in the riparian forests in southern Alberta suggested bidirectional hybridization between *P. angustifolia* and *P. balsamifera*, and unidirectional introgression between *P. balsamifera* and *P. deltoides* (Floate 2004). Molecular data has revealed a similar unidirectional introgression between *P. fremontii* and *P. angustifolia* in the Weber River drainage system in northern Utah (Martinsen et al. 2001). Although the hybrid nature and directionality of the hybridization has been successfully determined using morphological data, the maternal and paternal parental source of these natural hybrids remains unknown.

In this study, we used both nuclear- and chloroplast-based single nucleotide polymorphisms (SNPs), specific for *P. deltoides* and *P. balsamifera*, to test the hybrid status of morphologically identified hybrids, and determine the maternal and paternal parental species of the F₁ hybrids between these two species (known as *Populus* × *jackii* Sarg.) collected from natural stands, to infer the parentage of hybrids in natural populations.

Materials and methods

Plant material and DNA extraction

Fresh leaf samples were collected from morphologically distinct *P. deltoides*, *P. balsamifera*, first generation progeny (F₁) of controlled crosses between *P. deltoides* and *P. balsamifera*, and *P. ×jackii* occurring in natural stands (see supplementary data³, Tables S1 and S2). These hybrids are usually recognised by their broadly ovate leaves with heart-shaped bases (see supplementary data³, Fig. S1). In this study, we used natural hybrids that can be morphologically distinguished from the parental species. Thus, complex backcross hybrids that are morphologically indistinguishable from the parental species may not have been sampled for the present study. However, the exclusion of such hybrids in our samples was not expected to affect the interpretation of our results. The collected leaf samples were stored at –80 °C prior to extraction of DNA. The total genomic DNA was ex-

tracted from frozen leaf tissue using the methods of Doyle and Doyle (1987) and Dayanandan et al. (1997).

Selection and validation of SNPs

Molecular markers represent a powerful tool for identifying hybrid taxa (Rieseberg and Wendel 1993). Species-specific molecular markers derived from both biparentally inherited nuclear and uniparentally inherited organelles such as the chloroplast, which is maternally inherited in *Populus* (Rajora and Dancik 1995), are invaluable for detecting the parentage of putative hybrids. In particular, SNPs or DNA sequence variations that occur at a specific site in the genome (Cho et al. 1999; Griffin and Smith 2000) are useful for detecting the hybrids. Based on nucleotide sequence matrices of two noncoding regions of the chloroplast DNA (intron of *trnL* and intergenic region of *trnT-trnL*) and *ITS2* of the nuclear rDNA from a previous study of *Populus* (Hamzeh and Dayanandan 2004), four chloroplast and two nuclear SNPs specific to *P. balsamifera* and *P. deltoides* were identified (Table 1). The nrSNP1 and nrSNP2 were located within the *ITS2* region of the nuclear rDNA. The cpSNP1 was located within the intergenic region of *trnT-trnL*, and cpSNP2, cpSNP3, and cpSNP4 were located in the *trnL* intron of the chloroplast genome. The selected nuclear and chloroplast SNPs were further characterized for use as reliable molecular markers by genotyping 30 individuals of *P. deltoides* and 10 individuals of *P. balsamifera*. The inheritance pattern of SNPs was determined by genotyping 20 individuals selected from progenies of controlled crosses between *P. deltoides* and *P. balsamifera* and their parents.

Template preparation

Target genomic regions containing SNPs were amplified by the polymerase chain reaction (PCR). The oligonucleotide primers a, c, and d (Taberlet et al. 1991) and *trnCR* (Hamzeh and Dayanandan 2004) for the chloroplast genome and primers *ITS3* (Becerra and Venable 1999) and *ITS 28kj* modified from Culling (1992) for the *ITS2* region were used for PCR amplification. Amplification reactions contained 230 µmol/L dNTP, 2.5 mmol/L MgCl₂, 0.2 µmol/L of each primer, 1 unit of *Taq* DNA polymerase, and 2.5 µL buffer (0.2 mol/L Tris PH. 9.5; 0.25 mol/L KCl; 1 mg/mL BSA, 5 µL/mL Tween 20) in a total volume of 25 µL. PCR amplification was performed in a Mastercycler gradient thermal cycler (Eppendorf, Westbury, N.Y.) at 94 °C for 60 s, 55 °C for 30 s, and 72 °C for 60 s for 35 cycles. The residual primers and dNTPs were dephosphorylated using 0.5 unit of shrimp alkaline phosphatase (SAP, Fermentas, Burlington, Ont.) and 20 units of Exonuclease I from *E. coli* (Fermentas, Burlington, Ont.) at 37 °C for 2 h. Enzymes were inactivated at 80 °C for 20 min.

Single nucleotide primer extension reaction and genotyping

For each candidate SNP, an unlabeled oligonucleotide primer with its 3' end adjacent to the SNP (Table 1) was

³Supplementary data for this article are available on the journal Web site (<http://cjb.nrc.ca>) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5232. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Table 1. The single nucleotide polymorphism (SNP) locus, genotypes in *P. deltoides*, *P. balsamifera*, and *P. ×jackii*, and genotyping primer sequences of nuclear and chloroplast SNPs used in the present study.

SNP locus	Genotype			Primer name and sequence
	<i>P. deltoides</i>	<i>P. balsamifera</i>	<i>P. ×jackii</i>	
nrSNP1	GG	AA	GA	SNPBLIT2R: 5'-CGTGAGCCCGAGGGGAG-3'
nrSNP2	TT	CC	TC	SNPBLIT3R: 5'-GATGCCCGAGGGTCC-3'
cpSNP1	A	T	A	PBLIGIF: 5'-ACGTGAAAATGAAATATAGAAAAATGAATTGAATATA-3'
cpSNP2	G	T	G	SNPDLTNIIF: 5'-TAAAGGAATCCTTCTGTAAAGT-3'
cpSNP3	C	T	C	SNPBLTNIIF: 5'-ACCTATAAACATAATACATAGGAAA-3'
cpSNP4	A	T	A	PBLTN2R: 5'-ACGTACGTACGTACGTACGATCAAAAGCAATTTT-3'

Note: The nrSNP1 and nrSNP2 are located within the ITS2 region of the nuclear rDNA. The cpSNP1 is located within the intergenic region of *trnT-trnL*, and cpSNP2, cpSNP3, and cpSNP4 are located in the *trnL* intron of the chloroplast genome.

synthesized (Operon Biotechnologies, Huntsville, Ala.). A single nucleotide primer extension reaction was performed using an ABI SnapShot™ Multiplex Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and 0.2 μmol/L of genotyping primer. The thermal cycling was carried out on an Eppendorf Mastercycler gradient thermal cycler at 95 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s for 40 cycles. Unincorporated ddNTP was removed from the SnapShot™ PCR product by adding 1 unit of shrimp alkaline phosphatase (SAP) and incubating at 37 °C for 1 h followed by 20 min at 80 °C to inactivate the enzyme. Purified extension products were genotyped using an ABI 310 automated genetic analyzer (Applied Biosystems, Foster City, Calif.) and data were processed and analysed using GeneScan software (Applied Biosystems, Foster City, Calif.). Expected SNP genotypes for *P. deltoides* and *P. balsamifera* and sequences of SNP genotyping primers are listed in Table 1.

Results

Validation of SNPs

The genotypes of all *P. deltoides* and *P. balsamifera* individuals were consistent with the expected genotypes for all nuclear and chloroplast SNPs tested in this study, as listed in Table 1, confirming the reliability and sensitivity of the SNPs for the present study. The nuclear and chloroplast SNP genotypes of the parental individuals used in the controlled crossing experiment were also consistent with the genotypes of the *P. deltoides* and *P. balsamifera* individuals tested in this study. The nuclear SNP genotypes of all *P. ×jackii* from controlled crosses showed SNP alleles attributable to *P. deltoides* and *P. balsamifera* proving the Mendelian inheritance pattern of these SNPs. The chloroplast genotypes of all control-crossed *P. ×jackii* were consistent with the chloroplast genotype of *P. deltoides* and this confirmed the maternal inheritance of the chloroplast genome in *Populus*.

Genotype of natural *P. ×jackii*

The nuclear and chloroplast genotypes of all 23 natural *P. ×jackii* individuals tested were consistent with the genotypes of control-crossed *P. ×jackii*, confirming that these were hybrids of *P. deltoides* and *P. balsamifera*. The morphology (broadly ovate leaves with heart shaped bases; see supplementary data³, Fig. S1) and molecular identification of natural hybrids were congruent, suggesting that the natural hybrid *P. ×jackii* could be reliably identified using morphological features. All putative natural hybrid individuals tested had nuclear alleles representing both *P. deltoides* and *P. balsamifera* as parental taxa, and the chloroplast genotype corresponded to that of *P. deltoides*, suggesting an asymmetrical pattern of hybridization in the natural hybrids. Thus, our results clearly show that *P. deltoides* and *P. balsamifera* serve as ovule and pollen donors, respectively, in the *P. ×jackii* hybrid complex.

Discussion

All species of the genus *Populus* are dioecious and therefore cross-pollination through wind-dispersed pollen is needed for successful fruit set in *P. balsamifera* and *P. deltoides* (Dickmann and Stuart 1983). These two species

are somewhat reproductively isolated owing to different flowering times. Flowering of *P. balsamifera* generally occurs April–May and *P. deltoides* flowers from late February to April in the southern distribution range of the species in the United States (Schreiner 1974). In the Oldman river region of southern Alberta, both of these species and their hybrids are known to flower during May (Gom and Rood 1999). Flowering and pollen release may vary by as much as a month among trees in a stand (Farmer 1966; Gom and Rood 1999) and seasonal climatic fluctuations may shift the timing of flowering, leading to phenological overlap between these two species. Thus, some individuals of these two species can hybridize under natural conditions (Brayshaw 1965; Eckenwalder 1984b; Floate 2004).

The observed pattern of natural hybridization between *P. deltoides* and *P. balsamifera*, suggests an asymmetry in the reproductive isolation of these two species. The asymmetric reproductive barriers in general could be attributable to pre- and post-zygotic barriers. Various prezygotic mechanisms that may cause asymmetry in hybridization success include differences in style length (Kiang and Hamrick 1978; Sorensson and Brewbaker 1994), differential fruit abortion (Levin 1978; Howard et al. 1998), and self-incompatibility systems (Lewis and Crowe 1958). Controlled crosses involving female *P. deltoides* and male *P. balsamifera* showed a high success rate and produced viable seeds, whereas reciprocal crosses involving female *P. balsamifera* and male *P. deltoides* produced only a few seeds and the viability of these seeds was very low. For instance, in a crossing experiment performed in 1998, no seed set was observed in all 20 crossings between female *P. balsamifera* and male *P. deltoides*, whereas 5 out of 16 crosses between female *P. deltoides* and male *P. balsamifera* produced viable progenies. In another crossing experiment performed in 2004, only 2 out of 19 crosses between female *P. balsamifera* and male *P. deltoides* produced seeds, giving rise to only one seedling. Conversely, 4 out of 30 crosses between female *P. deltoides* and male *P. balsamifera* produced seeds and gave rise to over 600 seedlings. Similarly, the controlled crosses between female *P. trichocarpa*, and male *P. deltoides* yielded seeds with low viability, commonly associated with premature dehiscence of the capsule. Seedlings of these crosses are routinely produced through embryo rescue techniques (Dickmann 2001; Riemenschneider et al. 2001) suggesting a postzygotic incompatibility between these two species. *Populus trichocarpa* is evolutionarily closely related to *P. balsamifera* (Hamzeh and Dayanandan 2004) and, therefore, it is logical to assume that the asymmetric reproductive isolation observed in the *P. deltoides* and *P. balsamifera* complex may be mediated through postzygotic mechanisms. The evidence for asymmetric-cross incompatibility has also been found in a *P. angustifolia* and *Populus fremontii* S. Wats. hybrid complex in Utah. Restriction fragment length polymorphisms (RFLP) analysis in this hybrid complex showed that the hybrid population consisted of either F₁ hybrids or backcrosses to *P. angustifolia* (Keim et al. 1989). No trees attributable to crosses between F₁, or between F₁ and *P. fremontii* were found. The controlled backcrossing of F₁ hybrids to *P. fremontii* resulted in early death of seedlings,

suggesting a postzygotic developmental incongruity (Hogendoorn 1984; Keim et al. 1989).

We speculate that the observed asymmetries in hybridization between *P. deltoides* and *P. balsamifera* may be due to cytonuclear incompatibility resulting from divergent evolution of nuclear and cytoplasmic genes coding for proteins interacting in photosynthesis or respiration that affect the viability of hybrids (Michaelis 1954; Levin 1978; Wu et al. 1999). Alternatively, the cytonuclear incompatibility may result from the activity of transposable elements, which are abundant in many plant genomes (Tiffin et al. 2001). The activity of some transposable elements can be suppressed by maternally inherited factors (Engels 1989). Therefore, hybrid progeny with a cytoplasmic background that has not evolved with a transposable element may experience greater transposon activity leading to cytonuclear genetic incompatibility and reduced hybrid fitness or viability. Further studies focused on cyto-nuclear interactions in the *P. ×jackii* hybrid complex would be valuable to gain insights into mechanisms associated with asymmetric gene flow in forest trees.

In theoretical models of reproductive barrier reinforcement, the reciprocal crosses between lineages are assumed to be equally compatible (Felsenstein 1981; Liou and Price 1994; Kelly and Noor 1996). However, the asymmetries in gene flow between partially isolated taxa may greatly reduce the probability of evolution of reproductive barriers (Servedio and Kirkpatrick 1997) and are more likely to introgress. Under these circumstances introgression is likely to be directional, with genes moving mainly from one species to another, and to affect the phenotypic and genotypic variation in natural populations (Stebbins 1959). The proportion of parental genomes in hybrid species may serve as an indicator for assessing the directionality of introgression (Martinsen et al. 2001) and cytoplasmic and nuclear interactions in the hybrid zone (Rieseberg 1995).

In summary, our findings confirm the presence of asymmetric reproductive isolation in two interfertile plant species under natural conditions. Our data, based on both the biparentally inherited nuclear and the maternally inherited chloroplast genome, provide direct evidence for the long suspected presence of asymmetries in interspecific crossing barriers in *Populus* under natural conditions.

Acknowledgments

The authors thank Barbara Thomas of Alberta-Pacific Forest Industries (Alpac) and the University of Alberta, Alan Robertson (Alpac), David Kamelchuck (Alpac), Damase Khasa (Université Laval), Serge Morin (Ministère des Ressources naturelles et de la Faune, Québec) and François Caron (MRNFQ) for their help in collecting leaf samples; Chen Rae Hsu (Concordia University) for technical assistance; and Kevin Floate (Agriculture and Agri-Food Canada), Stewart Rood (University of Lethbridge), Paul Widden (Concordia University), and an anonymous reviewer for helpful comments that improved the manuscript. This research was supported by grants from Concordia University, Natural Sciences and Engineering Research Council of Canada, Le Fonds québécois de la recherche sur la nature et les technologies, and Canada Foundation for Innovation.

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