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Recombinant hybrids retain heterozygosity at many loci: new insights into the genomics of reproductive isolation in *Populus*

DOROTHEA LINDTKE,* C. A. BUERKLE,† THELMA BARBARÁ,*‡ BERTHOLD HEINZE,§ STEFANO CASTIGLIONE,¶ DENES BARTHA** and CHRISTIAN LEXER*‡
*Unit of Ecology and Evolution, Department of Biology, University of Fribourg, 1700 Fribourg, Switzerland, †Department of Botany, University of Wyoming, Laramie, WY 82071, USA, ‡Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3DS, UK, §Department of Genetics, Federal Research Centre for Forests, A-1140 Vienna, Austria, ¶Department of Chemistry and Biology, University of Salerno, 84084 Fisciano, Italy, **Department of Botany, West-Hungarian University, 9400 Sopron, Hungary

Abstract

The maintenance of species barriers in the face of gene flow is often thought to result from strong selection against intermediate genotypes, thereby preserving genetic differentiation. Most speciation genomic studies thus aim to identify exceptionally divergent loci between populations, but divergence will be affected by many processes other than reproductive isolation (RI) and speciation. Through genomic studies of recombinant hybrids sampled in the wild, genetic variation associated with RI can be observed *in situ*, because selection against incompatible genotypes will leave detectable patterns of variation in the hybrid genomes. To better understand the mechanisms directly involved in RI, we investigated three natural 'replicate' hybrid zones between two divergent *Populus* species via locus-specific patterns of ancestry across recombinant hybrid genomes. As expected, genomic patterns in hybrids and their parental species were consistent with the presence of underdominant selection at several genomic regions. Surprisingly, many loci displayed greatly increased between-species heterozygosity in recombinant hybrids despite striking genetic differentiation between the parental genomes, the opposite of what would be expected with selection against intermediate genotypes. Only a limited, reproducible set of genotypic combinations was present in hybrid genomes across localities. In the absence of clearly delimited 'hybrid habitats', our results suggest that complex epistatic interactions within genomes play an important role in advanced stages of RI between these ecologically divergent forest trees. This calls for more genomic studies that test for unusual patterns of genomic ancestry in hybridizing species.

Keywords: admixture, coadapted gene complexes, epistasis, hybrid breakdown, hybrid zone, speciation

Introduction

One of the big questions in evolutionary biology concerns the processes involved in the clustering of organisms into discrete species, and one central problem is to understand the origin and maintenance of reproductive barriers that reduce gene flow in sympatry (Coyne & Orr 1998; Turelli *et al*. 2001). Empirical data indicate that RI is often incomplete and evolves gradually (Coyne 1992; Mallet *et al*. 2007; Nosil *et al*. 2009b) as an incidental but important byproduct of differential adaptation (Rice & Hostert 1993). Thus, genomes are 'porous' to interspecies gene flow, and divergence at loci contributing to local

Correspondence: Dorothea Lindtke, Fax: + 41 (0) 263 009 698; E-mail: dorothea.lindtke@unifr.ch

adaptation can be high whereas gene flow may continue in the remainder of the genome (Wu 2001; Via 2009; Michel *et al.* 2010).

A wealth of theoretical and empirical studies has addressed the question as to how exactly speciation can proceed in the face of gene flow (reviewed in Rice & Hostert 1993; Turelli *et al.* 2001; Gavrilets 2003; Nosil *et al.* 2009b), and *hybrids* have provided invaluable clues about this process. The rough consensus is that, although hybrids between divergent populations may be formed, they frequently show reduced fitness compared to their parents (at least in the parental habitats) due to ecological maladaptation, reduced mating success or genetic incompatibilities. In particular, ecological isolation arises if intermediate forms fall between different adaptive peaks evolved by the parental populations, and hybrids suffer reduced fitness in both parental habitats, or hybrids show reduced reproductive compatibility due to intermediate trait values (Schluter 2001; Nosil *et al.* 2009b; Schluter & Conte 2009). Intrinsic isolation is most often attributed to genetic incompatibilities described by the Bateson-Dobzhansky-Muller (BDM) model, where substitutions that arise in different lineages cause incompatibilities when combined in hybrid genomes (Orr 1996; Gavrilets 2003). Fitness declines can be delayed until the second (or later) hybrid generations due to the greater severity of homozygous-homozygous BDM incompatibilities (Turelli & Orr 2000), or due to the breakdown of coadapted gene complexes (outbreeding depression; Lynch 1991; Edmands & Timmerman 2003; Landry *et al.* 2007).

Natural hybrid zones provide great opportunities for studies of RI. Genetic markers that show different rates of migration along a geographic transect have been widely used as indicators for genes that are involved in RI or adaptive introgression (geographic cline analysis; Barton & Hewitt 1985; Szymura & Barton 1986; Barton & Gale 1993). With the advent of genomic tools to assay large numbers of marker loci, novel analytical methods have been developed to examine introgression of particular loci as a function of genome-wide admixture (genomic cline analysis; Lexer *et al.* 2007; Gompert & Buerkle 2009, 2011). This has the advantage that it (i) facilitates the study of mosaic hybrid zones where no reasonable geographic transect can be placed; and (ii) because these methods are individual-based, they also facilitate the study of *heterospecific* genotypes (heterozygotes for alleles derived from different species) as a function of genome-wide admixture. This provides information on hybrid advantage or disadvantage through patterns of increased or decreased interspecific heterozygosity for individual genomic regions, and thus genomic studies of hybrid zones can contribute greatly to our understanding of RI. Unfortunately, most speciation genomic studies focus exclusively on divergence 'outlier' loci from genome scans of diverging populations or species (reviewed in Nosil *et al.* 2009a; Butlin 2010) and rarely test for the contribution of these loci to RI in hybrids (but see Gompert *et al.* 2012a). In addition, speciation is a continuous process, consisting of the initiation, strengthening, and completion of RI (Butlin *et al.* 2008). The questions on which barriers complete the process of speciation, and what maintains species boundaries after speciation is completed, remain open (Nosil *et al.* 2009b; The Marie Curie SPECIATION Network 2011).

Here, we aim at a better understanding of advanced stages of RI by investigating the genomic ancestries of natural hybrids between two highly divergent poplar species, *Populus alba* (White poplar) and *Populus tremula* (European aspen), using 77 mapped microsatellite markers. Three replicate hybrid zones were investigated to account for stochastic processes (such as drift) or locally varying ecological factors that might have influenced the outcomes of hybridization. We focused on the detection of different levels of interspecific heterozygosity as indication for locus-specific hybrid advantage or disadvantage across the genome, and their relation to locus-specific levels of genetic differentiation between parental populations. Three basic associations between these patterns were expected at the outset of our study:

Firstly, genome regions important for speciation are generally assumed to be associated with low fitness of heterospecific gene combinations (Orr 1995; Coyne & Orr 1998; Schluter & Conte 2009), resulting in *reduced interspecific heterozygosity* in hybrids at the respective loci and *increased genetic differentiation* between the parental populations. Secondly, *increased interspecific heterozygosity* resulting from heterosis at particular loci potentially leads to increased introgression of alleles from one species into the other and *reduced genetic differentiation* between species (Ingvarsson & Whitlock 2000; Charlesworth & Willis 2009). Note that heterosis often arises in F_1 hybrids, whereas later-generation hybrids may suffer from the breakdown of coadapted gene complexes (Lynch 1991; Barton 2001; Edmands & Timmerman 2003; Charlesworth & Willis 2009), so we expect to find few recombinant hybrids in the latter case. Thirdly, adaptive introgression of positively selected alleles from one species into another by means of *excess ancestry* (i.e. cline center shifts) in hybrids are expected to *decrease* genetic differentiation between species. Nevertheless, *excess ancestry* in hybrids coupled with *increased differentiation* between the parental populations can also point to loci important for species divergence, for example because these loci are involved in local adaptation or genetic incompatibilities (Nolte & Tautz 2010; Gompert & Buerkle 2011; Gompert *et al.* 2012b).

Populus alba and *P. tremula* are ecologically and morphologically well differentiated, easily delimited and recognizable species, although wide secondary hybrid zones are often formed between them (Lexer *et al.* 2005, 2010). Ecological species differences include habitat type (lowland flood-plains vs. upland), latitudinal amplitudes (Dickmann & Kuzovkina 2008) and leaf morphology, associated with ecology (Lexer *et al.* 2005, 2007, 2009). Diploid hybrids, sometimes referred to as *Populus* × *canescens* (Gray poplar), exhibit predominantly recombinant genotypes and are separated by marked genomic discontinuities from their parental species, indicating strong RI (Lexer *et al.* 2010). Although the conspicuous genomic gaps between parental species and hybrids might suggest some amount of spatial isolation, hybrids are often found sympatric with their parentals within large mosaic hybrid zones, frequently as pioneers in river flood-plains. In effect, hybrids do not have a readily recognizable, discrete 'hybrid habitat', as parental and hybrid genotypes grow intermingled with one another, root systems are extensive, and clonal ramets frequently reach sizes of up to 50 m (van Loo *et al.* 2008). Genomic studies in these species and hybrids are greatly facilitated by substantial genomic information available from the closely related *Populus trichocarpa*, the first completely sequenced forest tree (Tuskan *et al.* 2006).

We greatly extend a previous study on genomic ancestry in hybrids of European *Populus* (Lexer *et al.* 2010) by sampling many more loci (>47 000 new data points), thereby subjecting a total of 693 individuals to locus-specific analyses, and use detailed simulations to (i) examine the similarity of locus-specific outcomes of hybridization in three replicate hybrid zones; (ii) study locus-specific levels of interspecific heterozygosity in hybrids and their association to genetic differentiation among the parental species; and (iii) interpret the genomic patterns in terms of the mechanisms of advanced-stage RI between highly divergent but still hybridizing species.

Methods

Sampling and laboratory analyses

Leaf tissue was collected in mosaic hybrid zones and sympatric or adjacent parental populations of *Populus alba* and *Populus tremula* along three different river drainage systems, located in Italy (Ticino river), Austria (Danube) and Hungary (Tisza; Fig. 1). All three sites display strong signs of natural disturbance as expected for flood-plain forests. The three sites differ in their degree of human disturbance, as the Danube and Tisza rivers have been affected by flood protection measures from the 19th century onwards, whereas the Ticino has remained largely unaffected from such alterations. Sexually mature trees (generally >20 years) were sampled in all sites. The three populations were previously characterized by Lexer *et al.* (2010) with a smaller number of genetic markers. Here, we extend this molecular genetic dataset and analyze it comprehensively with a strong focus on locus-specific effects, and their similarity in replicate hybrid zones with differences in population history and ecology.

Individuals were genotyped at 77 mapped microsatellite loci distributed across all 19 chromosomes of the *Populus* genome (Table S1, Supporting information; http://www.ornl.gov/sci/ipgc/ssr_resource.htm; van der Schoot *et al.* 2000; Smulders *et al.* 2001; Tuskan *et al.* 2004; De Carvalho *et al.* 2010). In 10 out of the 77 loci, amplification fragments were not consistent with simple codominant inheritance patterns and these loci were thus scored conservatively as dominant markers via the presence or absence of a single amplicon of expected size. Laboratory procedures for DNA extraction and PCR amplification followed Lexer *et al.* (2005). Allele size polymorphisms were resolved and visualized on an Applied Biosystems (ABI) 3100 (Austria) and a 3130 Genetic Analyzer (Italy and Hungary). Fluorescence peaks were scored using ABI fragment analysis software (Genescan and Genotyper or GeneMapper) and were checked manually. Potentially clonal individuals (fewer than six mismatches at 77 markers) were removed from the dataset, resulting in 219, 288 and 186 individuals maintained in the final data set for Italy, Austria and Hungary, respectively.

Statistical analyses

As the use of different genotyping instruments and software can lead to shifts in DNA fragment sizes, each hybrid zone was treated separately in most statistical analyses. This was possible because adjacent parental reference populations were sampled for *each* hybrid zone, and genetic ancestry and other key parameters could be estimated independently for each locality (below). An exception was made for 11 codominant loci identified in previous work (Lexer *et al.* 2005, 2010) that showed clear fragment differences and few stutter bands (indicated with an asterisk in Tables S1–S4, Supporting information). For these markers, all hybrid zone datasets were combined by careful comparison of allele sizes and frequencies, using custom scripts. This allowed us to estimate genetic differentiation among the three replicate hybrid zones and to compare basic statistics directly between them. Unless noted otherwise, calculations were carried out in R (R Development Core Team 2011).

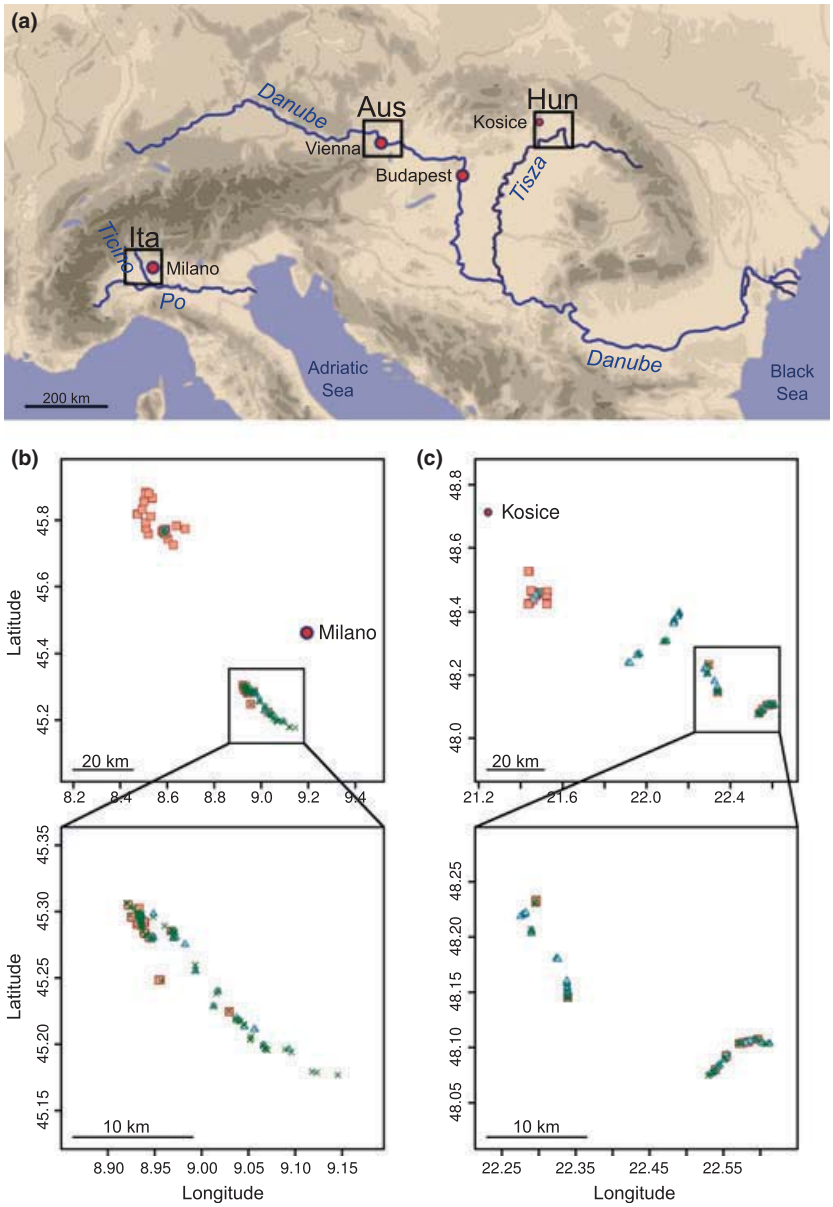

Fig. 1 Map of sampling localities. (A) Study sites in Italy (Ita), Austria (Aus) and Hungary (Hun). (B) Spatial arrangement of genotype classes for Italy. (C) Spatial arrangement of genotype classes for Hungary. Hybrid spots are further magnified. Brown squares, *Populus tremula*; blue triangles, *Populus alba*; green crosses, admixed individuals ($0.01 < Q < 0.99$). For details about the Austrian hybrid zone see Lexer *et al.* (2005) and van Loo *et al.* (2008). Physical map modified from www.euratlas.com.

Basic statistics for hybrid zone replicates. Parental populations (*P. alba*, *P. tremula*) as well as admixed individuals were characterized for 11 codominant microsatellite markers used in the combined dataset. Three genotypic classes in each locality were defined according to STRUCTURE (Pritchard *et al.* 2000) genome-wide admixture proportions (Q) in a rigorous way, yielding 'pure' parental ($Q \leq 0.01$ or ≥ 0.99) and genetically 'intermediate' ($0.25 \leq Q \leq 0.75$) individuals typical for hybrid zones of these two species (Lexer *et al.* 2010). Our main goal was to estimate genetic differentiation *within* each genotypic class (*P. alba*, *P. tremula*, hybrids) *between replicate localities*. To achieve this, F_{ST} and allele frequency differential ($\delta = \sum |f_{i1} - f_{i2}| / 2$, where f_{i1} and f_{i2} represent the ith allele frequencies in the two ancestral populations, respectively; Zhu *et al.* 2005) were computed among localities for each of the three genotypic classes to test for the degree of independence between hybrid zone replicates. In addition, observed (H_O) and expected heterozygosity (H_E) and inbreeding coefficients (F_{IS}) were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010).

Interspecific genetic differentiation between *P. alba* and *P. tremula within each locality* was estimated using all 67 codominant marker loci. Interspecific genetic differentiation was measured as F_{ST} (via AMOVA, Excoffier *et al.* 1992), complemented by within sample gene diversity (H_S), G'_{ST} (measure of G_{ST} standardized by H_S; Hedrick 2005) and allele frequency differential (δ).

These parameters were computed in ARLEQUIN 3.5 (F_{ST} values), FSTAT (Goudet 2001) (H_S), or manually (G'_{ST}, δ).

Although many speciation genomic studies focus on the identification of divergence 'outlier' loci from genome scans, we are skeptical towards their significance because of the well-known limitations of F_{ST} based genome scans for marker loci with variable mutation rates (Foll & Gaggiotti 2008; Butlin 2010; Feder & Nosil 2010; Michel *et al.* 2010), as are microsatellites (Ellegren 2004). Further, outlier scans based on deviations from putatively neutrally evolving regions will only be suitable to detect the most extreme regions (Michel *et al.* 2010) that are not necessarily associated with RI (Via & West 2008; Via 2009; Feder & Nosil 2010; Buerkle *et al.* 2011). Thus, we prefer to discuss the complete distributions of F_{ST} and δ as locus-specific measurements of genetic differentiation between the parental species in the main article, and we refer to the Supporting information for the results of complimentary outlier tests (Text S1, Supporting information).

Bayesian admixture analysis. A Bayesian model-based clustering method implemented in STRUCTURE 2.3.2.1 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007) was used to identify pure *P. alba* and *P. tremula* and to estimate admixture coefficients and locus specific ancestries (LSAs) in admixed individuals. In a first step, STRUCTURE was run under the admixture model using correlated allele frequencies to (i) verify that the data supported the inference of two clusters (k) for all three localities, (ii) identify pure parental individuals as reference samples for subsequent analyses and (iii) calculate the genome-wide admixture proportion (Q) for each of the admixed individuals, Q ranging from 0 (*P. tremula*-like) to 1 (*P. alba*-like). The analyses were carried out with 50 000 burn-in and 100 000 additional iterations and a prior mean for F_k of 0.7 to account for drift of each parental species population away from their common *Populus* ancestor. To detect the most likely number of clusters present in the data using the method of Evanno *et al.* (2005), MCMC estimates from three replicate chains of k from one to six were obtained.

In a second step, the linkage model implemented in STRUCTURE was used to estimate the ancestry of each allele at each locus. The linkage model incorporates information on the ancestry of neighbouring, physically linked markers (Falush *et al.* 2003), and can be used to yield estimates of LSA for each allele copy (the site-by-site output; probabilities that both, one, or none of the two allele copies at a locus are derived from one of the parental populations). These allow marginal, locus-specific estimates of interspecific homo- and heterozygosity across admixed individuals (described below). The linkage model was run with an additional burn-in of 25 000 iterations. Linkage information was obtained from the physical map in the *Populus trichocarpa* genome assembly v2 (http://www.phytozome.net/poplar) and from a controlled cross between *P. alba* and *Populus* × *canescens* (Table S1, Supporting information).

Null alleles (non-amplifying alleles) are common for genetic markers that are amplified by PCR. If present in only one of the parental species, null alleles can reduce estimates of interspecific heterozygosity in hybrids, thus the STRUCTURE linkage model was run with the recessive allele option (Falush *et al.* 2007) applied to all loci. To improve the estimation of LSAs for admixed individuals, parental individuals were flagged as 'learning samples' according to the results of the first step (admixture model; above), using a strict Q cut-off of ≤ 0.01 or ≥ 0.99. This strict cut-off was chosen to ensure that no potentially admixed individuals were included in the learning samples (see probability intervals in Fig. 2). The specification of 'learning samples' turned out to be an important step for the estimation of LSAs given parental allele frequencies, as these otherwise seemed to be distorted at loci with uncertainty about ancestry (including missing data, recessive alleles or shared alleles among parental species) by linked loci with high information content, probably due to the joint estimation of population allele frequencies and LSA.

Patterns of ancestry in admixed individuals. The estimates of LSA from STRUCTURE were used to compute two variables describing evidence for interspecific homozygosity and heterozygosity at each locus and individual following Lexer *et al.* (2010). Briefly, 'specific homozygosity' summarizes the evidence that both allele copies at a locus have ancestry from *P. tremula* (−1) vs. *P. alba* (+1), and 'interspecific heterozygosity' quantifies evidence that each allele copy at a locus was derived from the same (−1) vs. different (+1) parental species. Variables describing evidence for homo- and heterozygosity can range from −1 to +1, where values close to zero could result from low information about ancestry at a locus (e.g. due to low δ, missing data, or recessive alleles).

These characteristic values, computed per locus and individual, can be studied locus-by-locus and associated with the individual genome-wide admixture proportion (Q). This procedure is related to the concept of the genomic clines method of Gompert & Buerkle (2009), but instead of using discrete genotypes, continuous variables based on the evidence for interspecific homozygosity and heterozygosity are used for cline fitting. To summarize the results for each locus in a way that narrows the effects of unequal sampling of admixed individuals, we fitted the variables describing interspecific homozygosity and heterozygosity against Q, using cubic regressions (Fig. S1, Supporting information). The regression

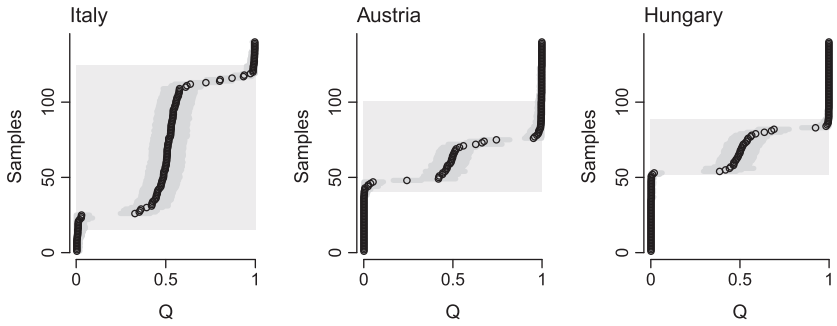

Fig. 2 Individual genome-wide admixture proportion, *Q* (circles), including 90% probability intervals (gray areas) estimated by the STRUCTURE admixture model for each of the three localities, ordered by *Q* value (0, *Populus tremula* like; 1, *Populus alba* like). Individuals with values within the light gray rectangle were classified as potentially admixed ($0.01 < Q < 0.99$), those outside as 'pure' species ($Q \leq 0.01$ or ≥ 0.99; not all parentals shown).

functions were integrated and the integrals normalized in such a way that any deviation from zero represents a measurement of over- or under representation of certain genotypes in the admixed individuals, relative to an idealized panmictic hybrid population. The observed integrals were subsequently scaled to possible maximum and minimum values at +1 and −1, respectively, simply by dividing by the maximum possible magnitude of deviation from zero, given the observed *Q* values. Note that although this scaling process diminishes the effect of *Q* on integral size, it cannot account for low information content at a particular locus, for which we still expect integral sizes close to zero. The integrals are particularly valuable for investigating the composition of recombinant hybrid genomes and how they might be shaped by the effects of over- and underdominance or excess ancestry. 'Excess ancestry' (quantified by specific homozygosity) characterizes the preponderance of alleles originating from only one parental species. This corresponds to a shift of the cline center, usually referred to as introgression of particular alleles into hybrid genomes (Payseur *et al*. 2004; Gompert & Buerkle 2009); we note that such cline center shifts do not necessarily point to introgression in the sense of gene flow between parental species (Gompert *et al*. 2012b). Positive values for interspecific heterozygosity correspond to the prevalence of heterospecific genotypes at a locus, as expected for F_1 hybrids or loci associated with overdominant selection, whereas negative values are indicative of processes that eliminate interspecifically heterozygous genotypes.

Simulations. Probability estimates for LSA might be influenced by low information content at a locus (above), decreasing the absolute value of the integral. To verify the quality of the STRUCTURE LSA values as well as our integral approach to quantify levels of interspecific hetero- and homozygosity along genomic clines, and to test whether admixed individuals are more compatible with F_1 or advanced hybrid generations, simulation studies were performed.

Eight categories of hybrids (F_1, F_2, F_5, F_{20} and four mixed hybrid classes, M_1 to M_4; $N = 500$ each) were simulated with QUANTINEMO 1.0.4 (Neuenschwander *et al*. 2008) for each locality. The number of loci, their map positions and parental allele frequencies (including null allele frequencies computed by STRUCTURE) were obtained from the original data set. Life history and mating system traits were modelled under simplified assumptions, which are not expected to strongly bias outcomes on genetic ancestries in hybrids. Simulations consisted of non-overlapping generations with the following life-cycle: (i) random mating between hermaphroditic adults within each patch resulting in a mean number of two offspring drawn from a Poisson distribution; (ii) migration of offspring; (iii) aging; (iv) regulation to carrying capacity. Recombination occurred according to the linkage map. To initiate the simulations, two patches with a carrying capacity of 1000 were filled with one or the other of the parental species. Offspring from both parental patches were allowed to migrate into a new, empty patch with a carrying capacity of 1200, where after one generation of random mating F_1 hybrids were identified and isolated. These F_1 hybrids were used to found a new patch with a carrying capacity of 500, where they reproduced with no further migration from the parental patches. F_1, F_2, F_5 and F_{20} hybrids were sampled after one, two, five and 20 generations, respectively. Populations of mixed hybrid classes (M_1 to M_4) were obtained by migration of 50% of offspring from each parental species into a new, empty patch with a carrying capacity of 500. From the second generation, one percent of the offspring arisen in each of the parental patches was allowed to migrate into the hybrid patch, resulting in approximately 3.8% immigrants per generation. Samples of the whole hybrid patch and thus consisting of different hybrid

generations, backcrosses and immigrants were taken at generation seven (M_1) and 22 (M_2). The simulations were repeated with twice the migration rate (approximately 7.4% immigrants per generation) (M_3 and M_4).

Subsequent to the quantiNemo simulations, null alleles and dominant alleles were substituted and missing data were introduced at the same percentage per locus as in the real data. For each hybrid category, 500 simulated individuals plus all parental references from the observed data were analyzed by structure using the same settings as for the real data. Because parental individuals were flagged as 'learning samples' as before, results for simulated and observed hybrids are directly comparable. Integral estimates were obtained for a random subset of simulated hybrids of the same size as in the real data set, plus the parental references. This procedure was repeated 1000 times to obtain confidence intervals (CIs) for the integral sizes.

Results

Characterization of hybrid zone replicates

Initial characterization of the three hybrid zones based on the structure admixture model classified 88, 27 and 29 individuals as genetically intermediate hybrids ($0.25 \leq Q \leq 0.75$) for Italy, Austria and Hungary, respectively. A combined dataset of 11 codominant microsatellites was used for a direct comparison of the three hybrid zones. As expected, observed (H_O) and expected heterozygosity (H_E) was highest in admixed, genetically intermediate individuals in all three localities, whereas inbreeding coefficients (F_{IS}) were negative (Table 1).

F_{ST} and δ among localities for each of the three genotypic groups allowed us to assess the degree of independence of the hybrid zone replicates. F_{ST} revealed low but significant genetic differentiation of the pure species populations between sampling sites, matching an isolation-by-distance pattern (Table 2). F_{ST} values of this magnitude are consistent with previous estimates for *Populus alba* and *Populus tremula*, and can be sufficient for the build-up of local adaptation in *Populus* (Castiglione *et al.* 2010; De Carvalho *et al.* 2010). Genetic differentiation between the three hybrid lineages is more obvious for δ, which is not affected by elevated within-population diversity typical for hybrids (Table 2). Interestingly, hybrids did not show the isolation-by-distance pattern found in the parentals.

Interspecific F_{ST}, Hedrick's G'_{ST} and δ, measured separately for each hybrid zone based on 67 codominant markers, was highly variable among loci but altogether substantial (e.g. F_{ST} between *P. alba* and *P. tremula* in Italy: mean 0.35, SD 0.25, range 0.00–0.97; Table 3), supporting advanced divergence and high levels of RI between *P. alba* and *P. tremula* (Lexer *et al.* 2010). Only a small proportion of loci showed low differentiation, potentially resulting from gene flow or shared ancestral polymorphisms (Fig. 3; Table S2, Supporting information).

Allelic diversities varied greatly across the 67 loci, with allele numbers ranging from 2 to 37 and within population gene diversities (H_S) from 0.00 to 0.92 (Table 3). This level of genetic diversity is generally problematic when using the statistic F_{ST} to quantify the level of genetic differentiation (Hedrick 2005) and is reflected by widespread deviations between estimates for δ and F_{ST}, particularly for loci with high numbers of alleles (Fig. 3; Table S2, Supporting information). Genomic patterns of genetic differentiation were highly concordant across localities, in line with high levels of intraspecific gene flow or similar architectures of RI between species (Fig. 3; Spearman correlation coefficients, averaged across pairwise comparisons for all

Table 1 Summary statistics for populations of *Populus alba*, *Populus tremula* and intermediate hybrids ($0.25 \leq Q \leq 0.75$) at three replicate localities, including number of individuals (N), inbreeding coefficient (F_{IS}), observed (H_O) and expected heterozygosity (H_E), estimated from 11 loci of the combined dataset. P-values for F_{IS} estimates, p_{FIS} ($F_{IS\ random} \geq F_{IS\ observed}$), are based on 20 022 permutations

Locality	Population	N	F_{IS}	p_{FIS}	H_O	H_E
Italy	*P. alba*	42	0.20	0.000	0.33	0.42
	P. tremula	68	0.17	0.000	0.35	0.43
	Hybrids	88	−0.14	1.000	0.58	0.52
Austria	*P. alba*	174	0.07	0.000	0.32	0.36
	P. tremula	54	0.05	0.090	0.45	0.50
	Hybrids	27	−0.13	0.995	0.62	0.56
Hungary	*P. alba*	93	0.11	0.000	0.33	0.37
	P. tremula	56	0.09	0.003	0.44	0.50
	Hybrids	29	−0.01	0.591	0.53	0.53

Table 2 Genetic differentiation among pairs of replicate localities within species, given by F_{ST} and δ. Measurements are based on 11 loci of the combined dataset. All F_{ST} values were significant from zero at $P < 0.05$

Population	Italy/Austria		Austria/Hungary		Hungary/Italy	
	F_{ST}	δ	F_{ST}	δ	F_{ST}	δ
Populus alba	0.060	0.160	0.016	0.082	0.085	0.209
Populus tremula	0.028	0.129	0.008	0.099	0.053	0.177
Hybrids	0.032	0.161	0.024	0.170	0.030	0.179

localities, using all codominant loci: 0.86 (δ); 0.80 (F_{ST}); all P-values $< 3 \times 10^{-12}$).

Patterns of ancestry in admixed individuals

Applying the STRUCTURE admixture model, the most likely number of clusters k was strongly supported to be two at all three localities (see Fig. S2, Supporting information, for L(k) and Δk), indicating that hybrids do not form a genetically divergent cluster. One-hundred-and-nine, 60 and 37 individuals (49.8%, 20.8% and 19.9% of all samples) were classified as potentially admixed for Italy, Austria and Hungary, respectively ($0.01 < Q < 0.99$; Fig. 2). A clear gap in the distribution of Q values is present in all three localities, consistent with previous findings for the Austrian population (Lexer *et al.* 2010).

More refined admixture analysis with the STRUCTURE linkage model provided estimates of LSA that were used to calculate evidence for specific homozygosity and interspecific heterozygosity for each locus and individual. These estimates were highly variable among loci, resulting in a mosaic-like pattern of genomic ancestries for admixed individuals, typical of advanced recombinant hybrid generations (Figs S3 and S4, Supporting information). Integration of regression functions of interspecific homozygosity and heterozygosity on Q provided an intuitive and useful measurement of over- or underrepresentation of certain genotypes in the three replicate hybrid zones. This approach captured the highly variable pattern of ancestry among loci, ranging from increased interspecific heterozygosity at a large number of loci to increased specific homozygosity at few loci (Fig. 4). The latter was often associated with excess ancestry of alleles that originated predominantly from one or the other parent (Fig. 5). Genomic patterns of LSA in admixed individuals were remarkably similar in all three localities (mean Spearman correlation coefficients across localities: interspecific heterozygosity, 0.68; specific homozygosity, 0.39; all P-values < 0.01; Table 4, Figs 4 and 5; Fig. S1, Supporting information), and often consistent across individuals as well (Figs S3 and S4, Supporting information).

The pattern of increased homozygosity is unlikely to be generated through the presence of null-alleles, as those were accounted for by the STRUCTURE recessive-allele model. Paralogs could increase interspecific heterozygosity but should be detectable due to a deviation from a codominant segregation pattern in hybrids, except possibly for duplicated loci that are fixed for the same allele within species. We identified few loci with an H_E in the parental species sufficiently small that this might have gone undetected during scoring (Table S3, Supporting information), but only two of them (G1255 and G1074) showed increased interspecific heterozygosities, and BLAST searches against the *P. trichocarpa* genome did not result in multiple hits. Thus, paralogs are extremely unlikely to be responsible for the increased interspecific heterozygosities observed.

The simulations confirm the rapid decay of interspecific heterozygosity expected beyond the F_1 generation (Anderson & Thompson 2002; Figs 4 and 6). The simulations indicate that moderate migration of pure

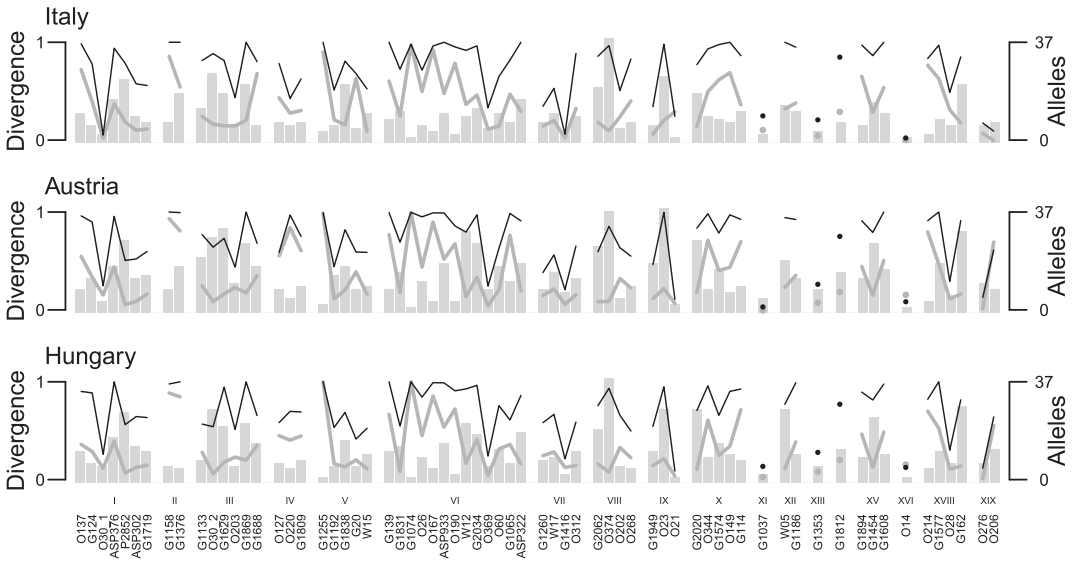

Fig. 3 Patterns of differentiation between *Populus alba* and *Populus tremula* at the three localities. Depicted are allele frequency differentials δ (black line), and F_{ST} values (bold gray line) for 67 codominant markers, ordered along chromosomes according to their map positions. Bars indicate numbers of alleles at each locus.

parental individuals into the hybrid zone does not increase probabilities of interspecific heterozygosity, but broadens the realized range of Q. Increased recombination time after initial admixture results in a smaller variance in interspecific heterozygosities as neighbouring markers become more independent (Fig. 6B–D, F–H, J–L). Figure 6 reveals that none of the observed admixed individuals shows a genome-wide probability of interspecific heterozygosity expected for F_1 hybrids. This indicates that our data set comprises advanced hybrid generations only, including some, probably advanced, backcrosses. However, individual genome-wide probabilities of interspecific heterozygosities are clearly in the upper range of the expected distribution for advanced hybrid generations.

Locus-specific interspecific heterozygosity fell below values expected for F_1 hybrids but considerably exceeded values from other simulated hybrid categories at many markers (Fig. 4). Loci with frequencies outside the 95% CIs (2.5–97.5% quantiles) of simulated data for recombinant hybrids were classified as 'outliers'. Note that this dichotomous classification is difficult for homozygote excess because drift can lead to an excess of either homozygote. Outliers of each category, except

Table 3 Total number of alleles (N_A), within sample gene diversity (H_S) and measurements of genetic differentiation (F_{ST}, Hedrick's G'_{ST} and δ) between *Populus alba* and *Populus tremula*. Given are averages over 67 codominant loci, with standard deviations and ranges in parentheses

Locality	N_A	H_S	F_{ST}	Hedrick's G'_{ST}	δ
Italy	9.7 (6.37; 2–36)	0.50 (0.23; 0.02–0.90)	0.35 (0.25; 0.00–0.97)	0.70 (0.33; 0.01–1.00)	0.72 (0.29; 0.02–1.00)
Austria	13.3 (8.01; 2–36)	0.52 (0.24; 0.00–0.90)	0.35 (0.27; 0.00–1.00)	0.69 (0.33; 0.00–1.00)	0.71 (0.28; 0.03–1.00)
Hungary	11.4 (7.49; 2–37)	0.54 (0.24; 0.00–0.92)	0.32 (0.25; 0.01–1.00)	0.67 (0.31; 0.02–1.00)	0.70 (0.26; 0.09–1.00)

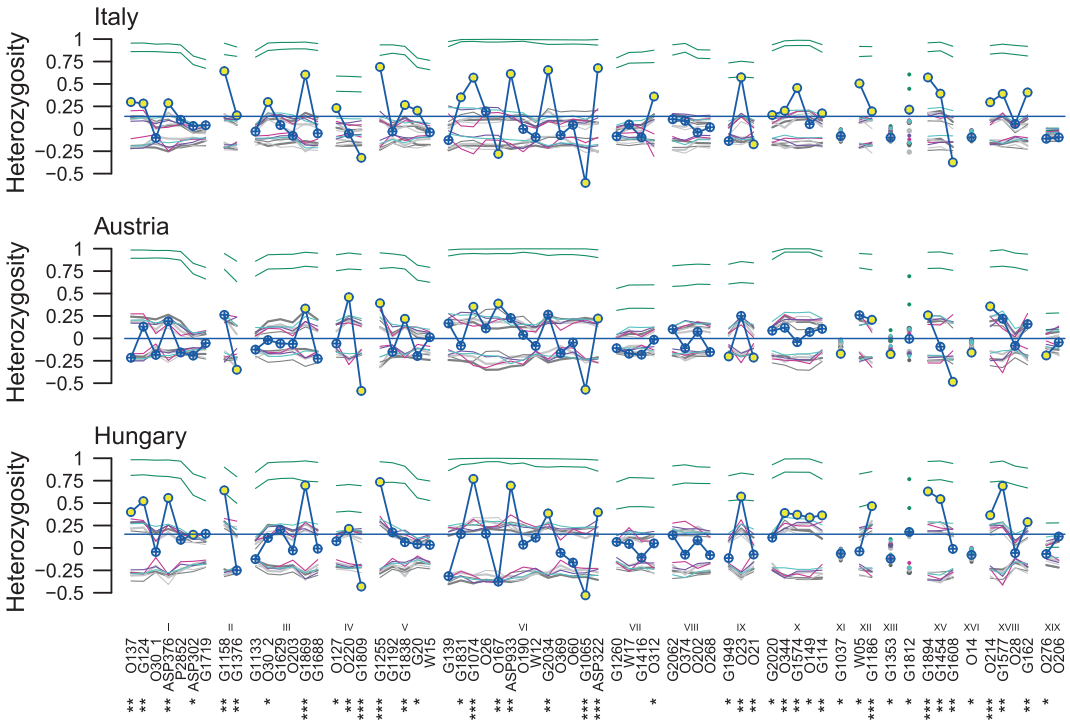

Fig. 4 Interspecific heterozygosity for each locus, measured by integrated regression functions of Q on evidence for interspecific heterozygosity, at the three localities (points connected by blue line; see main text for details). Depicted are values for 67 codominant markers, ordered on chromosomes according to their map position. Elevated positive or negative values indicate a surplus of interspecific heterozygote or homozygote genotypes relative to an idealized panmictic hybrid population. The solid horizontal line denotes the mean over all loci. Ninety-five per cent CIs of integral measurements for each of eight simulated hybrid categories are shown (green, F_1; turquoise, F_2; purple, F_5; violet-red, F_{20}; bold dark gray, mixed generation sample M_1; bold light gray, M_2; dark gray, M_3; light gray, M_4). Values exceeding all 95% CIs of simulated data (except F_1) are indicated by circles with yellow filling. Stars next to a locus name indicate that the locus has been identified as an outlier in one (*), two (**), or all three (***) localities.

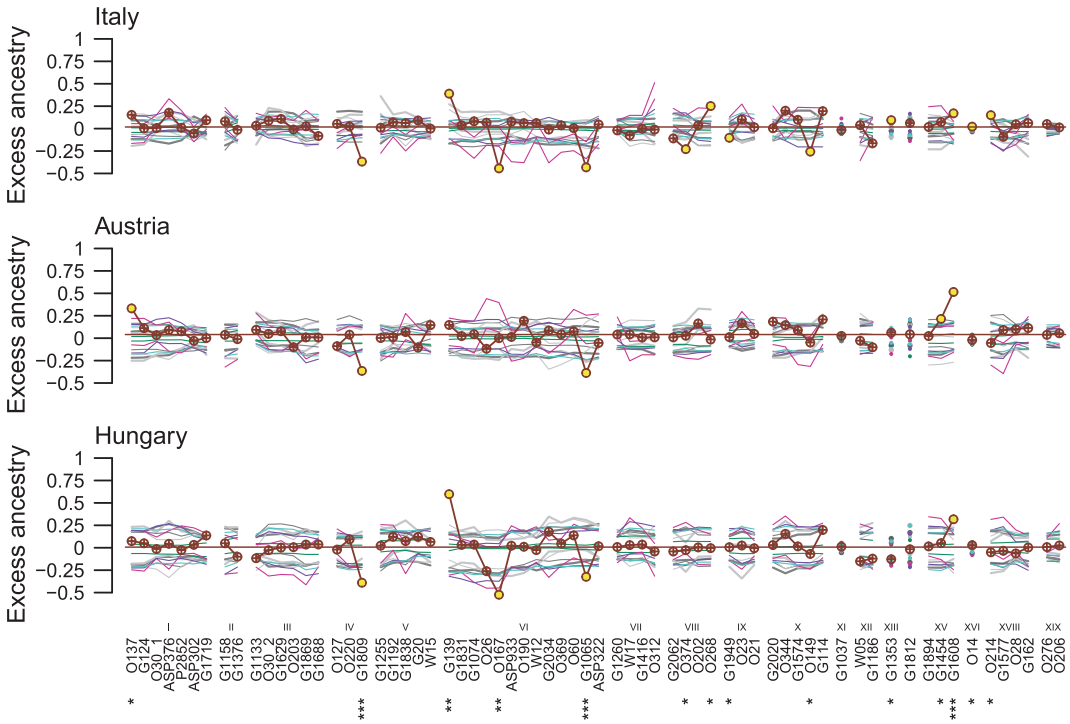

Fig. 5 Excess ancestry for each locus, measured by integrated regression functions of Q on evidence for specific homozygosity, at the three localities (points connected by brown line; see main text for details). Elevated positive or negative values indicate a surplus of *Populus alba* or *Populus tremula* homozygote genotypes, respectively. See Fig. 4 for details on other graphical elements.

for *P. alba* homozygotes, show significant associations among localities (Table 4).

Contrary to our expectations, there was a highly significant, positive association between loci with $\delta > 0.8$ (approximately one half of the markers) and outlier loci with *increased* interspecific heterozygosity. In contrast, there was no association between loci with $\delta > 0.8$ and outlier loci exhibiting *decreased* interspecific heterozygosity (Table 5). Although the power for detecting outliers might increase with δ, this should apply for both increased and decreased heterozygosities. No association between interspecific heterozygosity outliers and divergence (F_{ST}) outliers were found (Text S1, Tables S4 and S5, Fig. S5, Supporting information).

Discussion

Identifying the causes of RI between subspecies and species is a central topic in molecular ecology and evolutionary biology (Wu 2001; Via & West 2008; Nosil *et al.* 2009b; Via 2009; Michel *et al.* 2010). In contrast to speciation genomic studies that exclusively examine pairs of divergent populations or species, the genomic analysis of natural hybrid zones potentially makes feasible the identification of loci that currently contribute to RI. These loci will reduce fitness in hybrids and are expected to leave a detectable signature in recombinant genomes (Barton & Gale 1993; Gompert & Buerkle 2009, 2011; Payseur 2010), particularly as admixture is expected to increase the association between genetic markers and loci under selection due to the generation of large chromosome blocks (Briscoe *et al.* 1994).

In this article, we have focused on the detection of different levels of interspecific heterozygosity as indication for locus-specific hybrid advantage or disadvantage across the genome, and their relation to the extent of locus-specific levels of genetic differentiation between parental species. At the outset of our study, we expected to find *decreased interspecific heterozygosity* and *increased genetic differentiation* for genome regions important for RI, and *increased interspecific heterozygosity* and *reduced genetic differentiation* for loci associated with heterosis, as described in the introduction.

Differences in genomic background and linkage disequilibrium can complicate comparisons of patterns seen in hybrids and their parents (Lexer *et al.* 2007). However, because a remarkable aspect of our results is the great similarity of genomic patterns among different localities, we can focus on features that are repeatable, and thus are unlikely to be strongly affected by stochastic or locality-specific events. We discuss the most salient features of our results in the light of recent efforts to infer the genomic architecture of RI and differential adaptation from hybrid zones among wild species of animals and

Table 4 Correlation and association between replicate localities for interspecific heterozygosity and specific homozygosity, measured by integrated regression functions for 67 codominant markers as in Figs 4 and 5. Spearman's rank correlation tests (*P*-value and correlation coefficient ρ) for integral values; Fisher's exact tests (including *P*-value, odds ratio and 95% CI) and Phi coefficient ϕ for interspecific heterozygosity and homozygosity outlier loci.

Locality	Correlation (integral values)*			Association (only outliers)†					
		Spearman			Fisher's exact test				Phi
		P-value	ρ		*P*-value	Odds ratio	95% CI		ϕ
Italy/Austria	Heterozygosity	0.00000	0.62	Increased heterozygosity	0.03443	6.19	1.10	65.18	0.30
				Decreased heterozygosity	0.00127	33.42	2.78	1842.74	0.52
	Homozygosity	0.00213	0.37	Excess of *Populus alba* homozygotes	0.24872	5.62	0.08	127.12	0.18
				Excess of *Populus tremula* homozygotes	0.00678	Inf	2.11	Inf	0.56
Austria/Hungary	Heterozygosity	0.00000	0.63	Increased heterozygosity	0.00202	10.74	1.86	114.96	0.40
				Decreased heterozygosity	0.02035	Inf	1.13	Inf	0.42
	Homozygosity	0.00034	0.43	Excess of *P. alba* homozygotes	0.08820	25.92	0.27	2437.80	0.39
				Excess of *P. tremula* homozygotes	0.00136	Inf	5.05	Inf	0.81
Hungary/Italy	Heterozygosity	0.00000	0.77	Increased heterozygosity	0.00000	21.31	4.95	134.40	0.61
				Decreased heterozygosity	0.00452	Inf	2.63	Inf	0.62
	Homozygosity	0.00200	0.37	Excess of *P. alba* homozygotes	0.00678	Inf	2.11	Inf	0.56
				Excess of *P. tremula* homozygotes	0.00042	Inf	5.38	Inf	0.69

*Spearman correlation tests were conducted for each locality pair on locus-specific integral values for heterozygosity and homozygosity.

†Fisher's exact tests and Phi coefficients were computed from binary coded, locus-specific variables (outlier/non-outlier), separately for positive or negative deviations from neutral expectation, resulting in four tests of association for each locality pair.

plants. We note that our study system differs from many other hybridizing taxa not only by a high level of genetic differentiation, but also by the huge number of seeds produced per generation, which provides the potential to observe fit recombinant variants even if intense selection is acting on most genotypes.

Genomic patterns in parental populations

The high level of genetic differentiation between the parental species (mean across localities: $F_{ST} = 0.34$; $\delta = 0.71$) reflects their long divergence time of at least several million years (Stettler *et al.* 1996) and indicates the presence of strong RI. In particular, the high δ at most, but not all, markers is consistent with divergent species that still share a portion of their genomes via gene flow (Wu 2001; Fig. 3). The high similarity of patterns of genetic differentiation between *Populus alba* and *Populus tremula* across localities (Fig. 3) is in line with high levels of intra-specific gene flow or consistently acting, intrinsic mechanisms of isolation, which is in turn consistent with highly similar patterns of LSA in hybrids (below).

Genomic patterns of ancestry in hybrids

All three hybrid zones consist primarily of parental-like and genetically intermediate, advanced recombinant hybrid genotypes rather than F_1 hybrids (in fact, not a single admixed individual sampled in the hybrid zones possesses a genotype that is consistent with an F_1 sta-

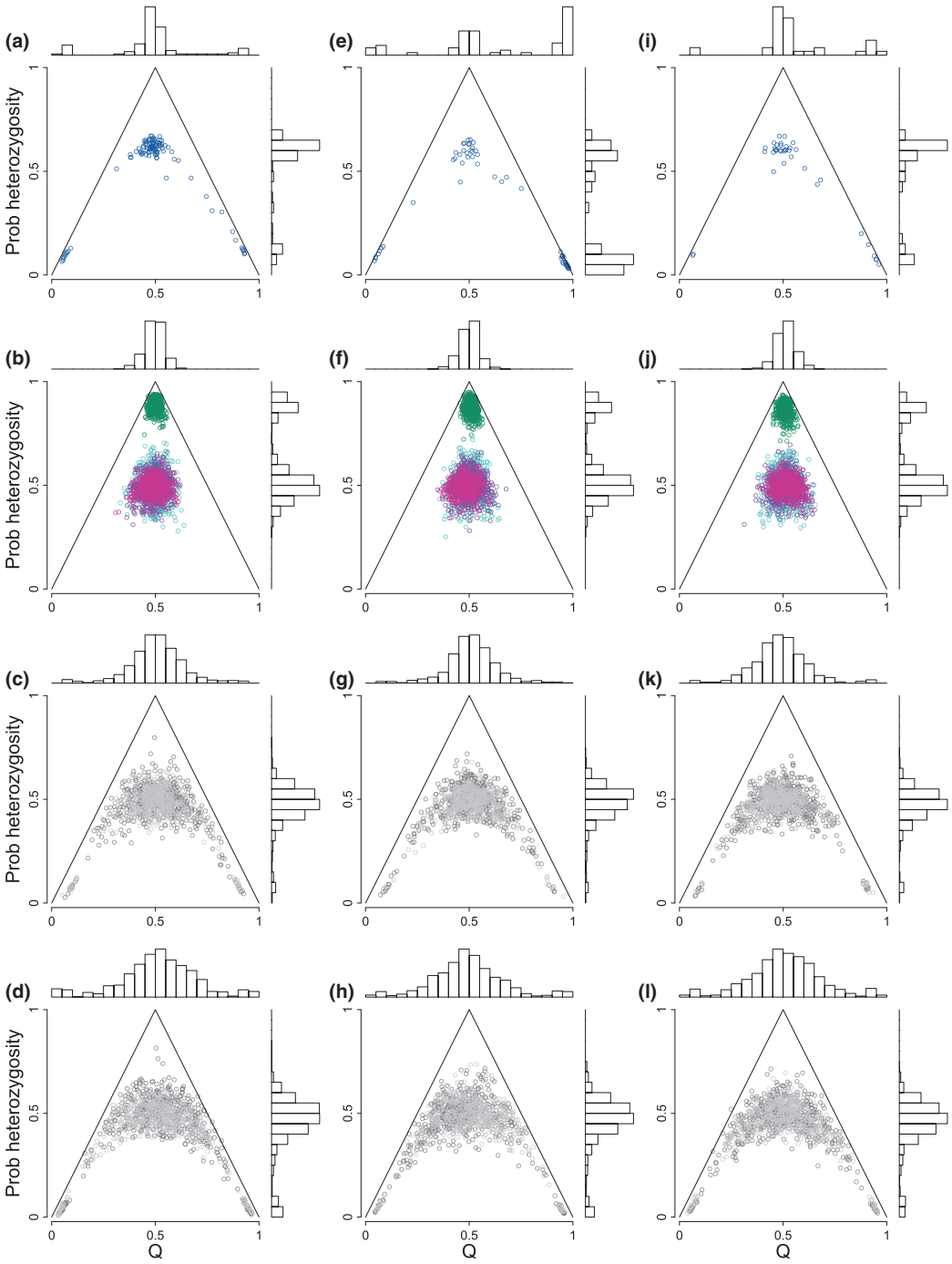

Fig. 6 *Q* vs. individual average probability of interspecific heterozygosity over all 77 markers for Italy (A–D), Austria (E–H) and Hungary (I–L). Lines indicate maximum possible interspecific heterozygosities given *Q*, where decreased interspecific heterozygosity is expected for advanced recombinant hybrids. Histograms at the top and the right of each plot show distributions of *Q* and probabilities of interspecific heterozygosity, respectively, pooled for all hybrid categories shown in the main plot. (A, E, I) Observed values for admixed individuals. (B, F, J) Simulated values for each sample of 500 F_1 (green), F_2 (turquoise), F_5 (purple) and F_{20} (violet-red) hybrids. (C, G, K) Simulated values for each sample of 500 mixed hybrids with 3.8% immigrants from the parentals per generation after seven (M_1; dark gray) and 22 generations (M_2; light gray). (D, H, L) Simulated values for each sample of 500 mixed hybrids with 7.4% immigrants from the parentals per generation after seven (M_3; dark gray) and 22 generations (M_4; light gray).

Table 5 Fisher's exact tests (including *P*-value, odds ratio and 95% CI) and Phi coefficient ϕ for association tests between loci with $\delta > 0.8$, and interspecific heterozygosity outlier loci*

	Association ($\delta > 0.8$, increased heterozygosity)					Association ($\delta > 0.8$, decreased heterozygosity)					Association ($\delta > 0.8$, increased/decreased heterozygosity)				
Locality	*P*-value	Odds ratio	95% CI		ϕ	*P*-value	Odds ratio	95% CI		ϕ	*P*-value	Odds ratio	95% CI		ϕ
Italy	0.0001	8.9	2.6	37.1	0.48	1.0000	1.2	0.1	14.7	0.02	0.0001	8.5	2.6	31.6	0.49
Austria	0.0002	Inf	3.3	Inf	0.45	0.3197	0.4	0.1	2.2	−0.14	0.0619	2.9	0.9	10.5	0.25
Hungary	0.0000	24.2	5.5	155.7	0.64	0.5016	0.0	0.0	7.0	−0.15	0.0000	13.9	3.8	61.8	0.57

*Fisher's exact tests and Phi coefficients were computed from binary coded, locus-specific variables, with $\delta > 0.8$ or outlier status coded as 1. Outlier status was set to 1 with respect to increased heterozygosity, decreased heterozygosity, or heterozygosity outlier regardless of the direction of the deviation.

tus; Fig. 6). The three genotypic classes are separated from each other by striking genotypic gaps (Fig. 2; Figs S3 and S4, Supporting information), indicating the presence of strong reproductive barriers between pure species and between each of them and their hybrids.

The high incidence of interspecific heterozygotes at numerous loci is not expected for advanced hybrid generations beyond the F_1 (Anderson & Thompson 2002). Our finding is also unexpected based on theory of RI achieved by low hybrid fitness through endogenous or exogenous selection against heterospecifics (Barton & Hewitt 1985; Gavrilets 1997; Coyne & Orr 1998; Schluter & Conte 2009) and contrasts with other empirical studies of recombinant hybrid zones (Nolte *et al.* 2009; Teeter *et al.* 2010).

In the following, we discuss our findings on locus specific patterns of ancestry in hybrids, together with estimates of genetic differentiation between the parental genomes. We will focus on (i) the surprising finding of elevated interspecific heterozygosity for many loci in recombinant hybrids and (ii) the similarities in genomic ancestry and genetic differentiation among the three replicate localities. We will touch only briefly on the more predictable findings of reduced interspecific heterozygosity in hybrids and excess ancestry at particular loci.

Loci with increased interspecific heterozygosity in hybrids. Interspecific heterozygosity outliers in admixed individuals were significantly associated with elevated genetic differentiation between the parental species, consistent across localities (Table 5). This combination of high genetic differentiation and increased heterozygosity was unexpected from speciation genetic theory (Orr 1995; Coyne & Orr 1998; Gavrilets 2003). The maintenance of interspecific heterozygosity across large parts of the genome even in advanced recombinant hybrid generations suggests that there is positive selection for these genotypes, as heterozygosity would decay otherwise. Simple additive effects of parental alleles on phenotype expression and ecological selection could explain the encountered pattern (Burke & Arnold 2001), if intermediate genotypes were selected in the hybrid (micro-) habitat, but selected against in the parental (micro-) habitats. However, because of the close geographic proximity between hybrids and parents (Fig. 1; coupled with the presence of extensive root systems and clonal ramet sizes of up to 50 m; van Loo *et al.* 2008), simple additive gene effects and single-locus heterosis seem insufficient explanations for the increased heterozygosities in the presence of the observed genomic discontinuities. Rather, heterozygote advantage depending on epistatic interactions between a set of loci (Barton 2001) appears to be more likely. In particular, coadapted gene complexes might exist within each species due to their long divergence time. Only those hybrids with two complete sets of coadapted gene complexes (one of each parent) might experience high fitness, corresponding to heterosis in F_1 but hybrid breakdown in F_2 generations (outbreeding depression; Lynch 1991; Edmands & Timmerman 2003). As F_1 hybrids are rare in these hybrid zones, combined selection pressures *against* (few) incompatibilities *between* species and *for* coadapted gene complexes *within* species might explain the surplus of heterozygotes at many loci, leading to genotypes that partially resemble F_1's. Furthermore, single genetic regions that are part of coadapted gene complexes will be less likely to introgress as this will separate them from their genomic background. Consequently, pronounced genetic differentiation between species will be found at coadapted loci (e.g. those with increased interspecific heterozygosities in recombinant hybrids), a pattern that we detected in our data (Table 5). Coadapted gene complexes have been suggested to be important in other empirical studies on hybrid fitness (Hegarty *et al.* 2009; Pritchard *et al.* 2011) and remain to be tested explicitly for *Populus* in the future. In addition, it would be worthwhile to conduct more detailed simulation studies in order to investigate if the observed genomic patterns could be recovered by

the mechanisms proposed here, and to perform experiments to test for associations between genotype and fitness. Common garden experiments with open pollinated progeny from both species and their hybrids have been established to address the latter issue.

We note that heterozygote advantage is an expected genomic feature of long-lived, widely distributed forest trees that experience enormous environmental variation in space and time (Petit & Hampe 2006), and heterosis has been demonstrated to be important in *Populus* (Li *et al.* 1998). It is thus likely that our results (i.e. maintenance of heterozygosity through two complete sets of coadapted gene complexes in advanced hybrids) can be explained in part by the longevity and sessile nature of trees such as *Populus* spp. in unstable and dynamic habitats.

Similarity across hybrid zone replicates. The highly variable patterns of ancestry across the genome are in stark contrast with the striking similarities of major LSA patterns across replicate localities (Figs 4 and 5, Table 4). These similarities are unlikely to stem entirely from high inter-population gene flow, as genetic differentiation (F_{ST}) between localities is significant and δ is appreciably high (Table 2). Also, as the Ticino (Italy) and Danube/Tisza (Austria/Hungary) river drainage systems were populated via different postglacial migration routes (Fussi *et al.* 2010), we assume a fair degree of independence between the hybrid zone replicates. The missing isolation-by-distance pattern among the three hybrid populations further indicates a lower connectivity between the hybrid localities compared to the parental species (Table 2). The geographic similarity of LSAs – and the uniformly strong discontinuities between the genomes of parents and hybrids (Fig. 2) – rather point to an important role for intrinsic selection in the maintenance of RI. The similarity in genomic patterns of LSA across all three hybrid zones suggests that only a limited number of interspecific allelic combinations are selected. The longevity of *Populus* trees (magnified further by clonality; Dickmann & Kuzovkina 2008; van Loo *et al.* 2008) coupled with abundant seed produced by a single tree in a single year (up to 50 million; Wyckoff & Zasada 2002) imply a great potential for postzygotic selection to 'filter' fit gene combinations in recombinant hybrids.

Loci with reduced interspecific heterozygosity and excess ancestry. Underdominance is an expected genomic feature of hybrids between divergent species, and several such cases of elevated specific homozygosity were indeed observed in our study, predominantly associated with excess ancestry from one or the other parental species (G1809, G139, O167, G1065, G1608; Figs 4 and 5). As selection against heterospecific genotypes can drive alleles from one parent to low frequency in the hybrids (e.g. the derived alleles for BDM incompatibilities, or because of drift), it is difficult to distinguish patterns arising from directional selection from those arising due to underdominance alone (Nolte & Tautz 2010; Gompert & Buerkle 2011; Gompert *et al.* 2012b). Simultaneously high genetic differentiation for three loci (G139, O167, G1608; $\delta \geq 0.96$ at all localities; Table S2, Supporting information) suggests that at least some genetic loci showing excess ancestry are linked to regions involved in species divergence rather than adaptive introgression. Our simulations showed that drift alone can generate magnitudes of homozygote excess very close to that of the observed values, although it is unlikely that this will affect the same loci at multiple localities.

RI between diploid hybrids and their parental species

The conspicuous genotypic gaps between admixed and parental individuals (Fig. 2), reflecting the fact that hybrids remain essentially distinct despite genetic contact with their parents, are reminiscent of patterns expected for an incipient hybrid species (Nolte & Tautz 2010). However, it remains unclear if recombinant *P.* × *canescens* can be regarded as an independent and stabilized hybrid lineage yet, particularly as (i) the most likely number of clusters detected by STRUCTURE was indeed two at all three localities; and (ii) hybrids occur in close geographic proximity to parental individuals and in the same flood-plain habitats as *P. alba*, a condition unlikely to result in homoploid hybrid speciation (Buerkle *et al.* 2000; Gross & Rieseberg 2005). Further, it is unknown whether hybrids are able to persist independently from their parentals. Potential differences in micro-habitat (i.e. niche breadths of hybrids and parentals) and the degree of reproductive independence of *P.* × *canescens* and their potential status as an incipient hybrid species require further study in the future, but will not change our findings on RI between the parental species. Homoploid hybrids are common in many organismal groups (Burke & Arnold 2001; Mallet *et al.* 2007), and thus our results on the genomics of RI obtained here, although certainly not generalizable, may be of relevance for the genetics of speciation in many other taxa.

The discontinuity between recombinant hybrids and their parental species is apparently associated with strong RI, in line with the absence of F_1 hybrids in our data. Because a large part of the genome of the admixed individuals is still heterospecific, a generally low fitness of intermediates is unlikely to be the cause. Rather, as only particular genetic recombinations prevail in hybrids, the presence of coadapted gene complexes within each species might explain a large proportion of RI that is maintained between these highly divergent taxa.

Conclusion

By comparing genomic patterns of ancestry in hybrids with genomic patterns of genetic differentiation between their parental species across three replicate localities, we were able to obtain insights into the genomic consequences of advanced stages of RI between two highly divergent, hybridizing forest trees. Many of the highly differentiated loci were not associated with RI through underdominance, but rather showed markedly increased interspecific heterozygosity in hybrids. Hybrid zone replicates exhibited very similar genomic compositions, pointing to an important role for intrinsic barriers. These appear to be built after initial divergence by complex epistatic interactions (e.g. coadapted gene complexes, or BDM networks), potentially in combination with ecological selection, where only certain genotypes are supported in hybrid genomes. Our results indicate that epistatic interactions within genomes might be more important in maintaining (and potentially, completing) RI than negative interactions between single genetic regions of divergent species of *Populus*. The strong genomic discontinuities seen between recombinant hybrids and their parents in all three localities indicate that *Populus* hybrids represent strong genotypic filters, maintaining integrity of their parental species in the face of gene flow.

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This study forms part of D.L. PhD project on the genetics of reproductive isolation and trait differences in European *Populus* in C.L. group. C.A.B. and C.L. share broad interests in the evolutionary genomics of adaptation and speciation, and T.B. contributed as a Post-Doc in the group. B.H., S.C. and D.B. have long-standing interests in forest tree biology, conservation genetics and breeding.

Data Accessibility

Microsatellite data are accessible as STRUCTURE input files in the Supporting information.

Supporting information

Fig. S1 Cline estimates for all 77 markers used in this study and all three localities.

Fig. S2 Most likely number of genetic clusters k at the three localities.

Fig. S3 Locus specific ancestry (LSA) described by evidence for interspecific heterozygosity at the three localities for all potentially admixed plus eight of each of the parental-like individuals (y-axis), ordered by Q value as in Fig. 2, main article (at each locality: top, *P. alba* like; middle, admixed; bottom, *P. tremula* like), analyzed with 77 markers across 19 chromosomes in map position (x-axis).

Fig. S4 Locus specific ancestry (LSA) described by evidence for specific homozygosity at the three localities for all potentially admixed plus eight of each of the parental-like individuals (y-axis), ordered by Q value as in Fig. 2, main article (at each locality: top, *P. alba* like; middle, admixed; bottom, *P. tremula* like), analyzed with 77 markers across 19 chromosomes in map position (x-axis).

Fig. S5 Summary for 67 codominant loci for the Italy hybrid zone, including interspecific heterozygosity and excess ancestry in admixed individuals, and divergence- and diversity-based genome scans involving the two parental species *P. alba* and *P. tremula*.

Table S1 General information about 77 microsatellite markers used in this study, including published name, position on scaffold (in bp), distance used for STRUCTURE linkage runs, marker type (codominant or dominant scoring), and primer sequences.

Table S2 Locus-specific measurements of genetic differentiation between *P. alba* and *P. tremula* for 67 codominant loci, including number of alleles (N_A), F_{ST}, Hedrick's G'_{ST}, and δ, for each of the three replicate localities.

Table S3 Locus-specific measurements of observed (H_O) and expected heterozygosity (H_E) for *P. alba* and *P. tremula* for 67 codominant loci at the three replicate localities.

Table S4 Results of outlier scans for 67 codominant markers at the three replicate localities.

Table S5 Fisher's exact tests (including P-value, odds ratio and 95% CI) and Phi coefficient ϕ for tests of association between loci identified as being under selection by genome scans involving the parental species using BAYESCAN, LOSITAN, or Ewens-Watterson tests (EW; results for *P. alba* and *P. tremula* are pooled), and loci showing interspecific heterozygosities exceeding simulated 95% CIs in admixed individuals (see main article, Fig. 4, and Text S1 for more details on outlier scans)

Text S1 Genome scans for parental populations.