Adaptive Introgression Shapes the Pan-genome of Populus Hybrid Zones

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Abstract

Poplar trees…

## 0.1 Introduction

Hybridization can lead to the exchange of genetic variation across species boundaries, a process known as introgression. Introgression is a powerful source of genetic novelty, playing a significant role in the evolutionary history of natural systems (Hedrick 2013; Suarez-Gonzalez, Lexer, and Cronk 2018), humans (Racimo et al. 2015) and key crop varietals (Burgarella et al. 2019; Gao et al. 2019; Kou et al. 2020; Cheng et al. 2019; Sun et al. 2020; Qiao et al. 2021; Zanini et al. 2022). Admixed individuals, the recipients of introgressed sequence, harbor novel combinations of alleles originating from both parental species, and may present phenotypic variation which transcends that observed in the parental species. In this sense, admixed individuals represent unique opportunities, not only to better understand the genetic basis of phenotype, but also to better understand the nature of molecular interactions between divergent haplotypes. Furthermore, when parental species inhabit differing environments, patterns of introgression may also reflect the interaction between genetic variation and environment. Ultimately, these GxG and GxE interactions define the contexts in which introgression is adaptive. Past research on natural admixed populations suggests that introgression can be an adaptive process, through which genetic variation related to locally-adaptive phenotypes is passed from one species to another (Hedrick 2013; Suarez-Gonzalez, Lexer, and Cronk 2018; Leroy et al. 2020; Rendón-Anaya et al. 2021; Hamilton and Miller 2016; Savolainen, Pyhäjärvi, and Knürr 2007; Kremer and Hipp 2020; Gaczorek et al. 2024). Closer and more extensive analysis of natural manifestations of adaptive introgression can reveal the types of genetic variation that are able to cross species boundaries without a fitness consequence, the regions of the genome that are receptive to the introduction of foreign sequence, and the degree to which environmental context influences the adaptive nature of introgression. Research in this area is valuable not only because it sheds light on important questions in evolutionary biology, such as the evolution of species boundaries and molecular basis of adaptive traits, but also because it gauges the overall potential for the introduction of divergent genetic variation into novel genetic and environmental background. Therefore, research in this area has great potential for broader impact for two main reasons. First, because the contribution of biotechnology to advances in the fields of medicine and agriculture increasingly depend on the introduction of genetic sequence into a novel background. Second, because introgression provides a potentially faster and more reliable route for the adaptive evolution of phenotype than de novo mutation, meaning that natural systems and breeding programs alike may depend on introgression in order to facilitate rapid evolution of adaptive traits in response to changing climates.

The size and molecular origin of the genetic variation that is exchanged between species through adaptive introgression is important to define, as a growing body of research suggests that genomic structural variation (SV), here defined as genetic variation larger than 50bp resulting for insertion, deletion, translocation or inversion of genomic sequence, is often the causal variation underlying ecologically and economically important traits in many taxa (*CITE*). For instance, SV may play a role in local adaptation to climate, and past research shows divergence between populations for SV genotypes resulting from local adaptation (Hämälä et al. 2021; Songsomboon et al. 2021; Y. Li et al. 2024; Z. Li et al. 2023). At a broader scale, research also shows that SV maintain genetic differentiation between species (L. Zhang et al. 2021; Ferguson et al. 2024). However, little is known about the adaptive exchange of SV genotypes between divergent populations and species. Introgression between crop varietals and wild relatives is a key factor in the breeding history of many crops, and recent work shows that SV are often the causal variants involved in this process (Gao et al. 2019; Kou et al. 2020; Cheng et al. 2019; Sun et al. 2020,; Qiao et al. 2021; Zanini et al. 2022). Introgression is also common in natural systems and recent work highlights introgression as an important source of adaptive genetic novelty in many species, particularly forest trees (Suarez-Gonzalez, Lexer, and Cronk 2018; Leroy et al. 2020; Rendón-Anaya et al. 2021; Hamilton and Miller 2016). However, past work on the genetics of such adaptive introgression in natural systems focused primarily on SNP genotypes. While hybrid genomes may be porous to variation at the single nucleotide scale, and introgressed SNP alleles may even present an adaptive advantage in admixed individuals, it remains unclear if the same is true for SV genotypes, which have greater potential for deleterious phenotypic effects. Recently, research has highlighted a potential role for SV in adaptive introgression. This work suggests that SV may play an important role in both adaptive introgression (Almarri et al. 2020; Xia et al. 2023; X. Zhang et al. 2021) and in the maintenance of species boundaries (L. Zhang et al. 2021). If SV is indeed involved in adaptive introgression, it is worth investigating the molecular nature (eg size, frequency, variant class, genomic location) of the SV alleles involved, as well as the consistency and directionality of adaptive introgression involving SV. Admixed individuals may present mosaics of SV alleles derived from both parental species, or introgression may be directionally biased toward one species. Similarly, some loci may act as barriers to introgression, while others are more receptive. Natural hybrid zones may be hotspots for the de novo generation of SV alleles through processes such as unequal crossing over. Here, we leverage extensive sampling of natural forest tree hybrid zones, cutting edge techniques for genotyping SV in admixed individuals and established landscape genomics analyses to investigate these areas of uncertainty.

The study of SV in natural hybrid zones is challenging, as it requires the ability to accurately genotype SV in a large number of admixed individuals. The majority of past research on SV in natural hybrid zones has relied on the use of reference genomes derived from a single individual from only one of the parental species, which can lead to reference bias (Bock et al. 2023; Song et al. 2023; Feng et al. 2024). Reference bias occurs when the genomes of resequenced individuals contain regions that are highly diverged from the reference genome sequence, causing reads originating from these regions to align incorrectly, or not align at all. This can lead to misinterpretation or under-representation of variation resulting from admixture (Secomandi et al. 2025). Reference bias is particularly problematic when studying SV, as large insertions and deletions may be longer than sequencing reads, making variation in their presence and absence (PAV) challenging to detect. Furthermore, SV is often species-specific and, because admixed individuals represent a mixture of genetic variation from two or more species, the presence of reference bias can obscure the effect that the presence (or absence) of genetic variation has on traits, and can hinder the identification of SV that is involved in introgression.

Recently, an increasing number of studies have relied on pan-genome assembly to overcome reference bias and to accurately genotype SV (Liang et al. 2025; Secomandi et al. 2025; Kang et al. 2023; Gao et al. 2019; Z. Li et al. 2023; Y. Li et al. 2024; Songsomboon et al. 2021). A pan-genome assembly is a non-redundant collection of sequences originating from multiple individuals. This genetic information can be represented as “nodes” in a pan-genome graph, while the linear sequence of each input genome is stored as a “path” connecting a series of nodes. In a pan-genome graph, each node describes the alignment between at least two sequences, given an expected level of sequence divergence (Kang et al. 2023). Pan-genome graphs can capture complex variation that is not present in a single reference genome, and can provide a more accurate representation of the genetic variation present in admixed individuals. Pan-genome assembly has been used to identify and genotype SV related to ecologically and economically important traits in a variety of taxa, and has been shown to be an effective tool for studying adaptive evolution in natural populations (Secomandi et al. 2025; Fang and Edwards 2024; Kang et al. 2023). Pan-genome assembly could help overcome the challenges of studying SV in admixed tree populations, and could provide new insights into the role of SV in adaptive introgression in natural populations of forest trees (Secomandi et al. 2025). Despite this potential, we know of only one study that used pan-genome assembly to examine introgression in the context of hybridization in the wild. Liang et al. (2025) use a pan-genome based approach to genotype SV in a range-wide sampling of the interfertile oak species *Quercus variabilis* and *Q. acutissima*, showing that SV harbor signals of adaptive introgression that differ from those detected from SNP data and identifying adaptive introgression of SV genotypes in a gene rich region of oak chromosome 9 (Liang et al. 2025). In light of this work, it is worth investigating if signatures adaptive introgression on SV exist in other interfertile tree species and asking not only how this adaptive signal is distributed across the whole genome but also how it varies with environmental context across the range of hybridizing species. Here, we add to this nascent line of research by leveraging pan-genome assembly and fine-scale sampling of natural hybrid zones to explore genome-wide relationship between structural variation and introgression between *Populus balsamifera* and *Populus trichocarpa*, two forest tree species that diverged much more recently than the oak species studied by Liang et al. (2025).

*Populus balsamifera* and *Populus trichocarpa* readily interbreed in nature, and early and advanced generation hybrids have been described in hybrid zones located where their ranges overlap (Geraldes et al. 2014; Suarez-Gonzalez et al. 2016, 2018; Chhatre et al. 2018). It is thought that hybridization between these two species facilitates introgression of genetic variation related to locally-adaptive phenotypes from one species to the other (Suarez-Gonzalez et al. 2016, 2018). However, studies on controlled inter-specific crosses in *Populus* have made it clear that introgression is often biased toward particular regions of the genome, particular *Populus* species, or specific environments (Lexer et al. 2005; Thompson et al. 2010; Meirmans et al. 2017). Therefore, the porosity of the genome to adaptive introgression between these species may be constrained by incompatibilities between the genomic variants involved as well as environmental context, two factors which warrant further investigation. The environmental context of adaptive introgression may be of particular importance in the case of *P. balsamifera* and *P. trichocarpa*. *P. balsamifera* is more commonly found in colder, continental climates while *P. trichocarpa* is more often associated with milder, coastal climates with longer growing seasons (Geraldes et al. 2014; Suarez-Gonzalez et al. 2018). Considering hybrid zones between these species often fall along the boundaries of their ranges (Chhatre et al. (2018); Fetter and Keller (2023)), it seems possible that adaptive introgression helps hybrid individuals persist in environments that are outside of the optimum for either parental species. Suarez-Gonzalez et al. (2018) showed support for this hypothesis, finding that introgressed regions in the genomes of hybrids harbored signs of selection and variation associated with adaptive traits, including phenology. However, the specific adaptive variants within these genomic regions, and the nature of their effects on fitness remain, for the most part, undiscovered. Identification of SV involved in adaptive introgression between these two species would not only contribute to a broader understanding of the molecular basis of adaptive introgression, but would also provide helpful insight into the conservation of these ecologically important species. In trees, long generations times are thought to limit the potential contribution of de novo mutation to adaptive evolution in response to rapid environmental change (Savolainen, Pyhäjärvi, and Knürr 2007; Feng et al. 2024). Therefore, adaptive introgression is viewed as one of the few routs for speicies like *P. balsamifera* and *P. trichocarpa* to adaptively evolve in response to climate change (Feng et al. 2024). If adaptive introgression does help admixed individuals persist in environments outside of their optimum, then the variation involved could guide genetic conservation efforts, such as assisted gene flow. Furthermore, industrial breeding of *Populus* varietals generally involves interspecific crosses, and breeding programs would benefit from a clearer understanding of the genetic variation that is involved in adaptive introgression between these two species.

Here, we take advantage of recent advances in pan-genome assembly methods to produce a pan-genome reference, comprising 24 diverse haplotypes from *P. balsamifera*, *P. trichocarpa* and their hybrids, facilitating unbiased analysis of the sequence variation that is segregating within natural *Populus* hybrid zones. Using this new pan-genome assembly, we genotype structural variation across 566 individuals sampled from within and outside of 6 distinct *P. balsamifera* and *P. trichocarpa* hybrid zones. We assess the extent of genomic diversity present in these species and their hybrids, identifying genomic variation that is not present in the *P. trichocarpa* reference genome. Furthermore, we describe structural variation involved both in introgression and in genomic divergence between the two species. We shed light on the role that introgression may play in shaping the pan-genome of these species, and the degree to which tree genomes are porous to the inter-specific exchange of structural variant alleles.

## 0.2 Methods

### 0.2.1 Sample collection and whole genome sequencing

In January 2020, we collected dormant branch cuttings from 546 poplar trees along 7 distinct latitudinal transects spanning natural contact zones between Populus trichocarpa and Populus balsamifera [Figure 1](#fig-1). Short read whole genome sequencing and subsequent bioinformatic analyses were performed as described in Bolte et al. (2024). Briefly, Genomic DNA libraries for all sampled individuals were constructed at the Duke University Center for Genomic and Computational Biology and sequenced using an Illumina NovaSeq 6000 instrument with an S4 flow cell with 64 samples per lane (Illumina Inc., San Diego, USA). De-indexing, QC, trimming adapter sequences, and sequence pre-processing were completed by the sequencing facility. In addition to short read sequencing, we also sequenced a subset of 40 sampled individuals with PacBio HiFi long reads. We harvested tissue from newly-expanded leaves grown under low light conditions to use for high molecular weight DNA extraction. After confirming extraction quality through gel electrophoresis and bioanalysis, we submitted HMW DNA to the University of Maryland Center for Integrative Biosciences Genomics Core Facility for library preparation and sequencing on the the PacBio Sequel system with two samples per SMRT flow cell. We sequenced one *P. balsamifera* sample (939) for an additional run on a full SMRT cell. We also sequenced this individual with Oxford Nanopore Technology (ONT) platform. We submitted high molecular weight DNA to the Vermont Integrative Genomics Resource Sequencing Center for library preparation and sequencing on XXX flowcell (two runs) and XXX flowcell (one run)

### 0.2.2 Genome assembly

Of the 40 individuals sequenced with HiFi long reads, we selected 16 for whole genome assembly [Figure 1](#fig-1). These samples ranged from 20 to 35x long read coverage. We performed de-novo genome assembly of the HiFi reads for these samples with HiCanu (Nurk et al. 2020). We set HiCanu parameters as follows: gSize=“400m”, lc=“5”, lcer=“0.055”, ovrlp=“350”, mincov=“9”. To detect potential contaminants in the raw assemblies produced by HiCanu, we used the program Kraken2 to compare the k-mer content of assembled contigs to the “PlusPFP” database of known taxon-specific k-mers representing archaea, bacteria, viral, human, protozoa, fungi and plant taxa (Lu et al. 2022). We then used a custom bioawk script to remove any assembled contig that Kraken2 assigned to a taxonomic unit other than *Populus*, leaving only unassigned or *Populus*- assigned contigs in each assembly. We assessed the accuracy and completeness of the decontaminated assemblies using QUAST and BUSCO (Gurevich et al. 2013; Simão et al. 2015). To further assess the contiguity and quality of these assemblies, we used minimap2 and BWA to map the original HiFi reads and additional Illumina short reads back to each assembled haplotype (H. Li 2018; H. Li and Durbin 2010). We passed these alignments to the program CRAQ (K. Li et al. 2023), which leverages read depth along assembled contigs for quality assessment. We repeated these quality assessment checks at each subsequent stage of genome assembly. Phasing, or the separation and concatenation of contigs belonging to the same parental haplotype is a key step in genome assembly for hybrid individuals, as divergent haplotypes likely contain important genetic information. We used mininmap2 to map reads back to assembled contigs and to map assembled contigs to themselves. We then used the purge haplotigs pipeline (Roach et al., 2018) to split diploid assemblies into two assembled haploytpes for each individual. We used RagTag (Alonge et al. 2022) to connect decontaminated, phased contigs into pseudo-chromosomal scaffolds, guided by alignments of assembled contigs to the Nisqually1 *P. trichocarpa* reference genome. We visually inspected minimap2 alignments of scaffolded assemblies to the reference genome to identify potential scaffolding errors. We used RepeatMasker to annotate and mask repetitive regions and annotated possible coding domains and predicted protein sequences for each assembly with AUGUSTUS (Smit et al., 2015; Hoff et al., 2019).

### 0.2.3 Pan-genome assembly

In a pan-genome graph, a non-redundant collection of all input sequences is represented as “nodes”, while the linear sequence of each input genome is stored as a “path” connecting a series of nodes. In this approach to graph construction, each node of the graph w describes the alignment between at least two sequences, given an expected level of sequence divergence. We constructed a pan-genome graph from an all-by all alignment of the 24 assembled haplotypes. Any contigs shorter than 100kb were dropped before alignment. We used wfmash (Guarracino et al., 2021) to perform all-by-all alignments between assembled chromosomes, and seqwish (Garrison and Guarracino, 2022) to project alignments into a graph pan-genome assembly. Pan-genome graphs constructed this way can have highly complex topography, which may hinder downstream applications such a sequence alignment. To combat this issue, we used the program smoothXG to smooth complex variation in these graphs. We also assembled a separate graph specifically for subsequent alignment-based analyses using the minigraph-cactus pipeline (Hickey et al. 2023). We used the program panacus to asses pan-genome growth and coverage statistics (Parmigiani et al. 2024). We used the program ODGI (Guarracino et al., 2022) to perform basic graph quality control and to partition the core ( sequence present in all individuals), shell (sequence variably present or absent across individuals) and singleton sequence (present in only one individuals) content for each graph and individual represented in the graphs. We used the program vg (Garrison et al. 2018) to deconstruct each graph assembly into a VCF containing the variation encapsulated in each graph. We passed this deconstructed VCF file to vcfbub (github.com/pangenome/vcfbub) to collapse nested SV sites into the top-level variant for all SV less than 10kb in length. Using bcftools (Danecek et al. 2021) we split biallelic sites into multiallelic records for this deconstructed vcf.

### 0.2.4 Genotyping Pan-genomic Variation

We aligned sequencing reads from additional samples to pan-genome graph assembly to genotype SNPs and SVs. We aligned PacBio HiFi reads for 40 total individuals (including the 16 used to construct the pan-genome graph) to the minigraph-cactus graph using the program GraphAligner (Rautiainen and Marschall 2020). To characterize sequence variation across a broader set of individuals, we also mapped Illumina short reads for 575 individuals to the pan-genome graph using the program giraffe (Sirén et al. 2021). We then called SV and SNPs from these alignments using the vg call algorithm (Garrison et al. 2018). We used bcftools to split multialleleic calls into separate records, normalize alleles against the *P. trichocarpa* reference and merge calls for all samples into one VCF representing SV and SNP genotypes in the *P. trichocarpa* reference coordinate space.

### 0.2.5 Analysis of Structural Variation and Introgression

We used ADMIXTURE results from Bolte et al (2024) to assign each individual an admixture proportion at K=2 and K=4. The genetic data used for this ADMIXTURE analysis came from SNP calls from mappings of the same illlumina reads used in this study, however they were mapped to the *P. trichocarpa* reference genome. We used this same genetic data set to perform local ancestry inference (LAI) using LOTR (Dias-Alves, Mairal, and Blum 2018). These ADMIXTURE and LAI data sets were used to visualize and analyze patterns of local and global genomic ancestry throughout this study.

We used ODGI to flatten the Nisqually1 *P. trichocarpa* reference genome path in the cactus graph. We then used a bed file representation of this path and reference gene annotations to genotype PAV of reference sequence across all paths of the cactus graph. We used R version 4.1.0 to identify core and shell gene sequences from this data and to perform PCA on the PAV genotypes (R Core Team 2024). We also ran a similar PAV PCA with the merged vcf representing all 575 sequenced samples in this study, using plink2 (Chang et al. 2015). We used the -–allele-weights flag to extract the loading of each allele for each SV on the first 3 principal components.

To find SV potentially involved in introgression, we employed custom scripts to identify ancestry blocks along the chromosomes of all 335 admixed individuals in this study. An individual was considered admixed if its k=2 ADMIXTURE score indicated at least 5 percent admixture genome-wide. We used Bedtools (Quinlan and Hall 2010) to find SV that overlapped ancestry blocks in each admixed individual. We then scored each SV allele based on its prevalence in ancestry blocks that contrasted the average global ancestry of an individual.

For all SV that were outliers in principal component or LAI analyses, we used Bedtools to check for overlap with annotated genes. We then used plantgenie to analyze PFAM enrichment of gene sets (Sundell et al. 2015).

## 0.3 Results

### 0.3.1 Whole genome sequencing and assembly.

Illumina sequencing yielded on average of 8358 MB of sequence, corresponding to an average sequencing depth of 21x. PacBio HiFi sequencing yielded on average 6368 MB of sequence, corresponding to an average sequencing depth of 16x. Sample 939 was sequenced to a depth of roughly 60X HiFi and roughly 20X ONT reads. Table 1 shows summary statistics for the 16 whole genome assemblies included in the pan-genome. Assembly quality and contiguity were generally high, however alternate haplotypes were not always fully assembled (Table 1).

### 0.3.2 Pan-genome assembly

The minigraph-cactus pangenome graph contained a total of 102,907,131 nodes comprising 1.2 Gbp of sequence. Of this, 243 Mbp was present across all individuals (core genome), while 423Mbp was present in some but not all individuals (shell genome). The remaining 537 Mbp represented singleton sequence. [Figure 2](#fig-2) shows how the size of the sequence classes changes as individuals are added to the pan-genome. [Figure 3](#fig-3) shows the proportions of core, shell and singleton sequence within each sample. **?@fig-4** shows each genome in the pan-genome graph plotted on the first two principal components of a PCA on PAV of reference sequence.

### 0.3.3 Pan-genomic Variation

3708 annotated genes were variably present or absent across the 17 genomes used to construct the pan- =genome. These genes were significantly enriched for various protein families including NB-ARC domain, D-mannose binding lectin, sulfotransferases, cupins, and Cytochrome P450. Of these shell genes, 167 were present in all of the pure *P. trichocarpa* individuals and none of the pure *P. balsamifera* samples making up the pan-genome graph. These *P. trichocarpa* - specific genes were enriched for protein families, including sulfotransferases, serine carboxypeptidases, Mlo family proteins, D-mannose binding lectin, Glycosyl hydrolases and absistic acid (ABA) induced proteins.

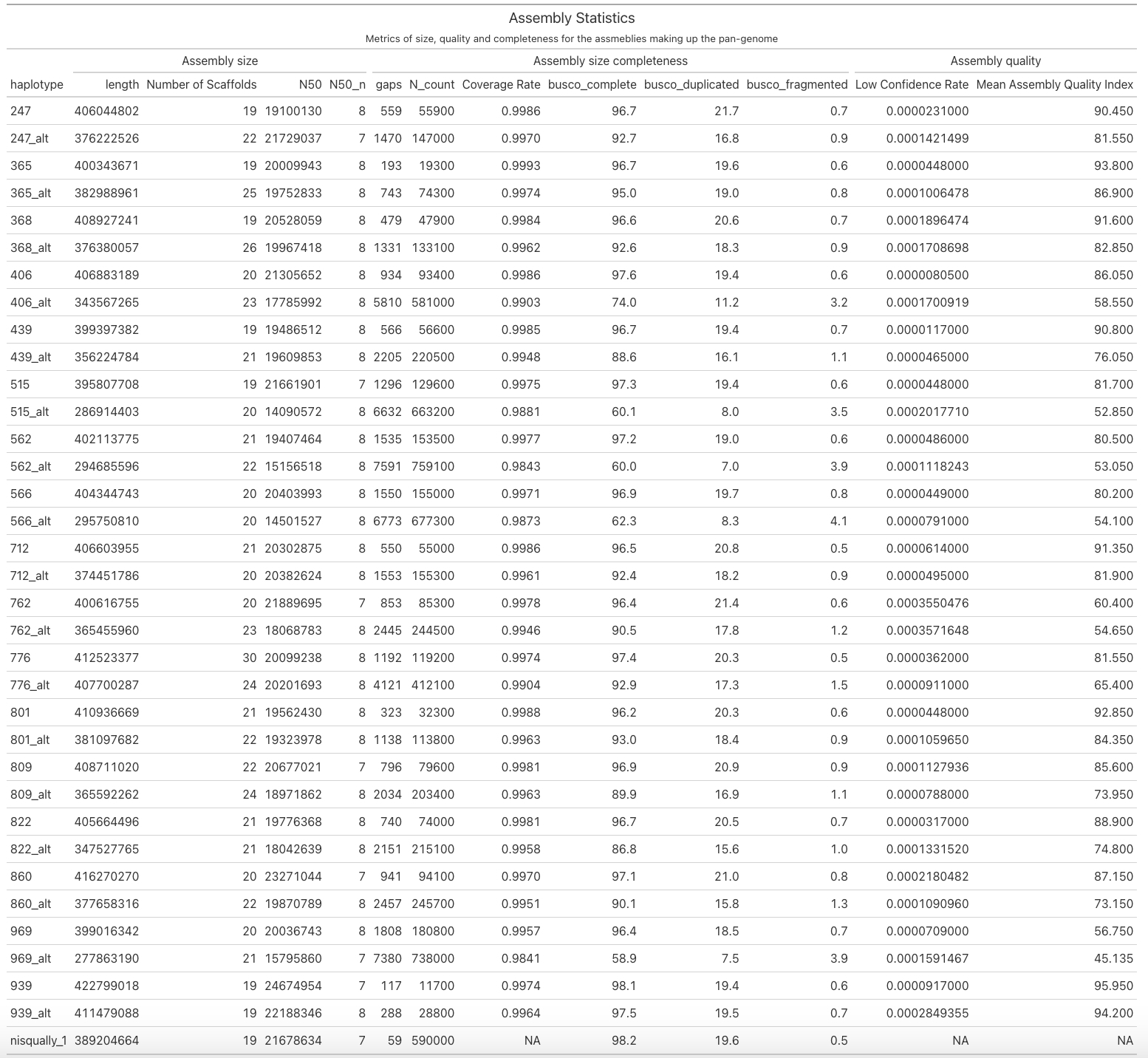
The cactus pan-genome graph contained 1,773,427 SV greater than 20bp in length. [Figure 4](#fig-5) shows the distribution of these variants across the genome. The distribution of frequencies of the non-reference allele for these SV is shown in [Figure 5](#fig-6).

The first two principal components of a PCA on PAV genotyped from short read alignments to the pan-genome graph are shown in [Figure 6](#fig-7). The loading of each allele for each SV on the first principal component of a PCA on PAV genotyped from short read alignments to the pan-genome graph is shown in **?@fig-8**. The SV that were outliers on PC1 overlapped 1362 annotated genes. These genes were significantly enriched for protein families involved in development and growth (K-Box proteins, KNOX proteins), cold tolerance (DEAD/DEAH box helicases), and phenology (SRF transcription factors).

### 0.3.4 SV and Introgression

We identified 126,973 SV that overlapped ancestry blocks in admixed individuals, and found that 30,721 of these were associated with blocks of either *P. trichocarpa* or *P. balsamifera* ancestry in admixed individuals.

### 0.3.5 Tables

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# 1. Figures

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Figure 1: (a): sampling locations for 575 individuals used in this study, color indicates ancestry based in ADMIXTURE analysis (b): A subset of 16 individuals used for whole genome assembly

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Figure 2: (a): Pan-genome growth curve visualizes the change in core and shell genome size as samples are added. (b): The relative length of the core, shell and singleton portions of the pan-genome for each sample represented

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Figure 3: The first two principal components of a PCA on presence/absence variation in the pan-genome. Results of PCA on common (a), rare (b) and all (c) PAV are shown Color indicates ancestry based in ADMIXTURE analysis.

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| Figure 4: The density of SV larger than 20bp in length across the genome (purple) compared to the density of annotated genes (green) |

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| Figure 5: The frequency distribution of the minor allele for SV of different size classes |

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| Figure 6: The first two principal components of a PCA of PAV genotyped from short read alignments to the pan-genome graph. Color indicates ancestry based on ADMIXTURE analysis. Shape indicates which of the latitudinal transects the individual was sampled from. |

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