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Genetic analysis of post-mating reproductive barriers in hybridizing European *Populus* species

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Molecular genetic analyses of experimental crosses provide important information on the strength and nature of post-mating barriers to gene exchange between divergent populations, which are topics of great interest to evolutionary geneticists and breeders. Although not a trivial task in long-lived organisms such as trees, experimental interspecific recombinants can sometimes be created through controlled crosses involving natural F_1's. Here, we used this approach to understand the genetics of post-mating isolation and barriers to introgression in *Populus alba* and *Populus tremula*, two ecologically divergent, hybridizing forest trees. We studied 86 interspecific backcross (BC_1) progeny and >350 individuals from natural populations of these species for up to 98 nuclear genetic markers, including microsatellites, indels and single nucleotide polymorphisms, and inferred the origin of the cytoplasm of the cross with plastid DNA. Genetic analysis of the BC_1 revealed extensive segregation distortions on six chromosomes, and $>90\%$ of these (12 out of 13) favored *P. tremula* donor alleles in the heterospecific genomic background. Since selection was documented during early diploid stages of the progeny, this surprising result was attributed to epistasis, cyto-nuclear coadaptation, heterozygote advantage at nuclear loci experiencing introgression or a combination of these. Our results indicate that gene flow across 'porous' species barriers affects these poplars and aspens beyond neutral, Mendelian expectations and suggests the mechanisms responsible. Contrary to expectations, the *Populus* sex determination region is not protected from introgression. Understanding the population dynamics of the *Populus* sex determination region will require tests based on natural interspecific hybrid zones.

Keywords: speciation; hybrid zone; *Populus*; introgression; segregation distortion; sex chromosome

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Introduction

The genetics of reproductive barriers is of great current interest in evolutionary genetics, because the extent of gene flow experienced by diverging populations or species depends crucially on it (Coyne and Orr, 2004). Reproductive isolation (RI) is a key feature of Mayr's Biological Species Concept, which includes the notion of whole genome isolation between divergent taxa as a hallmark of 'good' species. More recently, a 'genic view' of species and speciation has found wide acceptance, which recognizes that genomes can be porous and that RI is a property of individual loci or genomic regions, rather than the genome as a whole (Wu, 2001; Lexer and Widmer, 2008).

RI will first arise in genomic regions harboring 'speciation genes' or other isolation factors, leading to genomic islands of divergence (Wu, 2001; Emelianov *et al*., 2004; Nosil *et al*., 2009), and will subsequently spread across the genome. Nevertheless, many groups of taxa do not achieve complete genomic isolation for millions of years, as observed in *Helianthus* (sunflowers), *Populus* (poplars, aspen, cottonwoods), *Quercus* (oaks), *Silene* (campions) or *Iris* (lilies), to name just a few examples among plants (reviewed by Lexer and Widmer, 2008). Despite the shift in perception from whole genome isolation to a genic view of species, the genetics of RI is still at the center of attention in speciation genetics (Widmer *et al*., 2009). This is the case because the genetics of RI will determine how quickly gene flow ceases and which loci are affected first.

Plant speciation geneticists often use controlled crosses to study the genetics of post-mating components of RI (Fishman *et al*., 2001; Coyne and Orr, 2004; Bouck *et al*., 2005; Sweigart *et al*., 2006). Even in species with strong reproductive barriers, interspecific multi-generation crosses can sometimes be obtained in the laboratory. In such crosses, interspecific recombination in the meiosis of the F_1 will effectively start to break up the parental species' genomes, which allows geneticists to isolate and study chromosomal blocks with a role in moderating gene flow (Lexer and Widmer, 2008). One approach to achieve this goal is to search for loci or genomic regions with departures from Mendelian expectations, also known as segregation distortion (Fishman *et al*., 2001; Yin *et al*., 2004; Bouck *et al*., 2005; Sweigart *et al*., 2006;

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Lopez-Fernandez and Bolnick, 2007). As expected from theory (Barton, 2001; Wu, 2001; Turelli and Moyle, 2007), such studies tend to recover the full breadth of departures from Mendelian expectations, including genome regions that cause isolation and resist introgression (Fishman *et al*., 2001; Sweigart *et al*., 2006), and loci that cross the barrier more readily than expected based on Mendel's laws (Tiffin *et al*., 2001; Yin *et al*., 2004; Bouck *et al*., 2005).

Experimental cross-based approaches such as this (beyond the F_1) have rarely been used in forest trees because of the difficulty of creating recombinant hybrid generations in species with long generation times (but see Yin *et al*., 2004; Yin *et al*., 2008). The scarcity of experimental multi-generation crosses in forest trees is unfortunate, as long-lived trees provide the opportunity to dissect post-mating barriers in organisms with juvenile-adult phase change, that is potentially from gametes through early embryonic and juvenile stages to maturity. One way to circumvent the long time needed to generate experimental crosses in forest trees is to take advantage of natural hybrids (for example Woolbright *et al*., 2008), but this approach remains largely unexplored.

Populus alba and *Populus tremula* are two ecologically divergent European members of the 'model tree' genus *Populus*. Reproductive barriers between these species are incomplete, leading to the frequent formation of extensive 'mosaic' hybrid zones (Lexer *et al*., 2005, 2010). Ongoing genomic studies of natural populations indicate that the species boundary is porous, with some loci resisting introgression and others crossing the barrier more readily than predicted by genomic expectations (Lexer *et al*., 2007, 2010). Morphometric data indicate introgression of phenotypic traits from *P. tremula* into *P. alba* (Lexer *et al*., 2009), despite the presence of substantial RI involving both assortative mating and post-zygotic isolation in the form of epistatic interactions (Lexer *et al*., 2010). Studies of controlled interspecific progeny are useful for reducing the complexity of patterns of RI seen *in situ* in natural hybrid zones.

Since *P. alba* and *P. tremula* are dioecious and sex determination in *Populus* appears to be controlled by an incipient sex chromosome (Yin *et al*., 2008; Pakull *et al*., 2009; Paolucci *et al*., 2010), controlled crosses also provide the opportunity to study the role of the sex determination region in blocking interspecific gene flow, which is a topic of great current interest in evolutionary genetics (Qvarnström and Bailey, 2009). Our hypothesis at the outset of this study was that species isolation genes will have accumulated in the sex determination region of poplar, as recently observed for other taxa (Qvarnström and Bailey, 2009).

With this in mind, the questions of this study were as follows: (1) What do marker segregation data from a controlled interspecific backcross (BC_1) tell us about the strength and genetic architecture of post-mating reproductive barriers in these hybridizing forest trees? (2) How great is the potential for interspecific introgression across porous species boundaries in *Populus*? (3) What is the likely role of the *Populus* sex determination region in blocking or moderating interspecific gene flow? To address these questions, we analyzed and interpreted segregation patterns of alleles of known species origin for a genome-wide panel of molecular genetic markers, genotyped in a controlled interspecific BC_1 of *P. alba* and *P. tremula*, and compared the results to patterns of divergence and linkage disequilibrium (LD) in natural populations.

Materials and methods

Plant materials (BC_1 and natural populations)

To study post-mating reproductive barriers creating segregation distortions, we developed a controlled backcross of an F_1 natural poplar hybrid (*Populus tremula* × *alba*) with a pure *P. alba* (BC_1). The male parent of this cross was a known natural clone (J1) of *P. alba* from the Jalón river in the Ebro watershed (Northeast of the Iberian Peninsula), and the female parent an F_1 natural hybrid (BET3) from a hybrid population in the Tajo river headwaters (Central Iberian Peninsula). The F_1 hybrid status of BET3 was assessed through phenotypic features and confirmed prior to this study by genomic admixture analysis in STRUCTURE 2.2 following Lexer *et al*., 2005 (95% credible intervals of admixture coefficient *Q* did *not* include 0.25 or 0.75). The species origin of maternally inherited plastid DNA in BET3 was identified here by sequencing the *trnC-petN1* plastid DNA region in this interspecific F_1 hybrid and comparison with sequences from pure individuals of *P. alba* and *P. tremula*; plastid DNA haplotypes are known to be highly divergent between *P. alba* and *P. tremula* (Lexer *et al*., 2005; Fussi *et al*., 2010).

The controlled backcross was produced at INIA's nursery (Madrid, Spain), yielding 131 seedlings that were further grown under greenhouse conditions. Early mortality in first-year seedlings reduced the number of offspring available for DNA extraction and genotyping to 86 individuals, currently maintained in two clone banks planted in Central Spain.

Apart from the BC_1 used for mapping, up to 201 individuals from European populations of *P. tremula* and up to 167 individuals from European populations of *P. alba* were employed to determine the parental species origin of markers used in segregation analysis, and for additional population genetic analysis of markers located on chromosome XIX (below). Population genetic data for this purpose were taken from de Carvalho *et al*. (2010) and Lexer *et al*. (2010), where detailed documentation of populations and genotypic data can be found. Briefly, populations of *P. tremula* were from Spain, Scotland, Central Sweden and Austria (two populations, one from the Eastern Alps and one from the Bohemian Massif). Populations of *P. alba* were from Spain, the Austrian Danube and the Hungarian Tisza valley. In addition, 20 individuals of *P. alba* whose sex had been determined phenotypically during the flowering season were sampled in Spain (12 females and 8 males). Species assignment for each individual was achieved using STRUCTURE 2.2 as described above.

Molecular genetic markers and genotyping reactions

Genomic DNA was purified from young fresh leaves using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany). A genome-wide set of 98 nuclear markers was used for segregation analysis in the interspecific BC_1 (Supplementary Tables 1 and 4). These markers included microsatellite loci available from the *Populus* genome

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consortium (Van der Schoot *et al*., 2000; Smulders *et al*., 2001; Tuskan *et al*., 2006; Yin *et al*., 2009), microsatellites isolated *de novo* by our group from expressed sequence tags and from genomic sequence for contig 117 of *Populus trichocarpa* genome assembly v.1, homologous to chromosome XIX of *Populus* (Joseph and Lexer, 2008; de Carvalho *et al*., 2010), and single nucleotide polymorphisms as well as insertion–deletion (indel) markers isolated from expressed sequence tags representing candidate genes for traits involved in ecological divergence between *P. alba* and *P. tremula* (Joseph and Lexer, 2008). The *trnC-petN1* plastid DNA region was sequenced in the parents of the mapping cross using essentially the same protocols. All of the marker loci are reported and documented in detail elsewhere (Joseph and Lexer 2008; de Carvalho *et al*., 2010; Lexer *et al*., 2010), with the exception of a small number of new microsatellites from chromosome XIX, the incipient sex chromosome of *Populus*. Because of their special relevance to the objectives of this paper, all chromosome XIX loci are documented once more in detail in the supporting materials of the present paper (Supplementary Table 2).

Forward primers for all nuclear microsatellite markers (86 loci in total; Supplementary Table 1) were M13-tailed, and standard polymerase chain reaction protocols were used for DNA amplification following Lexer *et al*. (2005). Allele sizes were resolved using an Applied Biosystems (ABI) 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and FAM and JOE fluorescent dyes. Indel polymorphisms in five expressed sequence tags (Joseph and Lexer, 2008) were genotyped as length polymorphisms, using the same methods. For seven further expressed sequence tags (Supplementary Table 1), single nucleotide polymorphisms were identified by resequencing the parents of the BC_1 cross following Joseph and Lexer (2008), and the progeny was genotyped for each single nucleotide polymorphism using the ABI SNaPshot assay (Applied Biosystems) following the manufacturer's instructions.

Data analysis

Segregation analysis in the interspecific BC_1: Marker alleles segregating from the interspecific F_1 hybrid parent BET3 were analyzed in the interspecific BC_1 progeny. The focus of this study was on patterns of *single marker* segregation–linkage analysis was only used as a quality control (below). Both segregation and linkage analyses were carried out in MAPMANAGER QTX. This program uses χ^2 statistics to test for deviations of marker segregation from Mendelian expectations, equivalent to tests for *gametic* or allelic segregation distortion. In addition, tests for distortions of particular genotypic classes (= *zygotic* distortions) were carried out using χ^2 tests in JOINMAP 3.0.

Species origin of the hybrid parent (BET3) alleles: The parental species frequencies of alleles found in the F_1 hybrid parent (BET3) were estimated from natural populations of *P. alba* and *P. tremula* (see above—Plant materials). Estimating parental frequencies was possible despite the somewhat heterogenous geographic sampling of the parental species (above), because an overwhelming proportion of molecular variance in these European poplars and aspens resides *between* species, rather than between populations of the same species (Lexer *et al*., 2005; de Carvalho *et al*., 2010).

The odds-ratio tests were based on a contingency table constructed using the frequency of each allele in the two species, using proc FREQ in SAS version 9 (SAS Institute Inc, Cary, NC, USA). When the allele frequency was zero or one in any of the species, odds ratios were undefined. In that case, a Fisher's exact test was performed to test for significant differences in the contingency table. For a small number of loci for which the odds-ratio test was not significant, species origin was inferred based on linkage with markers with clearly assigned species origin (Supplementary Table 1; species origin for these markers is given in parentheses). Information about the species origin of each allele in the BET3 hybrid parent was used to determine the direction of segregation distortions in the interspecific BC_1, either toward *P. alba* or toward *P. tremula*.

Synteny with *P. trichocarpa*: Although the main objective of this paper was to study *single marker* segregation distortions, marker coverage on several chromosomes was suitable for defining linkage groups. This was the case because these markers had been picked to tag clusters of quantitative trait loci-controlling species differences in a related project on admixture mapping in *Populus* (Lexer *et al*., 2010). These markers provided a useful quality control for our segregation data, as they allowed us to assess levels of synteny of these linkage groups between our European *Populus* species and the sequenced genome of *P. trichocarpa*.

To examine synteny, linkage groups in the interspecific BC_1 were determined based on log-of-odds likelihood statistics calculated by MAPMANAGER QTX. Marker groupings detected at a log-of-odd threshold of 3.00 were ordered locally by a multi-point analysis using the 'Ripple' command, and map distances in centimorgans were estimated from recombination frequencies using the Kosambi mapping function. Synteny between marker order in the interspecific BC_1 and *P. trichocarpa* was evaluated using *P. trichocarpa* genome assembly v2 (available at http://www.phytozome.net/poplar). Physical positions of all markers in *P. trichocarpa* were determined using blast-n searches of the primer sequences against *P. trichocarpa* genome assembly v2, using an *E*-value threshold of 0.1.

Analysis of chromosome XIX in natural populations: Markers located on the putative sex chromosome XIX were of special interest to this study, because sex chromosomes are often seen as 'hotspots' for species isolation genes. Analyzing natural populations places greater demands on the ease of scoring and robustness of molecular markers than segregation analysis in a simple, controlled pedigree; eight out of nine microsatellites from chromosome XIX (Supplementary Table 2) yielded readily interpretable, fully codominant marker genotypes in the 20 individuals of known sex sampled from natural populations of *P. alba* in Spain (Table 2), and six loci did so across European populations of *both* species, *P. alba* and *P. tremula* (3; Supplementary Table 3).

The loci were characterized via the number of alleles (A), expected (H_E) and observed (H_O) heterozygosity and within-population inbreeding coefficients (F_{IS}) in populations using the computer program FSTAT (Goudet,

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1995). LD between pairs of loci was estimated using the common marker correlation and exact *P*-values for LD were computed with GENEPOP (Rousset, 2008). To keep the number of pair wise LD tests manageable, these analyses were restricted to four populations of particular interest to ongoing evolutionary genetics research in these species: Danube (Austria) and Tisza (Hungary) for *P. alba*, and Eastern Alps (Austria) and Central Sweden for *P. tremula*. In addition, the 20 Spanish individuals of known sex were characterized for their diversity and heterozygosity (H_E and H_O).

To yield insights into the role of the putative sex chromosome XIX in blocking gene flow between *P. alba* and *P. tremula*, interspecific genetic divergence for chromosome XIX markers was estimated in the form of F_{ST} and the results confirmed by G'_{ST}, a standardized differentiation measure that takes within-population heterozygosity into account (Hedrick, 2005). To relate interspecific divergence on chromosome XIX to the genome-wide average, F_{ST} values for chromosome XIX were compared with genome-wide expectations for 93 microsatellite- and sequence-based genetic markers reported by Lexer *et al*. (2010).

Results

Marker polymorphism and species origin of donor alleles

Ninety-eight (98) genetic markers (microsatellites, indels and single nucleotide polymorphisms) representing all 19 chromosomes of the *Populus* genome were analyzed in the interspecific BC_1 of *P. alba* and *P. tremula* (Supplementary Tables 1 and 4). Out of these, 39 (40%) were polymorphic in the female F_1 hybrid parent (BET3) only, 11 (11%) were polymorphic in the male *P. alba* backcross parent (J1) only and 26 (27%) were polymorphic in both parents, whereas 22 (22%) were monomorphic in both parents of the cross (Supplementary Table 1). The odds-ratio test and Fisher's exact test facilitated statistical assignment of species origin of alleles segregating from the female F_1 hybrid parent (BET3) for 47 of the markers. For 13 further loci, putative species origin of alleles segregating from the F_1 hybrid parent could be assigned based on linkage to markers with clear species assignments (Supplementary Table 1). With respect to organellar DNA, plastid DNA sequencing revealed a *P. tremula* haplotype for BET3, thus indicating the species origin of the cytoplasm of the interspecific BC_1 cross.

Segregation distortions

Thirteen markers on six chromosomes (20% of polymorphic markers) displayed significant segregation distortion of alleles segregating from the BET3 hybrid parent of the BC_1 (Table 1), compared with three markers expected by chance alone. All of these loci displayed genotypic (=zygotic) segregation distortion as well. For 12 of these loci, the *P. tremula* (=donor) allele was significantly overrepresented in the backcross progeny. Only for 1 of the 13 distorted markers, segregation distortion was against the introgressed *P. tremula* allele (that is the *P. alba* allele segregating from the F_1 hybrid parent was overrepresented instead; Table 1). The results allowed us to discuss the strength of post-mating reproductive barriers between these hybridizing species (see below).

Synteny with *P. trichocarpa*

All detected linkages were conserved between *P. trichocarpa* genome assembly v.2 and the present interspecific BC_1 of *P. alba* and *P. tremula* (Supplementary Table 1). Synteny is best exemplified by chromosome VI, known to exhibit normal levels of recombination (Yin *et al*., 2004), and chromosome XIX, known to exhibit suppressed recombination (Yin *et al*., 2008) (Figure 1). Marker order was completely conserved on chromosome VI, whereas no recombination event was observed between four markers on chromosome XIX in the interspecific BC_1, corresponding to >560 kb on the *P. trichocarpa* physical genome map (Figure 1; see also below).

Segregation distortion and diversity of chromosome XIX

Three loci on the proximal end of chromosome XIX, the incipient sex chromosome of *Populus*, displayed segregation distortion in the female F_1 hybrid parent (BET3) in the form of an overrepresentation of donor alleles from

Table 1 Genetic markers with segregation distortion in an interspecific BC_1 between *P. tremula* and *P. alba*, including chromosome assignment on *P. trichocarpa* genome assembly v.2, significance levels of segregation distortions in the BC_1, identity of the over-represented allele for each locus, odds ratios for parental species assignments of alleles in natural populations and inferred species assignment of the overrepresented allele

Locus	*P. trichocarpa chromosome*	*Distortion P. alba × tremula F1 parent (♀)*	*Overrepresented allele*	*Odds ratio P. alba/ tremula F1 (♀) allele 1*	*Odds ratio P. alba/ tremula F1 (♀) allele 2*	*Species origin of overrepresented allele from F1 (♀)*
GCPM 1274	1	****	2	1.55/0.45	0.93/1.32	(*P. tremula*)
ASP 112376	1	*	1	3.18/0.05	0.00/3.34	*P. alba*
GCPM 124	1	**	2	42.20/0.07	0.11/2.90	*P. tremula*
GCPM 1629	3	*****	1	0.62/5.02	1.24/0.00	*P. tremula*
Thau	9	**	2	2.44/0.00	NA	(*P. tremula*)
ORPM 23	9	*****	1	0.00/3.49	1.61/0.00	*P. tremula*
ORNL 149	10	*	1	0.00/3.8	14.00/0.00	*P. tremula*
ORPM 344	10	****	1	0.00/3.73	2.27/0.25	*P. tremula*
GCPM 1250	10	****	2	4.03/0.04	0.25/23.05	*P. tremula*
GCPM 154	12	*****	1	0.00/2.79	NA	(*P. tremula*)
Yin1	19	**	1	NA	1.28/0.73	*P. tremula*
Yin2	19	*	2	2.64/0.00	0.00/1.90	*P. tremula*
ORPM 206	19	***	1	0.00/4.06	10.27/0.00	*P. tremula*

Abbreviations: BC, backcross; NA, not applicable.
Species assignments supported by the genotype data, but not significant in the odds-ratio test are shown in parentheses.
Significance thresholds from χ^2 tests.
*$P<0.05$, **$P<0.01$, ***$P<0.005$, ****$P<0.001$, *****$P<0.00005$.

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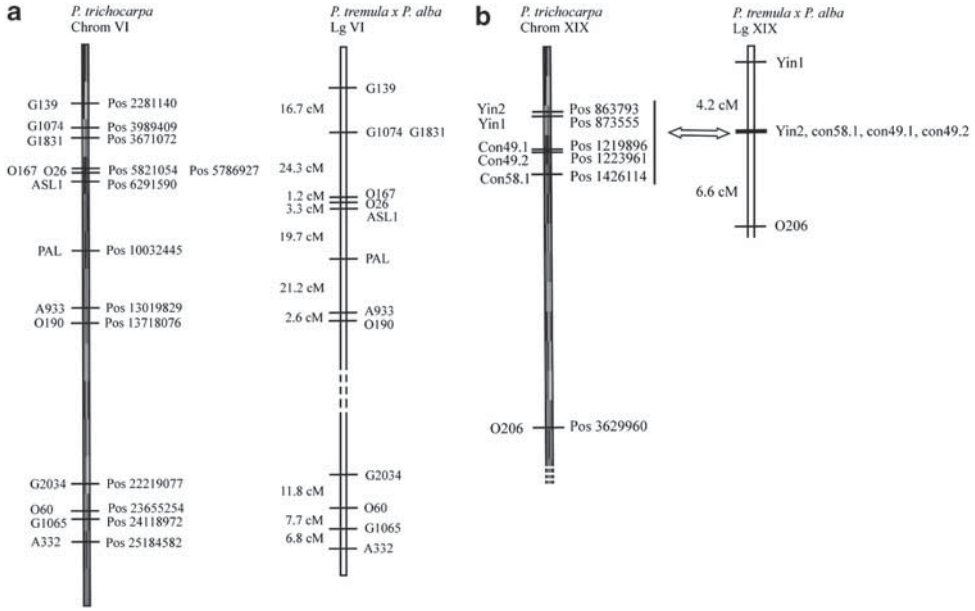

Figure 1 Comparison of the *P. tremula* × *P. alba* linkage map to *P. trichocarpa* genome assembly v.2 for chromosome VI (**a**), known to exhibit normal levels of recombination (Yin *et al*., 2004), and chromosome XIX (**b**), known to exhibit greatly reduced recombination (Yin *et al*., 2008). Complete synteny between the two maps is indicated by the conserved marker order on chromosome VI. On chromosome XIX, zero recombination was observed between markers Yin2, con58.1, con49.1 and con49.2 on the *P. tremula* × *P. alba* linkage map (indicated by the arrow), which corresponds to >560 kb on the *P. trichocarpa* genome assembly.

Table 2 Chromosome XIX diversity statistics for individuals of *P. alba* with known sex, including expected (H_E) and observed (H_O) heterozygosity and inbreeding coefficients (F_{IS}) in each group and the female/male ratio of H_O

Locus	*Females*				*Males*				H_O *ratio* ♀/♂
	A	H_E	H_O	F_{IS}	*A*	H_E	H_O	F_{IS}	
Yin2	4	0.645	0.273	0.589	4	0.692	0.625	0.103	0.437
Yin1	3	0.301	0.333	−0.114	3	0.492	0.625	−0.296	0.533
Con03.1	3	0.610	0.909	−0.527	3	0.689	1.000	−0.539	0.909
Con49.1	5	0.667	0.500	0.258	4	0.592	0.625	−0.061	0.800
Con49.2	5	0.825	0.500	0.411	5	0.842	0.375	0.571[a]	1.333
Con58.1	9	0.892	1.000	−0.128	9	0.858	0.750	0.134	1.333
O206	2	0.290	0.333	−0.158	2	0.325	0.375	−0.167	0.888
O276	5	0.656	0.750	−0.151	5	0.700	0.625	0.114	1.200

[a]Significantly different from zero at the 0.05 level.

P. tremula (Table 1). A survey of trees with known sex indicated autosomal behavior of this chromosome: there were no consistent departures from random mating (measured via F_{IS}) in known females and males and no consistent differences in heterozygosity between known females and males (Table 2). LD on chromosome XIX extended over >560 kb in natural populations of *P. alba* (Table 3), the species with the smaller effective population size, N_e (Lexer *et al*., 2005). The markers in LD corresponded to those loci with zero recombination in the interspecific BC_1 (see Figure 1 and above). LD was also observed in the Swedish population of *P. tremula*, whereas no LD was detectable in *P. tremula* from the Eastern Alps (Table 3).

Congruence between BC_1 segregation patterns and genomic divergence in natural populations

The increased introgression of *P. tremula* (=donor) alleles on chromosome XIX in the interspecific BC_1 (Figure 2a) was mirrored by reduced interspecific divergence in natural populations when measured as F_{ST} (Figure 2b); the median of interspecific F_{ST} was far below the genome-wide expectation of 0.369 reported by Lexer *et al*. (2010). For comparison, no such reduction in interspecific divergence was seen on chromosome VI, consistent with normal segregation on this chromosome (Figure 2). A congruent pattern among chromosomes XIX and VI was recovered when Hedrick's (2005) G'_{ST} was used as a measure of divergence ($G'_{ST} = 0.556 \pm 0.124$ on chromosome XIX vs 0.898 ± 0.049 on chromosome VI, respectively). The results allowed us to compare introgression patterns seen in the BC_1 and patterns of interspecific divergence observed in natural populations.

Discussion

Segregation distortions favor rather than impede introgression in a controlled cross of *P. tremula* and *P. alba*

Segregation distortions of genetic markers in interspecific crosses contain a wealth of information on the strength and genomic architecture of post-mating isolation between species (Fishman *et al*., 2001; Yin *et al*., 2004; Bouck *et al*., 2005; Sweigart *et al*., 2006; Lopez-Fernandez and Bolnick, 2007; Turelli and Moyle, 2007). This can point evolutionary biologists to genetic loci involved in speciation and to possible causes for asymmetries in reproductive barriers (Coyne and Orr, 2004; Turelli and Moyle, 2007) and breeders to genome regions that will resist introgression in marker-assisted breeding programs. Sometimes, however, the direction of segregation distortions in interspecific crosses also suggests the presence of mechanisms that *favor* rather than impede gene exchange (for example Tiffin *et al*., 2001; Yin *et al*.,

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Table 3 Linkage disequilibrium (LD) among markers on chromosome XIX in natural populations of *P. alba* and *P. tremula*

Locus[a]	*Yin2*	*Yin1*	*Con49.2*	*Con58.1*	*O206*
(a) P. alba/Austrian Danube valley					
Yin2 (863 793)	—	**0.331***	**0.154***	**0.208***	0.658
Yin1 (873 555)	**0.000***	—	**0.006**	**0.016**	0.532
Con49.2 (1 296 123)	**0.000***	**0.143**	—	0.278	0.587
Con58.1 (1 426 114)	**0.000***	**0.225**	0.148	—	0.363
O206 (3 629 960)	0.107	0.115	0.122	0.478	—
(b) P. alba/Hungarian Tisza valley					
Yin2 (863 793)	—	0.153	**0.168**	0.185	NC
Yin1 (873 555)	0.487	—	0.152	**0.032**	NC
Con49.2 (1 296 123)	**0.008**	0.134	—	**0.008**	NC
Con58.1 (1 426 114)	0.441	**0.160**	**0.165**	—	NC
O206 (3 629 960)	NC	NC	NC	NC	NC
(c) P. tremula/Eastern Alps					
Yin2 (863 793)	—	0.156	0.123	0.118	0.980
Yin1 (873 555)	0.593	—	1.000	0.936	0.510
Con49.2 (1 296 123)	1.000	0.121	—	0.271	0.903
Con58.1 (1 426 114)	0.346	0.118	0.165	—	0.677
O206 (3 629 960)	0.084	0.118	0.113	0.124	—
(d) P. tremula/Sweden					
Yin2 (863 793)	—	0.197	**0.186**	0.192	0.482
Yin1 (873 555)	0.284	—	0.111	0.111	0.784
Con49.2 (1 296 123)	**0.034**	0.166	—	**0.010**	**0.007**
Con58.1 (1 426 114)	0.323	0.188	**0.154**	—	0.723
O206 (3 629 960)	0.101	0.149	**0.142**	0.148	—

Abbreviation: NC, not calculated.
Marker correlations are shown above the diagonal and exact *P*-values are shown below the diagonal. LD extending up to 562 kb (assuming *P. trichocarpa* map distances) is detectable in populations of *P. alba* (a and b), the species with the smaller effective population size (N_e), whereas LD is less readily detectable in populations of *P. tremula* (c and d).
Significant tests at the 0.05 level are indicated by bold type, and significant tests after Bonferroni correction are indicated by an asterisk.
[a]The physical location of each marker in *P. trichocarpa* genome assembly v.2 is shown in parentheses.

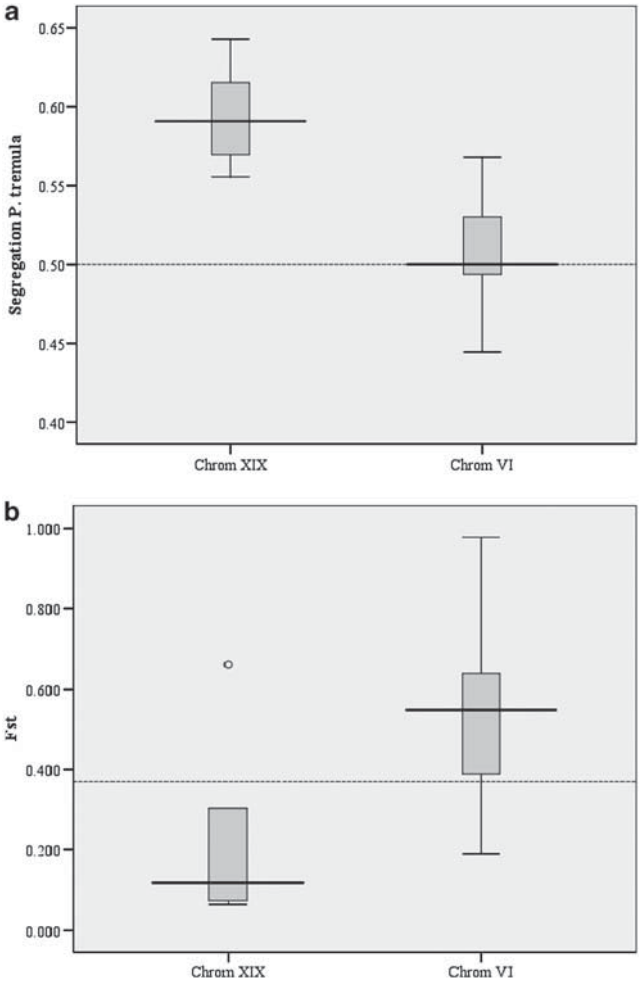

Figure 2 Box plots showing introgression and divergence of genetic markers on chromosomes VI and XIX relative to genome-wide expectations. **(a)** Segregation of *P. tremula* alleles in the interspecific BC_1 (dotted line, Mendelian expectation of 0.5). **(b)** Interspecific divergence (F_{ST}) in natural populations (dotted line, genome-wide expectation of 0.369; Lexer *et al*., 2010). Increased introgression of *P. tremula* alleles in the controlled interspecific BC_1 (**a**) and reduced interspecific divergence in natural populations (**b**) are visible for chromosome XIX.

2004; Bouck *et al*., 2005), a pattern reminiscent of adaptive introgression. These cases are of great interest to our understanding of the genetics of porous species boundaries (Barton, 2001; Tiffin *et al*., 2001; Wu, 2001; Lexer and Widmer, 2008; Widmer *et al*., 2009), but understanding the causes of these patterns is not a trivial task.

Here, we uncovered extensive segregation distortions (20% of polymorphic loci) in a controlled interspecific BC_1 of *P. alba* and *P. tremula*, two naturally hybridizing, ecologically divergent forest trees, and almost all of these distortions (12 out of 13; >90%) *favored* introgression of donor (*P. tremula*) alleles into the heterospecific *P. alba* genomic background (Table 1). These allelic distortions manifested themselves also at the zygotic (=genotypic) level, and high levels of mortality (34%) were observed during early diploid life stages of the progeny (first-year seedlings, a critical life stage in long-lived forest trees). Thus, the observed patterns are most easily explained by three non-exclusive hypotheses: (1) epistatic interactions between the hybridizing genomes (Coyne and Orr, 2004), (2) overdominance or heterozygote advantage in hybrids (Hartl and Clark, 1997), (3) cyto-nuclear coadaptation (Galloway and Fenster, 1999; Futuyma, 2009). The data allow us to address each of these hypotheses in turn.

Hypothesis 1, *epistasis*, refers to a central question in current speciation genetics, namely the relative role of Bateson–Dobzhansky–Muller incompatibilities vs other mechanisms in post-mating RI (Coyne and Orr, 2004; Turelli and Moyle, 2007). Evidence for epistasis comes from a recent analysis of genomic admixture in hybrid zones between these species: heterospecific genomic interactions clearly contribute to steep genomic clines in localities, where these species co-occur (Lexer *et al*., 2010). Nevertheless, epistasis alone is unlikely to generate the observed unidirectional bias in genotype frequencies observed here, consistently favoring alleles of the same species across multiple loci (Fishman *et al*., 2001). In fact, epistasis may be expected to produce the opposite pattern (a bias toward overrepresentation of *P. alba* alleles), so additional mechanisms must be invoked.

Hypothesis 2, *heterozygote advantage* (Hartl and Clark, 1997), is less frequently invoked in speciation genetic studies, but represents a mechanism of great interest in ecological and conservation genetics (Conner and Hartl,

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2004). Under this hypothesis, selection favoring interspecific heterozygotes will elevate the frequency of *P. tremula* donor alleles in the backcross. This hypothesis is supported by high mortality (34%) during early life stages of the BC_1 progeny and by the low level of heterozygosity of the *P. alba* backcross parent (only 38%), compared with 67% of heterozygous loci in the F_1 hybrid parent of the backcross. This suggests that increased heterozygosity due to introgression can ameliorate the negative effects of biparental inbreeding (that is of recessive deleterious alleles in homozygous state) in *P. alba*; biparental inbreeding in *P. alba* becomes apparent from the great magnitude of short-range kinship coefficients among individuals (F_{ij}) in recent studies of spatial genetic structure in this species (van Loo *et al*., 2008), and from the extra-ordinary clone sizes of genets of *P. alba* in Southern Europe (Brundu *et al*., 2008; González–Martínez and coworkers, unpublished data).

Hypothesis 3, increased introgression due to *cyto-nuclear interactions* (Galloway and Fenster, 1999; Tiffin *et al*., 2001; Futuyma, 2009), is equally supported by our data: our plastid DNA data indicate a *P. tremula* cytoplasm for the female F_1 hybrid parent of our interspecific BC_1. Maternal inheritance of cytoplasmic genomes implies that all BC_1 progeny will carry cytoplasmic genes from *P. tremula*, so nuclear *P. tremula* alleles segregating in the interspecific BC_1 will be favored by selection in combination with the maternally derived conspecific cytoplasm present in each individual. In effect, cyto-nuclear coadaptation (or genomic conflict between heterospecific cyto-nuclear combinations) will 'pull' *P. tremula* nuclear alleles into a *P. alba* genomic background, resulting in a pattern that resembles, but should not be mistaken with adaptive introgression.

In the absence of reciprocal crosses, it is impossible to reject or accept either of these hypotheses with certainty. The present study was based on a single successful interspecific BC_1 obtained by crossing an individual of *P. alba* with a natural F_1 hybrid. A reciprocal crossing design (each species used as pollen *and* seed donor) would allow us to distinguish between cyto-nuclear and purely nuclear effects (Galloway and Fenster, 1999). While the absence of reciprocal crosses represents a caveat, congruent results (see below) from controlled progeny *and* natural populations indicate the generality of our findings beyond the successful cross used for segregation tests.

The well-documented selection episode in first-year seedlings and the clear consistency with genetic data for natural populations also allow us to interpret our results in the context of those obtained for many other groups of plants (Tiffin *et al*., 2001). Our results suggest that asymmmetries in post-mating barriers in these forest trees may result in introgression rather than the evolution of reinforcement upon secondary contact. In fact, two of the loci with significant overrepresentation of *P. tremula* alleles in the controlled cross (GCPM 1629 on chromosome 3 and ORPM 149 on chromosome 10; Table 1) are already known to exhibit greater than neutral introgression of *P. tremula* alleles in a well-studied natural hybrid zone (Lexer *et al*., 2010). Of course, other mechanisms may be operating in parallel, slowing down introgression and strengthening the 'filter' to interspecific exchange (Coyne and Orr, 2004; Futuyma, 2009; Lexer *et al*., 2010). Thus, our study adds to the ongoing debate regarding the effects of asymmetric barriers on the evolutionary dynamics of species interactions upon secondary contact (Tiffin *et al*., 2001; Coyne and Orr, 2004; Lopez-Fernandez and Bolnick, 2007; Turelli and Moyle, 2007; Veltsos *et al*., 2008; Widmer *et al*., 2009).

The sex determination region of *Populus* is not protected from interspecific gene flow

Sex chromosomes (or sex determination regions more generally) have received considerable attention as 'hotspots' of genes or other genetic factors involved in species isolation (Qvarnström and Bailey, 2009). Particular attention has been paid to the mechanisms underlying 'Haldane's rule,' that is the observation that in hybrid zones between divergent populations or species, the heterogametic sex will often be rare, absent or sterile (reviewed by Coyne and Orr, 2004). More recently, the role of suppressed recombination on sex chromosomes has attracted much attention, triggered by the finding that reduced recombination greatly facilitates the accumulation of speciation genes on sex chromosomes (Qvarnström and Bailey, 2009). Both of these phenomena speak for an important role of sex chromosomes in speciation, and this should manifest itself in reduced introgression and increased divergence of these genome regions in recently diverged species (Nosil *et al*., 2009; Qvarnström and Bailey, 2009).

Our present results for the sex determination region of *Populus* are not consistent with this expectation. The proximal end of chromosome XIX exhibits suppressed recombination in our interspecific BC_1 (Figure 1) and increased LD in natural populations (Table 3); note that LD normally decays within 1 kb in these species (Ingvarsson, 2008; Joseph and Lexer, 2008), and that patterns of LD vary with differences in effective population size (N_e) and metapopulation structure (Lexer *et al*., 2007). Still, interspecific divergence (F_{ST} and G'_{ST}) of this region in natural populations is not greater than elsewhere in the genome (on the contrary, the opposite appears to be the case, see Figure 2 and Results). Hence, this genome region is not protected from interspecific gene flow. Consistent with this observation, genetic markers in this region display significant segregation distortion, consistently favoring *P. tremula* donor alleles in our interspecific BC_1 toward *P. alba* (Table 1). These consistent but counter-intuitive results may be explained by characteristic features that distinguish the *Populus* sex determination region from other, better-studied sex chromosome systems.

First, the *Populus* sex determination region interrogated by our markers on chromosome XIX carries clusters of nucleotide-binding site–leucine rich repeat resistance (R-) genes (Yin *et al*., 2008). The fact that this important functional class of plant R-genes has been amplified in the *Populus* genome to form large clusters (Kohler *et al*., 2008) highlights their functional importance in these trees. If these nucleotide-binding site–leucine rich repeat genes are indeed under balancing selection as widely assumed for plant R-genes (Futuyma, 2009), then introgression may be favored by selection, especially in long-lived forest trees for which levels of standing variation are limited by notoriously low rates of

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molecular evolution per unit time (Petit and Hampe, 2006).

A possible alternative explanation for increased introgression on chromosome XIX is based on the known variability of sex determination systems in *Populus*. Whereas genomic data for the North American *P. trichocarpa* are suggestive of a ZW sex chromosome system involving a female-specific chromosomal segment located in the proximal end of chromosome XIX (Yin *et al*., 2008), genetic mapping of the sex locus in *P. alba* and in a cross *P. tremula* × *Populus. tremuloides* indicates the presence of at least two loci-controlling sex in a non-terminal position on this chromosome (Pakull *et al*., 2009; Paolucci *et al*., 2010). Interestingly, the sex locus maps to the female genetic map in *P. alba* (Paolucci *et al*., 2010) and to the male map in *P. tremula* × *P. tremuloides* (Pakull *et al*., 2009), consistent with the presence of two or more sex-controlling loci with different degrees of dominance. This would suggest that the *Populus* sex chromosome is at a very early step of its evolution, in which pairs or groups of sexually antagonistic mutations have accumulated, but full differentiation into heteromorphic sex chromosomes has not yet been achieved (Charlesworth *et al*., 2005). Our data are consistent with this hypothesis, since microsatellites in this region behave like codominant, autosomal markers and show no consistent pattern of reduced heterozygosity in either sex (Table 2). The apparent variation present in the poplar sex determination system (Yin *et al*., 2008; Pakull *et al*., 2009; Paolucci *et al*., 2010) provides a possible alternative explanation for increased introgression of the sex determination region (below).

Conclusions and hypotheses for future work

Our study demonstrates the value of experimental crosses involving natural hybrids of known genomic composition in understanding the genetics of species boundaries and barriers to introgression in *Populus* and other long-lived forest trees. Ongoing studies of natural hybrid zones between *P. alba* and *P. tremula* by our group have started to reveal patterns of genomic admixture and RI in multiple 'replicate' hybrid zone localities of these species across Europe (Lexer *et al*., 2007, 2010), which now provides a basis for picking natural hybrids and parental genotypes for controlled crossing experiments, such as those presented here. Experiments involving reciprocal crosses and both backcrossing directions (toward *P. alba* and *P. tremula*) will provide a more refined picture of post-mating and post-zygotic barriers to gene flow in these ecologically important trees.

With respect to the apparent lack of interspecific isolation of the sex determination region (above), a novel hypothesis has recently been put forward to explain the spread of novel sex-specific genome segments across hybrid zones (Veltsos *et al*., 2008). According to this hypothesis, a new chromosomal sex-determination system can spread across hybrid zones, even though it would normally be selected against within a single, isolated population (Pannell and Pujol, 2009). This apparently is made possible by the interplay between 'identity' disequilibria, commonly observed in hybrid zones, and the sexually antagonistic selection pressures affecting pairs or groups of sex-controlling loci (Veltsos *et al*., 2008; Pannell and Pujol, 2009).

Although Veltsos *et al*. (2008) modeled the specific case of a XY replacing a X0 sex determination system, their model may be more widely applicable to other cases of asymmetric selection pressures in hybrid zones caused by dominance effects (that is 'dominance drive'; Mallet, 1986). The variable genetic architecture of sex determination in poplar (Yin *et al*., 2008; Pakull *et al*., 2009; Paolucci *et al*., 2010) could provide a suitable substrate for this type of process. Tests of the mode of spread and ecological impact of the sex determination region in hybrid zones between these species are currently underway.

Conflict of interest

The authors declare no conflict of interest.

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