Climate and hybridization shape stomatal trait evolution in *Populus*

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Abstract

● Stomata play a critical role in regulating plant responses to climate. Where sister species
differ in stomatal traits, interspecific gene flow can influence the evolutionary trajectory
of trait variation, with consequences to adaptation.

● Leveraging six latitudinally-distributed transects spanning the natural hybrid zone
between *Populus trichocarpa–P. balsamifera*, we used whole genome resequencing and
replicate common garden experiments to test the role that interspecific gene flow and
selection play to stomatal trait evolution.

● While species-specific differences in the distribution of stomata persist between *P.*
balsamifera and *P. trichocarpa*, hybrids on average resembled *P. trichocarpa*. Admixture
mapping identified several candidate genes associated with stomatal trait variation in
hybrids including *TWIST*, a homolog of *SPEECHLESS* in *Arabidopsis*, that initiates
stomatal development via asymmetric cell divisions. Geographic clines revealed
candidate genes deviating from genome-wide average patterns of introgression,
suggesting restricted gene flow and the maintenance of adaptive differences. Climate
associations, particularly with precipitation, indicated selection shapes local ancestry at
candidate genes across transects.

● These results highlight the role of climate in shaping stomatal trait evolution in *Populus*
and demonstrate how interspecific gene flow creates novel genetic combinations that may
enhance adaptive potential in changing environments.

Keywords: ecophysiology, introgression, trees, evolution, hybrids, adaptation

Introduction

Hybridization is an important source of variation that enhances adaptive evolution by
creating new recombinant genotypes and increasing standing genetic variation (Rieseberg &
Wendel, 1993; Burke & Arnold, 2001; Brauer *et al.*, 2023). The movement of alleles from one
species into the genetic background of another via repeated backcrossing can facilitate adaptive
evolution (Hamilton & Aitken, 2013; Hamilton *et al.*, 2013; Hamilton & Miller, 2016; Suarez-
Gonzalez *et al.*, 2018b,c; Menon *et al.*, 2021). This is particularly notable in plant species, where
adaptive introgression has been associated with traits related to water use that are critical to
maintaining physiological function under changing environmental conditions (Welch &
Rieseberg, 2002; Campbell, 2004; Wu & Campbell, 2007; Whitney *et al.*, 2010; Campbell &
Wendlandt, 2013; Suarez-Gonzalez *et al.*, 2018c). Despite potential benefits from introgression,
fine-scale assessments of the genetic variation underlying adaptive trait variation and the role of
extrinsic selection to introgression across different genomic backgrounds remains limited. This
study aims to understand how natural selection shapes the movement of genetic variation across
species boundaries that is essential for predicting how recombinant genotypes may respond to
climate change.

Stomatal traits are fundamental to photosynthesis, transpiration, and overall water
balance, and exhibit substantial variation across species (Hetherington & Woodward, 2003;
Drake *et al.*, 2013; Marek *et al.*, 2022; Chen *et al.*, 2024). These microscopic pores on the leaf
surface regulate CO_2 uptake and water vapor loss, making them key targets of selection under
changing environmental conditions (Chen *et al.*, 2024). Stomata have evolved variation in size,
density, and distribution across upper and lower leaf surfaces to optimize trade-offs associated
with carbon uptake, water-use efficiency, and pathogen exposure across environments
(Hetherington & Woodward, 2003; Dittberner *et al.*, 2018; Chen *et al.*, 2024). Natural hybrid
zones provide ideal systems to understand how varying genomic and environmental backgrounds
shape the broad and fine-scale evolutionary trajectory of stomatal traits. Genomic recombination
via hybridization and subsequent introgression can produce new heritable phenotypes, including

traits intermediate or in excess of the range observed in either parent species that may be

beneficial for adaptation under changing environmental conditions (Campbell, 2004; Campbell

& Wendlandt, 2013; Hamilton & Miller, 2016; Janes & Hamilton, 2017). Thus, identifying genes

underlying stomatal variation and tracking their movement across species boundaries may reveal

how interspecific gene flow facilitates adaptive trait evolution required for climate adaptation.

One approach to assessing the impact of gene flow into a contact zone and selection

against recombination in a hybrid zone is geographic cline analysis, which investigates spatial

gradients in traits or allele frequencies where species interbreed (Barton, 1979; Barton & Gale,

1993). Comparing geographic clines across repeated hybrid zones for traits, genome-wide

ancestry, and candidate genes underlying adaptive traits allows an evaluation of how gene flow

between species varies across genomic and environmental backgrounds (Stankowski *et al.*, 2017;

Schield *et al.*, 2024). However, as clines may arise from either intrinsic or extrinsic selection,

geographic cline analysis alone cannot distinguish the mechanisms facilitating or limiting genetic

exchange (Hamilton & Aitken, 2013; Janes & Hamilton, 2017; Capblancq & Després, 2020). To

address this gap, environmental associations may be used to identify the role extrinsic forces

may play in adaptive introgression. Integrating geographic cline analyses with environmental

associations can help identify traits or genes that move across both environmental and genomic

backgrounds and are critical to adaptation under future climates.

Populus is an evolutionary and ecological model tree that has become an invaluable

system for understanding how interspecific gene flow and natural selection shape trait variation

(Taylor, 2002; Lexer *et al.*, 2004; Buerkle & Lexer, 2008). In this study, we focus on the natural

hybrid zone between *P. trichocarpa × P. balsamifera*, two sister species that have evolved in

response to contrasting environmental conditions. The geographic distribution of *P. trichocarpa*

comprises mild, moist environments of the Pacific Northwest, in contrast to *P. balsamifera,*

whose distribution is confined to colder, drier boreal regions that experience greater seasonal

extremes (Suarez-Gonzalez *et al.*, 2018b,a). These environmental differences have contributed to

the evolution of species-specific stomatal trait variation. On average, *P. trichocarpa* has large

stomata on both the upper (adaxial) and lower (abaxial) leaf surfaces, a pattern consistent with

amphistomaty (McKown *et al.*, 2014, 2019). In contrast, *P. balsamifera* typically exhibits

smaller, more densely packed stomata that are mostly restricted to the lower leaf surface,

reflecting hypostomaty (Soolanayakanahally *et al.*, 2009; Pointeau & Guy, 2014). Previous work

has indicated that the movement of genomic regions from *P. balsamifera* into the genomic

background of *P. trichocarpa* have contributed to adaptive trait evolution linked to persistence in

colder and drier environments (Suarez-Gonzalez *et al.*, 2018b). However, less is known about

how genetic variation underlying stomatal traits moves across species boundaries (but see, Fetter

& Keller, 2023), or how climate may influence that movement.

Here, we identify genetic variation underlying stomatal traits and compare the broad- and

fine-scale role of geography and climate in shaping the movement of genetic variation across

repeated contact zones between *P. trichocarpa* and *P. balsamifera*. Specifically we ask: 1) How

do hybrids vary in stomatal traits relative to *P. trichocarpa* and *P. balsamifera*? 2) To what

extent do stomatal traits vary across repeated contact zones, and are these patterns shaped by

local climatic gradients? 3) What genes underlie stomatal trait variation, and how does the

environment interact to influence the extent and direction of introgression for candidate genes

across contact zones? This work identifies key candidate genes related to stomatal trait variation

in *Populus* and their relationships to climate gradients providing a basis to predict whether and

how genetic variation underlying adaptive traits may shift across species and environmental

gradients under climate change.

Material and Methods

Study Approach

Sample collection and library preparation

In this study, we analyzed 574 genotypes collected within the natural contact zone

between *Populus trichocarpa* × *P. balsamifera* hybrid zone (Fig. 1, Table S1; see Bolte *et al.*,

2024 for full details). Dormant vegetative cuttings were sampled from six contact zones spanning

a latitudinal gradient, referred to here as Alaska, Cassiar, Chilcotin, Jasper, Crowsnest, and

Wyoming transects, respectively. Cuttings were propagated clonally under greenhouse

conditions (24 °C day/15.5 °C night, no supplemental lighting) at the Virginia Tech Reynold's

Homestead Forest Resource Research Center (FRRC) in Stuart, VA, USA. Once established,

100 mg of fresh leaf tissue was collected from each genotype for DNA extraction and whole-

genome resequencing. Methods for propagation, DNA extraction, library preparation,

sequencing, and quality filtering are fully described in Bolte et al. 2024. Genomic libraries were sequenced on an Illumina NovaSeq 6000 using an S4 flow cell in 2×150 bp paired-end format, with 64 samples per lane. Illumina reads for each genotype were aligned to the *Populus trichocarpa* reference genome (v4.0), generating SAM files that were subsequently converted to BAM format using SAMtools (Li *et al.*, 2009). Individual gVCF files were generated using the HaplotypeCaller algorithm in GATK v3.7 and subsequently combined into a single VCF file using the GenotypeGVCFs function. The initial dataset included ~82 million variants and was filtered based on mapping quality (MQ < 40.00), strand bias (FS > 40.000, SOR > 3.0), mapping quality rank sum (MQRankSum < –12.500), read position bias (ReadPosRankSum < –8.000), and depth of coverage (QD < 2.0). INDELs and SNPs with more than two alternate alleles were excluded and then filtered to remove SNPs with a minor allele frequency below 5% and more than 10% missing data, resulting in a final dataset of 7,361,750 biallelic SNPs for downstream analyses. Additionally, thirty individuals were excluded based on ancestry estimates from Bolte et al. (2024) as their genomic composition did not correspond to either parental species.

Common gardens design

In March 2020, the 574 clonally propagated rooted *Populus* cuttings were planted across three common garden environments (Fig. 1). The common gardens, located at the FRRC in Stuart, VA (36° 37' N and 80° 09' W, elevation 359 m), Burlington, VT (44° 26' N and 73° 11' W, elevation 130 m) and Fargo, ND (46° 53' N and 96° 48' W, elevation 272 m) were established spanning both latitudinal and longitudinal gradients in temperature and precipitation. At each site, genotypes were planted in a randomized complete block design with three replicates per genotype, resulting in a total of 1,722 individuals per site.

Quantifying stomatal traits in replicated common gardens

During the summer of 2022, stomatal traits and associated measures were assessed for each genotype across three common garden experiments (see Table1 for list). For each genotype, the first fully expanded leaf on the dominant shoot was used to assess stomatal conductance, size, density, and distribution on both the adaxial (upper) and abaxial (lower) leaf surfaces. This leaf was selected to minimize variation due to leaf age and environmental effects between samples (Fetter *et al.*, 2021). Stomatal conductance (g_{sw}, mmol m^{-2} s^{-1}) was measured using a

LI-600 porometer in three non-overlapping areas per each fully expanded leaf without detaching

the leaf from the plant. Individual g_{sw} values represent the average of these three measurements.

Following g_{sw} measurements, leaves were collected to assess stomatal density, size, and

distribution. A layer of Newskin liquid bandage was applied to the upper and lower leaf surfaces

to make two stomatal impressions to capture variation across leaf surfaces. Stomatal impressions

from one leaf per individual were mounted on slides without a cover slip, and images were

captured using an Olympus BX-53 microscope equipped with an Olympus DP23 digital camera.

Micrographs were standardized to a 0.37×0.25 mm grid for analysis. Stomatal size traits were

measured and analyzed independently for each leaf surface using ImageJ (Abràmoff *et al.*,

2004). These included adaxial (G_U, µm) and abaxial (G_L, µm) guard cell length, as well as

adaxial (P_U, µm) and abaxial (P_L, µm) pore length. Stomatal measurements were obtained by

overlaying four equally spaced lines across each micrograph, selecting five stomata per image.

Individual values represent the average of five measurements per leaf surface. Stomatal density

was estimated as the number of stomata per unit leaf area for the adaxial (D_U, mm^{-2}) and abaxial

(D_L, mm^{-2}) leaf surfaces using automated counts in LeafNet (Li *et al.*, 2022). To validate

automated density measures, micrographs from 100 random genotypes were manually counted

and measured. A strong correlation was observed between automated and manual counts ($\rho =$

0.95, $p < 0.05$, Fig. S1); therefore, automated values generated by LeafNet were used for all

genotypes. Total stomatal density (D_T) was also calculated as the sum of D_U and D_L, representing

the overall number of stomata per unit leaf area. To estimate the degree of amphistomy, stomatal

ratio (SR) was calculated as the ratio of adaxial stomatal density divided by the total stomatal

density. SR values range from 0 to 1, with 0 indicating stomata are present only on the abaxial

surface, 0.5 indicating equal density on both surfaces, and 1 indicating stomata are exclusive to

the adaxial surface (Fetter *et al.*, 2021). To capture variation in adaxial stomatal occurrence (O_U)

among genotypes we assessed the presence (1) or absence (0) of adaxial stomata.

In addition to stomatal trait variation, intrinsic water-use efficiency ($\delta^{13}C$, ‰) was

assessed using the second fully expanded leaf. Leaves were collected and dried at 60°C until a

constant mass was reached. Dried leaf tissue was then homogenized into a fine powder using a

TissueLyser II (Qiagen, Hilden, Germany). Approximately 2–3 mg of the homogenized tissue

was placed into a tin capsule (Costech, Valencia, CA, USA) for analysis. Carbon isotope

composition (δ¹³C, ‰) was measured at the Central Appalachians Stable Isotope Facility (CASIF), Appalachian Laboratory (Frostburg, Maryland, USA).

Comparing variation in stomatal traits among hybrid genotypes relative to parental species

To test whether hybrids exhibit intermediate, transgressive, or parental-like stomatal traits, we first estimated the genetic values of each genotype for each trait by calculating best linear unbiased predictors (BLUPs) using R 4.3.1 (R Core Team 2024). BLUPs were estimated for each trait for each genotype across the three common gardens experiments using the following linear mixed model.

$$y_{ijkl} \sim \mu + E_i + B_{i(j)} + G_k + \varepsilon_{ijkl}$$

Where y_{ijkl} is the observed trait value for clonal replicate l, E_i is the fixed effect of the common garden experiment, $B_{i(j)}$ is the random effect of block j nested within garden i, G_k is the random effect of genotype, and ε_{ijkl} is the residual term. We then extracted the genotype random effects (G_k), which represents each genotype's estimated genetic contribution to the trait after accounting for garden and block effects for use in downstream analyses.

Individuals were classified into three genotypic classes based on admixture proportions from Mead *et al.*, (2025) to evaluate species-specific and hybrid differences in stomatal traits. Using ancestry estimates ($K = 2$), genotypes with Q > 0.98 were assigned to *P. trichocarpa* ($n = 132$), and those with Q < 0.02 to *P. balsamifera* ($n = 91$). Remaining genotypes ($n = 323$) were categorized as hybrids. To test for differences in stomatal traits among *P. trichocarpa*, *P. balsamifera*, and their hybrids, we first assessed homogeneity of variance using Levene's test in R 4.3.1 (RStudio Team 2024). For traits that met the assumptions of homogeneity, we used one-way ANOVA followed by Tukey's HSD for post hoc comparisons. For traits that violated this assumption, we applied the non-parametric Kruskal–Wallis test with Dunn's post hoc comparisons. Four out of eleven stomatal traits, including g_{sw}, D_U, SR, and O_U, did not exhibit homogeneity of variance across groups and were therefore analyzed using the non-parametric tests.

Admixture mapping to identify the genetic basis of stomatal variation

To identify the genetic basis of stomatal trait variation and water-use efficiency, we

performed admixture mapping using local ancestry inferred with Loter (Dias-Alves *et al.*, 2018)

and phenotype-genotype association conducted with GEMMA (Zhou & Stephens, 2014). Local

ancestry inference relies on phased haplotypes from reference parental genotypes to infer the

ancestral origin of specific genomic regions in admixed genotypes (Dias-Alves *et al.*, 2018) Prior

to local ancestry inference, the VCF file was phased and missing data imputed using BEAGLE

(Browning *et al.*, 2018, 2021). Parental reference genotypes for local ancestry inference were the

same as those used for trait comparisons as defined above. These phased reference haplotypes

were used to infer local ancestry across the genomes of 323 admixed individuals.

To associate genomic variation with trait variation we used Univariate Linear Mixed

Models implemented in GEMMA v.0.94.1 (Zhou & Stephens, 2014). A relatedness matrix

(estimated with the -gk 1 option) was included as a covariate to account for relatedness among

individuals. Global ancestry, estimated as the average local ancestry across all loci, was also

included as a covariate to account for population structure (Fetter & Keller, 2023). To account

for multiple testing, the significance threshold was adjusted using the admixture burden

(0.05/2719), which estimates the number of independently recombining chromosomal segments

within a genotype. Admixture burden was estimated following the method described by Shriner

et al., (2011). Specifically, an autoregressive (AR) model was fitted to the local ancestry

sequence of each genotype, and the spectral density of local ancestry values at frequency zero

was estimated using the spectrum0.ar function from the coda package in R 4.3.1. The number of

independent ancestry blocks per individual was then summed and averaged across all individuals

to estimate the mean effective number of independent tests in our dataset (Shriner *et al.*, 2011).

This value was used to adjust the significance threshold for admixture mapping. Manhattan plots

were generated using the qqman package in R to visualize admixture mapping results, with

significance assessed based on the admixture burden threshold (Turner, 2018).

To identify candidate genes associated with stomatal traits, we mapped loci that passed

the admixture burden threshold to the *P. trichocarpa* v4.1 gene annotation. To account for

upstream regulatory regions, gene boundaries were extended by 2 kb upstream (positive strand)

or downstream (negative strand). We performed manual BLAST searches on TAIR

(www.arabidopsis.org) to identify orthologous genes in *Arabidopsis thaliana*, and selected the

best hits based on sequence similarity. When multiple genes were located within the same

window, all were initially retained as candidates. Each gene was then evaluated for its potential

role in stomatal development and function. Candidate gene-specific ancestry values were

extracted from the ancestry matrix using gene positions from the *P. trichocarpa* v4.1 reference

genome, including a ±500 bp flanking region. For each admixed individual, locus-specific

ancestry at each gene was classified as homozygous *P. trichocarpa* (1), homozygous *P.*

balsamifera (0), or heterozygous (0.5).

Geographic clines to identify barriers to gene flow for stomatal traits, candidate genes and

genome-wide ancestry

To quantify the extent and direction of introgression for stomatal traits and associated

candidate genes, we fitted geographic clines separately for each of the six contact zones.

Geographic distances from the coast for each genotype within each transect were calculated

using the Haversine formula and scaled between 0 and 1 to allow comparisons across contact

zones (see Supplementary Methods). Geographic cline parameters, including center and width

(1/ maximum slope, Derryberry *et al.*, 2014), were estimated using genotype-specific BLUP

values for traits and ancestry genotypes for candidate genes using HZAR (Derryberry *et al.*,

2014).

For each trait, six different cline models were fit for each contact zone using different

tails (both, left, right, mirrored, none, null). Each model was run with a burn-in of 100,000

iterations followed by 500,000 Monte Carlo iterations to estimate posterior distributions of cline

parameters. The best-supported model for each trait in each contact zone was identified using

corrected Akaike Information Criterion (AICc), selecting the model with the lowest AICc score.

Cline parameter estimates based on the best-fit model were compared across contact zones.

For each candidate gene, ten different cline models using different combinations of

scaling (free or fixed) and tails (both, left, right, mirrored, or none), in addition to the null model

(no cline) were fit for each geographic transect. The inclusion of scaling and tail combinations

allowed us to capture the full range of potential variation in ancestry genotypes across the

contact zones. As with the trait clines, each gene cline model was run with 100,000 burn-in

iterations and 500,000 Monte Carlo iterations. AICc scores were used to select the best-fitting

model for each candidate gene and contact zone, and cline parameter estimates from these

models were compared across contact zones. We compared fine-scale introgression of individual

candidate genes with genome-wide ancestry, therefore cline parameters were estimated for

genome-wide ancestry within each contact zone.

Modeling the influence of climate on stomatal traits, candidate genes and genome-wide
ancestry

To complement geographic cline analyses, which identifies barriers to gene flow across

space, we modeled climatic clines to test whether climate predicts variation in stomatal traits,

candidate genes, and genome-wide ancestry within each contact zone. Twenty-five annual

climate normals (1961–1990) were obtained from ClimateNA (Wang *et al.*, 2016) using the

latitude, longitude, and elevation of each genotype origin. Due to correlations among climate

variables, we conducted a principal component analysis (PCA) using the prcomp function in R

4.3.1 to reduce multicollinearity and summarize climatic variation across genotypes based on

climate of origin. PC1 explained 55% of climate variation, and captured temperature-related

gradients, with high loadings for mean annual temperature and degree-days above 5 °C and

18 °C. PC2 explained 26% of climate variation, and captured moisture-related gradients, with

high loadings for mean annual precipitation, climate moisture index, and relative humidity

(Table S1, Figure S2). Given that PC1 and PC2 encompassed the major axes of climate variation,

subsequent analyses focused on these two PC axes.

For each trait and contact zone, linear regressions were fit to quantify the relationship

between climate and trait variation. Genotype-specific BLUP values were used as the response

variable, and the two first climate principal components (PC1 and PC2) as predictors within the

lm function in R 4.3.1 (R Core Team, 2024) following the equation:

$$y_i \sim \mu + E1_i + E2_i + \varepsilon_i$$

where y_i is the BLUP-estimated trait value for genotype *i*, μ is the intercept, and $E1$ and

$E2$ represent the scores for PC1 (temperature-related gradient) and PC2 (moisture-related

gradient), respectively, and ε_i is the residual error term. An interaction term between PC1 and

PC2 was included in initial models, but was not significant for the majority of trait–transect

combinations. To assess whether the direction and strength of climate–trait relationships varied

across transects, slopes were compared across contact zones.

292   For each candidate gene, we tested whether variation in gene ancestry was associated

293 with climatic gradients using logistic regression models implemented within the glm() function

294 in R 4.3.1. We fit a single model for each gene in each transect, including both PC1 and PC2 as

295 well as their interaction (PC1 × PC2) as predictors following the equation:

296        $$logit(P(y_i)) \sim \mu + E1_i + E2_i + E1_i * E2_i + \varepsilon_i$$

297   Where, where y_i is the BLUP-estimated trait value for genotype i, μ is the intercept, and

298 $E1$ and $E2$ represent the scores for PC1 (temperature-related gradient) and PC2 (moisture-related

299 gradient), respectively. $E1_i * E2_i$ represents the interaction term between PC1 and PC2, and ε_i is

300 the residual error. To compare climatic associations between candidate genes and the genome-

301 wide average, the same model was fit using genome-wide ancestry as the response variable.

302 Slope values from candidate gene models were compared to those from the genome-wide models

303 within each contact zone to evaluate differences in the strength and direction of climate–ancestry

304 relationships. Slopes were also compared across contact zones for each candidate gene to

305 evaluate whether climate–gene ancestry relationships vary.

306 **Results**

307 **Species-specific and hybrid variation in stomatal traits between *Populus balsamifera* and *P.**

308 *trichocarpa***

309   Parental genotypes of *Populus trichocarpa* and *P. balsamifera* differed significantly in

310 several stomatal traits, with hybrids exhibiting intermediate values or resembling one of the

311 parental species (Fig. 2A, Table 1). On average, *P. trichocarpa* exhibited reduced abaxial guard

312 cell length (G_L) compared to *P. balsamifera* ($p < 0.05$, Fig. 2A, Table S2), while adaxial guard

313 cell length (G_U) did not differ significantly between parental species (Fig. S3). In hybrids, G_L

314 was similar to *P. balsamifera* ($p > 0.05$, Fig. 2A); however, hybrids exhibited a broader range in

315 G_L than either parental species. Overall, G_U ($F_{2,496} = 9.36$, $p > 0.05$) and adaxial pore length (P_U;

316 $F_{2,386} = 2.91$, $p > 0.05$, Fig. S3) were not significantly different between parental and hybrid

317 genotypes. In contrast, abaxial pore length (P_L) differed significantly among genotype classes

318 ($F_{2, 496} = 4.39$, $p < 0.05$, Fig. S3); post hoc comparisons showed that hybrid genotypes had

319 significantly greater P_L than *P. trichocarpa* ($p < 0.05$) but did not differ from *P. balsamifera* ($p >$

320 0.05). Hybrids also exhibited a greater range of P_L values relative to parental species.

P. balsamifera had significantly lower total stomatal density (D_T) than *P. trichocarpa* (p

< 0.05; Fig. 2A). Hybrid genotypes did not differ significantly from *P. balsamifera* ($p > 0.05$),

but their D_T was significantly lower than *P. trichocarpa* ($p < 0.05$) and their range of phenotypic

values exceeded both parental species. Abaxial stomatal density (D_L) did not differ significantly

between the parental species ($p < 0.05$), although *P. trichocarpa* tended to exhibit higher D_L than

P. balsamifera (Fig. S3). Hybrid genotypes had significantly lower D_L than *P. trichocarpa* ($p <$

0.05) but did not differ from *P. balsamifera* ($p > 0.05$). Compared to both parental species, the

range of hybrid D_L values was greater. *P. trichocarpa* exhibited significantly greater adaxial

stomatal occurrence (O_U) and density (D_U) than *P. balsamifera* ($p < 0.05$, Fig. 2A). Hybrid

genotypes did not differ significantly from *P. trichocarpa*, with greater O_U and a comparable

range of traits values. *P. trichocarpa* exhibited a more even distribution of stomata across leaf

surfaces, reflected in a significantly higher stomatal ratio (SR) compared to *P. balsamifera*,

which had most stomata restricted to the lower surface ($p < 0.05$, Fig. 2A). Hybrids resembled *P.*

trichocarpa, with a higher SR ($p > 0.05$) and similar range of values. *P. trichocarpa* showed

significantly lower stomatal conductance (g_{sw}) and higher water-use efficiency ($\delta^{13}C$) than *P.*

balsamifera ($p < 0.05$; Fig. 2A). In hybrids, g_{sw} was intermediate between parents but exhibited a

broader range of trait values. In contrast, $\delta^{13}C$ values for hybrids more closely resembled *P.*

balsamifera ($p > 0.05$) and the range did not exceed that observed in both parentals.

Transect-specific climatic gradients influenced stomatal traits despite high interspecific

gene flow

Geographic cline analyses suggested limited barriers to gene flow for stomatal traits (Fig.

S4-S5), yet these traits were consistently associated with climatic gradients of origin, particularly

precipitation (PC2) across the six latitudinal contact zones (Fig. 2B, Table S3). Opposing trait–

climate relationships were observed between northern and southern contact zones for D_U, O_U,

and SR. Drier environmental origins (PC2) were associated with significant decreases in D_U, O_U,

and SR in the northern contact zones of Alaska, Cassiar, and Chilcotin, but significantly

increased along PC2 in the southern contact zones of Jasper, Crowsnest, and Wyoming (Fig. 2B,

Table S3, $p < 0.05$). Genotypes from warmer environments (PC1) were associated with increased

D_U and lower O_U across the Jasper and Chilcotin contact zones, but reduced density and

occurrence in the Wyoming contact zone ($p < 0.05$), while no significant associations were

observed in the remaining contact zones (Fig. S6). Neither precipitation nor temperature

gradients of origin were significantly associated with D_L or D_T across most contact zones ($p >$

0.05, Fig. S6), except for Alaska and Jasper, where warmer and drier origins were associated

with increased D_T ($p < 0.05$). Together, these results suggest that transect-specific selection

associated with climatic gradients have influenced density and distribution of stomata across the

species' contact zones.

Genotypes from drier origins (PC2) were associated with reduced G_L and P_L across most

contact zones ($p < 0.05$), except for Alaska and Wyoming, where similar, but not significant,

associations were observed ($p > 0.05$, Fig. 2B). In contrast to the abaxial surface, G_U and P_U did

not vary significantly with precipitation gradients from site of origin ($p > 0.05$, Fig. S6)

suggesting that stomatal size on the upper surface is influenced less by precipitation.

Temperature gradients (PC1) were not significantly associated with guard cell length or pore

length on either the adaxial or abaxial surface ($p > 0.05$, Fig. S6). These patterns suggest that

variation in stomatal size across contact zones is more strongly influenced by precipitation than

by temperature.

In contrast to stomatal density and size traits, g_{sw} was positively associated with

temperature (PC1) and precipitation (PC2) gradients of origin across multiple contact zones (Fig.

2B, Table S3). Genotypes from cooler environments exhibited significantly greater g_{sw} ($p < 0.05$)

with exception of Alaska and Cassiar. However, in Alaska, genotypes from drier environments

had higher g_{sw} ($p < 0.05$). Although no significant associations were detected between climatic

gradients and water-use efficiency ($\delta^{13}C$) in most contact zones, warmer genotype origins tended

to be associated with reduced $\delta^{13}C$ (Fig. S6). Drier genotype origins were negatively associated

with $\delta^{13}C$ in Cassiar and Wyoming ($p < 0.05$), but not in other contact zones (Fig. S6, Table S3),

suggesting a trend toward lower water-use efficiency in drier environments for these contact

zones.

Admixture mapping reveals common and trait-specific candidate genes

Admixture mapping identified multiple ancestry blocks significantly associated with

stomatal trait variation in hybrid genotypes (Table S4), revealing both trait-specific and shared

regions of genomic association. The strongest associations were found for D_U, SR, and O_U,

which were all associated with the same genomic region on chromosome 15 spanning

approximately 66.5 kb. Multiple loci within this region exceeded the admixture burden-corrected significance threshold (Fig. 3A), suggesting a shared genetic basis (e.g., pleiotropy) for these traits. The region contained several candidate genes (Fig. 3B), including *TWIST* (a homologue of *SPEECHLESS* in *Arabidopsis*, a regulator of stomatal development, Peterson *et al.*, 2010; Song *et al.*, 2023), *TRY* (controls adaxial leaf surface trichome patterning, Matías-Hernández *et al.*, 2016), *AGP22* (involved in cell wall structure via arabinogalactan peptides, Seifert & Roberts, 2007), *SF-RPO* (a plastid RNA polymerase sigma factor involved in chloroplast gene expression), *DFA* (*dihydrofolate synthetase*, involved in folate and nucleotide biosynthesis), and *MTM1* (a mitochondrial carrier domain protein involved in metal ion homeostasis, Su *et al.*, 2007). In addition, a region on chromosome 12 associated with both D_U and SR contained *EPR1*, an early phytochrome-responsive transcription factor (Kuno, 2003, Fig. 3B). A third region on chromosome 9, containing *NF-YC10*, a transcription factor that regulates ABA signaling and contributes to drought tolerance (Swain *et al.*, 2017), was associated with SR (Fig. 3B). Although no other traits passed the genome-wide significance threshold, we discuss suggestive associations for several additional traits in the Supplemental Material.

Transect-specific geographic clines suggests barriers to gene flow at candidate loci for stomatal traits

Geographic cline analyses revealed variation in cline centers and widths for candidate genes across the six *Populus* contact zones (Fig. 4, Table S5). In the Alaska contact zone, all stomatal candidate genes exhibited steep clines that mirrored genome-wide ancestry (w= 0.04, c = 0.64). Cline centers for these candidate genes were clustered towards the geographic distribution of *P. balsamifera* ($c \approx 0.65$), and cline widths were narrow across candidate genes (w < 0.03). These patterns suggest that overall genetic exchange is restricted in this region. In contrast, geographic clines in Jasper were broader (w = 0.23–0.58), with centers ranging from c = 0.23 to 0.35 for both candidate genes and the genome-wide mean indicative of minimal barriers to gene flow.

In the Cassiar, Chilcotin, Crowsnest, and Wyoming contact zones, several candidate genes exhibited steeper clines when compared to the genome-wide background, indicating that potential barriers to gene flow have evolved at regions of the genome critical to species-specific adaptive trait variation (Fig. 4, Table S5). In Cassiar, the genome-wide ancestry cline was

relatively broad ($w = 0.34$) and centered at $c = 0.51$. In contrast, several candidate genes

associated with adaxial stomatal density, conductance, and guard cell size exhibited steeper and

displaced clines (center differs from genome-wide cline), consistent with restricted introgression.

For instances, *NF-YC10, ABA1,* and *GRAS transcription factor* exhibited steep clines ($w = 0.03$–

0.09) with centers ($c = 0.40$–0.44) consistently displaced toward the *P. trichocarpa* range,

suggesting asymmetric introgression. In Chilcotin, the genome-wide ancestry cline was

extremely narrow ($w = 0.02$), but clines at candidate genes varied widely, reflecting candidate

gene-specific patterns of introgression. Several genes associated with adaxial stomatal density,

TRY, TWIST, and *DFA,* exhibited broad clines ($w = 1.00$), suggesting extensive genetic exchange

with centers displaced toward the *P. trichocarpa* range ($c = 0.38$, 0.37, and 0.34, respectively). In

contrast, *AGP22* (adaxial stomatal density) and *WD repeat protein 91* (total stomatal density)

showed narrower clines ($w = 0.14$ and 0.16) with centers at $c = 0.77$ and 0.80, respectively,

consistent with localized barriers to introgression and displacement toward the *P. balsamifera*

range. In Crowsnest, candidate genes associated with adaxial stomatal density including *TRY* (w

$= 0.05$), *TWIST* ($w = 0.04$), *DFA* ($w = 0.05$), and *AGP22* ($w = 0.08$) exhibited narrower clines

than the genome-wide average ($w = 0.33$). These genes had cline centers similar to the genome-

wide center ($c = 0.90$), ranging from 0.91 to 0.95. Likewise, *WD repeat protein 91* (total stomatal

density, $w = 0.04$, $c = 0.95$) and a *GRAS transcription factor* (upper guard cell size, $w = 0.04$, $c =$

0.93) showed similarly steep clines with centers also located within the *P. balsamifera* range,

consistent with restricted introgression at genes influencing stomatal traits. In Wyoming, the

genome-wide ancestry cline was relatively broad ($w = 0.42$), but most candidate genes exhibited

much narrower transitions, with cline widths ranging from 0.02 to 0.18. With the exception of

the *GRAS transcription factor* ($w = 0.59$) and *NF-YC10* ($w = 0.47$), which displayed cline widths

comparable to or broader than the genome-wide pattern, most genes showed evidence of stronger

barriers to gene flow. Cline centers were largely consistent across candidate genes, ranging from

0.87 to 0.95, and closely aligned with the genome-wide center ($c = 0.92$), with the exception of

ABA1, which exhibited a slightly lower center ($c = 0.84$). Collectively, these results suggest that

divergence is maintained at specific candidate genes involved in stomatal structure and function,

but the extent and distribution of these barriers vary across contact zones, potentially due to

differences in transect-specific selective pressures.

Climatic associations suggest extrinsic selection shape local ancestry at genes associated with stomatal traits

Precipitation gradients associated with climate of origin had the strongest influence on local ancestry at stomatal trait candidate genes across contact zones (Fig. 5, Table S6). Precipitation gradients (PC2) were associated with reduced *P. trichocarpa* local ancestry ($p <$ 0.01) at most candidate genes across Alaska, Cassiar, Chilcotin, Jasper, and Crowsnest contact zones (Fig. 5-S11). The probability of *P. trichocarpa* ancestry declined in genotypes originating from drier environments (*slopes*: –0.25 to –4.2), highlighting strong climate-mediated selection favoring *P. balsamifera* alleles where water availability was reduced. In Cassiar, genes associated with adaxial stomatal density and conductance, such as *ABA1*, *SF-RPO*, *TRY*, and *TWIST*, exhibited the steepest declines in *P. trichocarpa* ancestry along precipitation gradients (*slopes* = -2.8 to -4.23).

Temperature gradients (PC1) based on genotype origin were significantly negatively associated with local ancestry for candidate genes across several contact zones, although these effects were generally weaker and less consistent than those driven by precipitation (PC2, Fig. 5-S11). In the Chilcotin, Jasper and Crowsnest contact zones, colder environmental origins were consistently associated with reduced presence of *P. trichocarpa* alleles at candidate genes, with slopes ranging from –0.27 to –1.29. In Crowsnest, genes associated with adaxial stomatal density and conductance, such as *ABA1*, *SF-RPO*, *TRY*, and *TWIST*, exhibited the steepest declines in *P. trichocarpa* ancestry along temperature gradients (*slopes* = -0.66 to -1.29). In Alaska and Cassiar, temperature gradients had limited influence on local ancestry, with only a few genes (*NF-YC10*, *AGP22*, *ATMTM1*, *ABA1*) showing significant but moderate declines in *P. trichocarpa* ancestry in cooler environments (slopes: –0.33 to –1.06).

Local ancestry at stomatal trait candidate genes was influenced by the interaction between temperature and precipitation gradients from the climate of origin in the Cassiar and Chilcotin contact zone (Fig. S11, Table S6). In Cassiar, genes involved in stomatal conductance and patterning such as *ABA1*, *AGP22*, and *TWIST* exhibited significant negative PC1 × PC2 interactions ($p < 0.05$), indicating selection for *P. balsamifera* ancestry under cold and dry conditions. In Chilcotin, similar interactions at multiple genes, including *NF-YC10*, *EPR1*, and *GRAS TF*, were found. Interestingly, when individual candidate genes were compared with

471 genome-wide ancestry, consistent declines in *P. trichocarpa* allele frequencies were observed

472 under colder and drier conditions across the six contact zones (Fig. 5).

Discussion

As climate change accelerates, understanding how gene flow and selection interact to

shape the evolution of adaptive traits will be critical to guiding species management. In this

study, we investigated how interspecific gene flow and climate-mediated selection influence the

evolution of stomatal traits that are crucial to plant growth, survival, and water-use efficiency,

across six natural contact zones between *Populus trichocarpa* and *P. balsamifera*. Hybrid

genotypes exhibited intermediate or parent-like phenotypes for key stomatal traits, but there was

also substantial genetic variation mediated via hybridization. At a trait scale, geographic cline

analyses indicated high genetic exchange, however, analysis of candidate genes underlying

stomatal traits showed steep clines aligned with climatic gradients. Such gene-specific patterns

indicate that extrinsic selection plays an important role influencing the movement of specific

alleles across environmental gradients even in the presence of species barriers associated with

adaptive trait differences. Together, our results provide new insight into how extrinsic selection

impacts the movement of genetic variants into novel genomic and environmental backgrounds

that may be critical to adaptive trait evolution.

Species-specific differences in stomatal traits influence hybrids

Stomatal trait differences between *P. balsamifera* and *P. trichocarpa* reflect evolutionary

responses to their home environments. In this study, traits associated with water conservation,

such as reduced stomatal conductance, a high density of small stomata, and increased water-use

efficiency were observed in *P. trichocarpa* compared to *P. balsamifera*. This contrasts with

earlier findings that associated *P. balsamifera* with more water conservation strategies (Pointeau

& Guy, 2014). One possibility is that *P. trichocarpa* may have experienced lower water

potentials in the common gardens, leading to stomatal closure to avoid cavitation, consistent with

a more drought-sensitive strategy. In contrast, *P. balsamifera* may have maintained turgor and

kept stomata open under mild stress, reflecting greater drought tolerance (Groover *et al.*, 2025).

Differences may also arise from broader geographic sampling in our study, which captured more

intraspecific variation than previous studies (Pointeau & Guy, 2014). However, patterns of

amphistomy were consistent with previous findings in which *P. balsamifera* generally exhibited

none or fewer stomata on the adaxial leaf surface, while *P. trichocarpa* showed greater variation,

with more genotypes displaying adaxial stomata (McKown *et al.*, 2014, 2019; Fetter *et al.*,

2021).

Stomatal traits for hybrid genotypes were on average, intermediate or more similar to one

of the parental species but showed greater ranges of phenotypic values suggesting that

hybridization has generated a wide spectrum of trait variation upon which selection may act.

Stomatal conductance in hybrids on average was intermediate to *P. balsamifera* and *P.*

trichocarpa, consistent with additive genetic contributions from both parental species (Orians,

2000). Such additive trait expression may offer functional benefits in transitional environments

where hybrids encounter conditions not occupied by either parental species (Janes & Hamilton,

2017). In our study, hybrid genotypes largely resembled *P. trichocarpa* in adaxial stomatal

density, occurrence, and stomatal ratio with the presence of stomata on both leaf surfaces.

Previous studies have reported adaxial stomata in *P. balsamifera* admixed genotypes only in

regions of hybridization with *P. trichocarpa*, *P. deltoides*, or *P. angustifolia* (McKown et al.

2014, 2019; Pearce et al., 2005), suggesting that amphistomaty arises through introgression. In

hybrids, amphistomaty may provide an advantage, enhancing photosynthetic capacity across

transitional environments (Muir, 2019). In contrast, in *P. deltoides* × *P. angustifolia* hybrids,

amphistomatous traits from *P. deltoides* were not retained, and both *P. angustifolia* and hybrids

remained largely hypostomatous (Zanewich et al., 2018). These contrasting outcomes suggest

that the expression of amphistomaty in hybrids may be shaped by differences in parental trait

dominance or directional selection (Pfeilsticker et al., 2022, 2023).

Climate shapes genetic variation underlying stomatal traits despite high interspecific gene

flow

Despite geographic clines indicating limited barriers to gene flow at a trait level across

the six latitudinal contact zones, trait variation was strongly associated with climatic gradients,

particularly precipitation. Adaxial stomatal density, occurrence, and stomatal ratio were strongly

associated with precipitation gradients although the direction of the relationship varied across

several contact zones. In the northern contact zones, wetter origins were associated with higher

adaxial stomatal density with more genotypes with amphistomatous leaves, while drier origins

showed reduced adaxial stomata and more genotypes with hypostomatous leaves. This suggests

that, in the north, selection may favor amphistomaty under wetter conditions to support rapid

growth during short growing seasons, but is selected against in drier climates to limit water loss

(McKown *et al.*, 2019). Southern contact zones showed the opposite pattern with lower stomatal

ratio for genotypes sourced from wetter environments and higher from drier origins. In the south,

selection may favor lower adaxial stomatal density to reduce pathogen entry, reflecting a trade-

off between gas exchange and disease resistance which is typically higher in warmer, humid

regions of the south compared to the north (McKown *et al.*, 2014; Fetter *et al.*, 2021).

Stomatal size traits, including guard cell and pore length, showed clear associations with

precipitation across the hybrid zone. In most contact zones, genotypes from drier environments

were linked to smaller abaxial guard cells and shorter pore lengths, supporting the hypothesis

that reduced stomatal size reduces water loss under drought stress while allowing faster stomatal

closure (Niemczyk *et al.*, 2019; Volk *et al.*, 2022; Franks & Beerling, 2009). Contrary to

expectations that genotypes originating from cooler and drier climates would exhibit reduced

stomatal conductance (Chen *et al.*, 2024), we observed that these genotypes maintained higher

stomatal conductance when evaluated in common gardens. Lower evaporative demand in cooler

environments may reduce the cost of water loss, allowing higher stomatal conductance with

minimal risk. In drier regions, genotypes may exploit brief water availability by maximizing

carbon gain during short favorable periods (Buckley, 2019). However, observed patterns may

also reflect environmental differences between the common gardens and the climates of origin

(Oubida *et al.*, 2015).

Climate-driven selection limits gene flow at loci underlying stomatal traits

Although trait-scale geographic clines suggested minimal barriers to genetic exchange for

stomatal traits, finer-scale cline analyses of candidate genes underlying stomatal traits revealed

variable restrictions to gene flow, depending on genomic context and environmental conditions.

Most contact zones exhibited evidence of gene-specific constraints to genetic exchange;

however, the Alaska contact zone with coincident patterns of limited introgression for adaptive

and neutral loci may reflect broad ecological constraints. In this contact zone, the cline for

genome-wide ancestry was extremely narrow ($w = 0.04$) and all candidate genes showed

similarly steep transitions ($w < 0.03$), suggesting barriers to gene flow reflect genome-wide

divergence (Schield *et al.*, 2024). While steep coincident clines can also emerge from strong

genetic drift (Jofre & Rosenthal, 2021), the consistent decline in *P. trichocarpa* ancestry toward

drier climates (Fig. 5) supports the idea that precipitation gradients are shaping local ancestry at

candidate genes for stomatal traits. Similar studies in hybrid zones have shown that precipitation

can act as a key selective force influencing patterns of genetic variation and introgression

(Hamilton & Aitken, 2013; Menon *et al.*, 2021).

In contrast to Alaska, the Cassiar, Chilcotin, Crowsnest, and Wyoming contact zones

exhibited more porous species boundaries, where barriers to gene flow were locus-specific and

strongly influenced by climate of origin. In Cassiar, clines at candidate genes associated with

adaxial stomatal density and conductance including *ABA1*, *NF-YC10*, and *TRY* were markedly

steeper ($w < 0.09$) than the genome-wide average ($w = 0.34$), consistent with divergent selection

limiting introgression at adaptive loci underlying traits that show strong species-specific

divergence (Barton & Gale, 1993; Suarez-Gonzalez *et al.*, 2018c). Notably, this fine-scale

restriction contrasts with the broader clines ($w = 0.33$ -0.52) seen in other candidate genes and

genome-wide ancestry ($w = 0.35$) indicating that while much of the genome can move freely

across species boundaries, regions underlying traits like stomatal conductance and adaxial

stomatal density are subject to divergent selection. Consistent with climate-driven selection, we

found strong ancestry–climate associations, including significant temperature × precipitation

interactions, suggesting steep climatic gradients may impact recombination in Cassiar. Evidence

from chloroplast haplotype distributions in this zone also suggests introgression is climate-

mediated (Zavala-Paez *et al.*, 2025). These results highlight the role of extrinsic selection in

maintaining species differences where there might otherwise be weak barriers to reproduction

(Abbott, 2017). Unlike other contact zones, Jasper showed little evidence of barriers to gene

flow, either genome-wide or at candidate genes. Clines at both genome-wide loci ($w = 0.58$) and

candidate genes ($w = 0.23$–0.58) were broad, indicating gene flow and a lack of reproductive

isolation. Yet, consistent climate associations, especially with precipitation, suggest that

selection is acting, but currently the strength is insufficient to overcome the homogenizing effect

of gene flow.

Taken together, comparisons across the six latitudinal contact zones reveal that climate-

mediated selection, particularly variation in precipitation, constrains introgression both across the

genome and at functionally important candidate genes. However, the strength of this selection

varies with local environmental conditions, resulting in spatial differences in allele movement. Despite widespread interspecific hybridization, our findings demonstrate that environmental selection can maintain divergence at key loci. These results offer insight into the mechanisms that both limit and facilitate the movement of adaptive alleles, ultimately shaping the evolution of adaptive traits in hybrid zones.

Conclusion

This study reveals that stomatal trait evolution in *Populus* hybrid zones is shaped by the interplay of gene flow and climate-driven selection. Hybrids displayed a wide range of trait values, reflecting admixture between divergent parental species. Variation in stomatal traits was strongly associated with environmental gradients, particularly precipitation, suggesting that water availability plays a central role in influencing trait variation across this hybrid zone despite high levels of gene flow. However, although much of the genome exhibits high levels of interspecific gene flow at a trait-scale, several candidate genes associated with amphistomy and stomatal conductance exhibited restricted gene flow. Variation in the movement of genetic variants underlying adaptive traits was largely influenced by climate of origin shaping local ancestry distributions. These findings highlight that hybridization can generate novel genetic recombinants and facilitate genetic exchange in areas where barriers to reproduction are limited. But overall, the movement of adaptive genetic variation across species and environmental gradients will be strongly influenced by environmental selection.

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Competing interests

None declared.

Author contributions

Michelle Zavala-Paez led data collection, research design, data analysis and
interpretation, and manuscript writing. Stephen Keller contributed to data collection, data
analysis and interpretation, and manuscript writing. Jason Holliday and Matthew Fitzpatrick
contributed to data collection and manuscript writing. Jill Hamilton contributed to data
collection, research design, data analysis and interpretation, and manuscript writing.

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Data availability

The genomic data analyzed in this study were previously published by Bolte et al. (2024)
and are available on NCBI under accession number PRJNA996882. All data and scripts used for
the analyses will be deposited in Dryad upon acceptance and are provided in the supplementary
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Figure 1. Genotypes were collected across six east-west contact zones within the natural hybrid zone between *P. trichocarpa* and *P. balsamifera*. Light and dark gray represent the distribution range of *P. trichocarpa* and P. *balsamifera*, respectively. Georeferenced sampling locations are color coded by genomic ancestry (Mead et al, 2025; K = 2). Clonally replicated genotypes were planted at the common gardens in Stuart, VA (purple square), Burlington, VT (orange square), and Fargo, ND (green square).

Figure 2. A) Boxplots showing best linear unbiased predictors (BLUPs) for abaxial guard cell length (G_L), total stomatal density (D_T), adaxial stomatal density (D_U), stomatal ratio (SR),

intrinsic water-use efficiency (δ^{13}C), and stomatal conductance (g_{sw}), for *P. balsamifera*, hybrid, and *P. trichocarpa* genotypes. Each point represents a genotype, and colors indicate the proportion of *P. trichocarpa* genomic ancestry. Letters denote statistically significant differences among groups based on post-hoc comparisons ($p < 0.05$). B) Relationship between genotype BLUPs values for abaxial guard cell length (G_L), stomata ratio (SR), stomatal conductance (g_{sw}), and the first two principal components of climate at genotypes' origin. PC2 reflects a gradient from wetter to drier environments (increasing values = drier), while PC1 reflects a temperature gradient (increasing values = warmer). Shaded areas indicate 95% confidence intervals for the fitted linear regression. Lower panels show trait-transects slopes (±95% CI) extracted from linear models fit separately within each transect.

Figure 3. A) Manhattan plot showing results from GWAS by admixture mapping for stomatal ratio. The black dashed line indicates the genome-wide significance threshold ($-\log_{10}(P) \approx 4.73$), corrected for admixture burden (0.05/2719). B) Zoomed-in views of significant associations with stomatal ratio on chromosomes 9, 12, and 15. Significant SNPs are highlighted in red. Candidate genes and their orientations are indicated by arrows.

Figure 4. Geographic clines for candidate genes associated with stomatal trait variation and genome-wide ancestry across six contact zones in the *Populus balsamifera* × *P. trichocarpa* hybrid zone. Best-fit cline models estimated using HZAR illustrate the relationship between scaled geographic distance and ancestry (proportion of *P. trichocarpa* ancestry). Each colored line and dot represent a candidate gene, with cline shape determined by the model that best fits the observed ancestry frequency transition across the landscape. Lower panel provides a summary of cline parameters for each candidate gene. Points represent the estimated cline center (geographic midpoint of ancestry frequency change), and horizontal bars denote cline width (the spatial scale over which the transition occurs). Variation in cline shape, center, and width highlight differences in patterns of introgression and potential barriers to genetic exchange across transects.

A)

B)

Alaska

Cassiar

Chilcotin

Jasper

Crowsnest

Wyoming

Warmer --------- PC1 --------- Cooler

Wetter --------- PC2 --------- Drier

NF–YC10
EPR1
TRY
SF–RPO
TWIST
DFA
AGP22
ATMTM1
WD repeat protein 91
Ubiquitin–like
ABA1
GRAS TF
Genome-wide ancestry

Figure 5. Relationship between climate and nuclear ancestry at stomatal trait candidate genes across six *Populus* contact zones. (A) Ancestry as a function of PC1, representing a gradient from warmer (low values) to cooler (high values) environments. (B) Ancestry as a function of PC2, representing a gradient from wetter (low values) to drier (high values) environments. Each point represents an individual genotype, colored by gene. Lines represent fitted values from generalized linear models (GLMs), fitted separately for each gene within each contact zone, with ancestry coded as 0 (*P. balsamifera* homozygotes), 0.5 (heterozygotes), or 1 (*P. trichocarpa* homozygotes).

Table 1. Summary of stomatal traits and associated measures assessed across the common gardens, including trait names, abbreviations, units, and symbology. Trait differences among parental species and hybrids based on results from our study indicating whether hybrids showed intermediate values or resembled one parent. N.S = no significant differences across groups.

Trait	Units	Symbology	*P. balsamifera*	Hybrids	*P. trichocarpa*
Adaxial guard cell length (G_U)	µm		N.S	N.S	N.S
Abaxial guard cell length (G_L)	µm		Greater	*balsamifera-like*	Smaller
Adaxial pore length (P_U)	µm		N.S	N.S	N.S
Abaxial pore length (P_L)	µm		Greater	*balsamifera-like*	Smaller
Adaxial stomatal density (D_U)	mm^2		None or less dense	*trichocarpa-like*	Denser
Abaxial stomatal density (D_L)	mm^2		Less dense	*balsamifera-like*	Denser
Total stomatal density (D_T)	mm^2		Less dense	*balsamifera-like*	Denser
Stomata density ratio (SR)	None		More hypostomatous	*trichocarpa-like*	Higher degree of amphistomy
Adaxial stomatal occurrence (O_U)	None		Lower	*trichocarpa-like*	Higher
Stomatal conductance (g_{sw})	mol m^{-2} s^{-1}		Higher	Intermediate values	Lower
Intrinsic water-use efficiency ($\delta13C$)	‰	High/Low WUE	Lower	*balsamifera-like*	Higher