Botanical Journal of the Linnean Society, 2026, **210(2)**, 185–202
https://doi.org/10.1093/botlinnean/boaf036
Advance access publication 19 May 2025
Original Article

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Solving a century-old conundrum: genetic integrity of a rare and local endemic shrub facing introgression with a widespread congener

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ABSTRACT

Hybridization is a key driver of evolutionary processes and speciation. Advances in sequencing technology provide unprecedented opportunity to study discriminating phenotypic characters at the molecular level and identify candidate loci associated with the speciation process. *Salix chlorolepis* is a rare and threatened endemic shrub species restricted to the upper slopes of Mount Albert (Canada) proposed to hybridize with *S. brachycarpa*, a locally abundant geographically widespread congener. We aimed to characterize rangewide genetic variation of *S. chlorolepis*, establish whether it actually hybridizes with *S. brachycarpa*, and assess whether leaf pilosity is an indicative variable phenotypic trait related to introgressive hybridization. Using single nucleotide polymorphism data, we inferred patterns of genetic structure and diversity. We tested for genetic associations with environmental distance, taxonomic identity, or phenotypic variability in leaf hair density using populations genomics approaches at the multilocus (partial Mantel tests and generalized dissimilarity models) and single-locus (latent factor mixed model, pRDA, PCAdapt, OutFLANK) levels. Although a common genetic ancestry persists within *S. chlorolepis*, fine-scale spatial genetic structure reflects its fragmented distribution. Interspecific genetic admixture in sympatry zones corroborates the existence of introgressive hybridization. Leaf pilosity is an important variable explaining multilocus genotype variation between the two taxa. We flagged nine candidate loci that are both strongly associated with leaf pilosity phenotype and with divergent selection between taxa. The existence of such loci 'impermeable' to introgression ensures maintenance of interspecific barrier and clear phenotypic differentiation between species. Such genomic heterogeneity alleviates assimilation risk of the rare and threatened species by its more abundant congener despite ongoing introgression.

Keywords: DArTseq; endemic species; F_{ST} outlier; generalized dissimilarity modelling; genotype–phenotype association; hybridization; introgression; leaf pilosity; rare species; *Salix*

INTRODUCTION

Hybridization is a key driver of evolutionary processes and speciation in plants (Mallet 2005, 2007, Arnold 2015, Goulet *et al.* 2017). Increasing availability of new molecular techniques and recent developments of advanced genomic analyses enable the study of natural hybridization on a whole-genome scale and offer an unprecedented analytical framework for understanding the origin of species and the maintenance of their integrity (Seehausen *et al.* 2014). Hybridization may result in at least three evolutionary processes among breeding populations (Abbott *et al.* 2013): (i) progressively increasing whole-genome resistance to introgression and reinforcement of interspecific reproductive isolation (Wu 2001, Bierne *et al.* 2011, Seehausen *et al.* 2014, Wolf and Ellegren 2017), (ii) adaptive divergence between populations through homoploid hybrid speciation (Buerkle *et al.* 2000, Gross and Rieseberg 2005, Mavárez *et al.* 2006) or allopolyploid speciation (Mallet 2007), and (iii) a balance between selection and hybridization mechanisms, whereby introgression is limited to certain regions of the genome (i.e. genomic heterogeneity; Harrison and Larson 2014, 2016, de Lafontaine *et al.* 2015, Pfennig 2021). Indeed, some genetic variants transmitted through introgressive hybridization may result in a transfer of adaptive genes already filtered out by natural selection (i.e. adaptive introgression, Shaw and Mullen 2011, Abbott *et al.* 2013, Suarez-Gonzalez *et al.* 2018). Conversely, some alleles recalcitrant to introgression contribute to maintaining interspecific

Received 2 October 2024; revised 18 March 2025; accepted 10 April 2025

differences, and therefore species integrity, by accumulating genetic divergences that may ultimately result in distinct phenotypes (Barton 2001, Shaw and Mullen 2011). Yet, plant species identification relying solely on phenotypic criteria might hinder the formal distinction of hybrids from their parental species (Rieseberg *et al.* 1993, Hardig *et al.* 2000). Recent advances in sequencing technology have offered an unprecedented opportunity to study discriminating phenotypic characters at the molecular level (Pinheiro *et al.* 2016, Bersweden *et al.* 2021, Pérez-Pedraza *et al.* 2021) and identify candidate loci associated with the speciation process (Ravinet *et al.* 2017, Campbell *et al.* 2018).

The evolution of willows (*Salix*, Salicaceae) is strongly driven by hybridization and introgression (Brunsfeld *et al.* 1992, Fogelqvist *et al.* 2015, Wagner *et al.* 2021, Marinček *et al.* 2023). The genus includes 400–500 species primarily distributed in the northern hemisphere among which 113 are native to North America (Argus 2010a). Owing to their reduced and poorly differentiated floral structures, their dioecious habit, and the intrinsic variability of their phenotypes exacerbated by a high propensity for hybridization, willow species represent a major challenge for taxonomists (Azuma *et al.* 2000, Argus 2006). Several barriers to interspecific reproduction operate within the genus such as phenological mismatches (Mosseler and Papadopol 1989), incompatibility between pistillate and staminate flowers (Mosseler 1989), and the low survival among F1 individuals (Argus 2006). Despite these limitations, some 120 taxa resulting from hybridization are currently recognized in North America (Argus 2006) albeit the delimitation among species remains elusive in several cases (Wagner *et al.* 2021).

The green-scaled willow (*Salix chlorolepis* Fernald) is an endemic shrub whose range is restricted to the serpentinized area (derived from the weathering of ultramafic peridotite rocks) of Mount Albert, eastern Canada (Fig. 1A, B). First described in the early 20th century by Merritt Lyndon Fernald (Fernald 1905), *S. chlorolepis* is strictly found in a few late-melting snowbeds located between 900 and 1000 m a.s.l. (COSEWIC 2006, 2020). The species exclusively coexists in sympatry with the short-fruited willow (*Salix brachycarpa* Nutt.), a boreal shrub species widespread in North America (Schneider 1918, Argus 2010b), albeit geographically restricted to the Mount Albert area in Eastern North America, where it is locally abundant and grows ubiquitously in the subalpine meadows and alpine tundra (Fig. 1C, D). *S. brachycarpa* has a variably pilose foliage and ovaries whereas *S. chlorolepis* is completely glabrous. Shortly after the discovery of *S. chlorolepis*, Schneider (1918) suggested that it hybridized with *S. brachycarpa*. The putative hybrid (*Salix* × *gaspeensis* Schneider) would resemble *S. chlorolepis* but has a sparsely pilose foliage and clearly pilose ovaries (Argus 1965). This hybrid designation, based on morphological traits intermediate between presumed parental species, has never been corroborated by molecular assays. The whole *S. chlorolepis* population is estimated at <300 individuals (COSEWIC 2006, 2020), which is much fewer than *S. brachycarpa* that is commonly

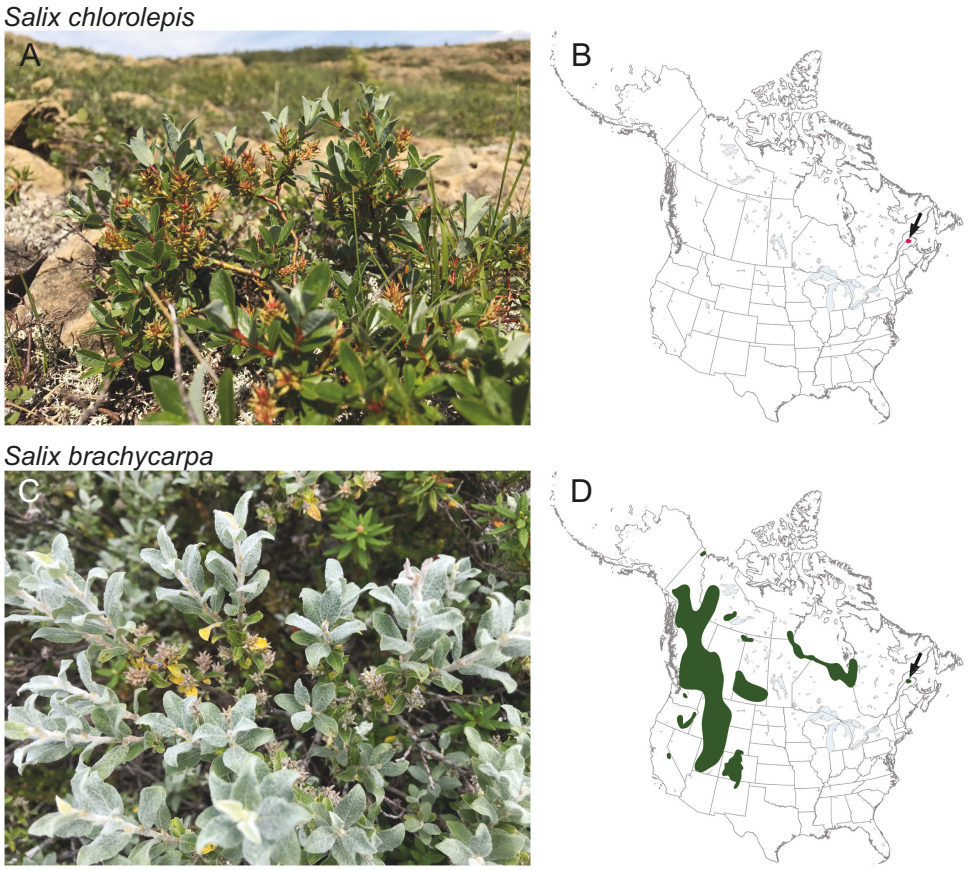

Figure 1. Photograph and geographical range of *Salix chlorolepis* (A, B) and *Salix brachycarpa* (C, D). Arrow indicates the location of Mount Albert, Canada.

encountered in the serpentinized area of Mount Albert, even within the limited range of *S. chlorolepis*. This local endemic is a threatened species in Canada (D1 status since 2006, confirmed in 2020; COSEWIC 2006, 2020) and qualifies as a critically imperilled species at the global scale (G1 status) according to NatureServe (2020). Because of the demographic precarity of *S. chlorolepis*, there is an urgent need to clarify its propensity to hybridize with *S. brachycarpa* and assess the risk this could pose for the preservation of the species' genetic integrity (Environment Canada 2015, COSEWIC 2020).

This study aims to evaluate the genetic variability of *Salix chlorolepis* over its entire geographical range and its potential genetic interactions with *Salix brachycarpa*. Specifically, we (i) evaluated the pattern of genetic diversity among the local colonies of *S. chlorolepis*; (ii) probed the existence of introgressive hybridization between *S. chlorolepis* and *S. brachycarpa* as suggested by Schneider (1918) using genomic, environmental, and phenotypic data; and (iii) assessed whether phenotypical variability can be attributable to environmental factors and/or associated with genetic diversity induced by hybridization. The restricted geographical range of *S. chlorolepis*, estimated to 7.9 km^2, provided high spatial resolution information on the eco-evolutionary dynamics associated with the hybridization process occurring in the strongly selective habitat of serpentine outcrops (Pillon *et al.* 2019). As hybridization and genetic swamping raise several concerns for biodiversity conservation (Pfennig *et al.* 2016, Vallejo-Marín and Hiscock 2016), our study provides original insight into the genetic status of a rare endemic species and the genomic architecture of its key diagnostic phenotype.

MATERIAL AND METHODS

Study area and species identification

The study area includes the summit plateau and upper slopes of Mount Albert, a serpentinized rock massif culminating at 1151 m a.s.l. in eastern Canada (48.9° N, 66.2° W) that was glaciated until the Upper Wisconsinan, some 10 400 years ago (Richard *et al.* 1997). The total population of *Salix chlorolepis* (258 confirmed individuals) is divided into 11 colonies ranging from 1 to 136 individuals (COSEWIC 2020). *S. chlorolepis* is an endemic shrub species whose habitat is restricted to late-melting snowbeds at the top of steep slopes, generally formed of rocky scree located on the margins of the Mount Albert plateau (Québec, Canada). This willow species is exclusively found on serpentine soils, which are generally toxic to generalist plant species (Alexander *et al.* 2007), and emerges late in the spring following delayed melting of heavy snow accumulations in its habitat. Typical individuals of the species are described as having brick-red twigs, green, glabrous, glossy foliage, a green involucral bract, and glabrous ovaries (Fig. 1A). They are also known to flower from July to early August (Argus 2010c).

Salix brachycarpa is a shrub species with a transcontinental boreal distribution that extends to the summit of Mount Albert. Although this willow is globally and locally more common than *S. chlorolepis*, it has never been formally recorded on other, non-serpentinized mountains of the region. *S. brachycarpa* has variable leaf pilosity, giving it a silvery appearance when strongly pubescent (Fig. 1C). *S. brachycarpa* is found in sympatry with *S. chlorolepis* at elevations between 825 and 1050 m a.s.l., while being roughly evenly distributed in allopatry on the summit plateau and its slopes, down to lower elevations (Argus 2010b).

The formal delimitation of parental taxa and putative hybrids remains uncertain a priori, we thus used an agnostic approach for our field sampling. For the sake of clarity, individuals morphologically described as *S. chlorolepis* according to this definition will be named as such. We will also refer to *S. brachycarpa* for any individuals bearing the characteristics detailed above for this species. Individuals resembling *S. chlorolepis* in all respects, except for pilose ovaries and abaxial surface of the leaves, are hereby referred to as putative hybrids.

Sampling design

We collected samples for morphological and genomic analyses from a total of 258 individuals identified as *Salix chlorolepis*, *Salix brachycarpa* or their putative hybrids on Mount Albert (Table 1). For each individual sampled, 2–3 leaves were harvested starting from the fourth node of the twig apex, dried pressed, and stored flat for later phenotypic analysis. Also, up to eight juvenile leaves (or buds) per individual were collected, preserved in silica gel, and stored in a freezer at −20°C until DNA extraction for genomic analysis. Finally, a series of environmental data were recorded for each sampled individual, including altitude, slope inclination and orientation, percent cover of lichens, mosses, herbaceous and shrub strata, organic layer thickness, and canopy height.

Up to 10 individuals identified as *S. chlorolepis* were sampled from six colonies (total n = 51 individuals) for analysing the species' genetic structure (Table 1, Fig. 2A). Note that three colonies including <10 *S. chlorolepis* individuals were collected exhaustively (i.e. all individuals were sampled). To assess the presence of introgressive hybridization between the two species, sampling was carried out in areas where *S. chlorolepis* and *S. brachycarpa* coexist in sympatry. We focused on the two *S. chlorolepis* colonies with the largest numbers [North colony (C_{NORTH}) and South colony (C_{SOUTH}); Fig. 2A]. From the centre of each of these two colonies, we systematically sampled along a transect oriented towards the top of the Mount Albert plateau (Fig. 2B, C). At each transect [North transect (T_{NORTH}) and South transect (T_{SOUTH})], a regular sampling step was applied to collect 15 individuals identified as *S. brachycarpa* in the sympatric zone, and then beyond for ~30 additional individuals in the allopatric zone where *S. brachycarpa* exists in the absence of *S. chlorolepis* (Table 1, Supporting Information, Note S1). Sampling of putative hybrids (n = ~20 individuals per transect) was also carried out in the sympatric zone (no putative hybrids were recorded outside this zone). Finally, to distinguish genetic and ecological factors associated with variations in leaf pilosity in *S. brachycarpa*, we sampled an additional 72 individuals distributed as close as possible to 100 intersection points of a systematic orthogonal grid covering the entire plateau (Fig. 2B). Where no specimens were found within 20 m of the grid intersection point, no individuals were sampled.

Phenotypic analysis

A leaf hair density index was designed using an image analysis method modified from Nielsen *et al.* (2014). The 'Threshold

Table 1. Details on the datasets used for the analyses.

Datasets	n_{total}	n_{loci}	K	Sampled species
Six *S. chlorolepis* colonies	51	14 046	4	
C_1	6			*S. chlorolepis*
C_2	9			*S. chlorolepis*
C_{SOUTH} (south colony)	10			*S. chlorolepis*
C_{NORTH} (north colony)	10			*S. chlorolepis*
C_5	10			*S. chlorolepis*
C_6	6			*S. chlorolepis*
Colonies + sampling grid	123	15 143	4	*S. chlorolepis* (n = 51) *S. brachycarpa* (n = 72)
T_{NORTH} + T_{SOUTH} + grid	219	12 651	3	*S. chlorolepis* (n = 20) *S. brachycarpa* (n = 149) putative hybrids (n = 50)
T_{NORTH} (north transect)	69	13 263	2	*S. chlorolepis* (n = 10) *S. brachycarpa* (n = 32) putative hybrids (n = 27)
T_{SOUTH} (south transect)	78	13 203	2	*S. chlorolepis* (n = 10) *S. brachycarpa* (n = 45) putative hybrids (n = 23)
Sampling grid	72	14 109	1	*S. brachycara*

Notes: n_{total} and n_{loci} represent the number of individuals and the number of loci retained after the filtration process. K is the number of genetic groups retained from sNMF analysis.

Color' function of ImageJ software v.1.53a (Schneider *et al.* 2012) was used as a tool for measuring leaf pilosity. Each leaf was photographed using a camera (Leica IC90 E, Wetzlar, Germany) mounted on a stereomicroscope (Leica M60, Wetzlar, Germany) at ×32 magnification, under constant illumination set to maximum. The adaxial side of each leaf occupied the full width of the image, with the primary vein positioned in the centre of the frame. In the 'Threshold Color' function, we selected the 'HSB color' model in 'Threshold' mode, using the following settings: hue 53–122, saturation 0–255, brightness 0–255. Images were converted into matrices of black or white pixels (Fig. 3). The percentage of white pixels (i.e. pixels corresponding to hair) in relation to the total number of recorded pixels provides the leaf hair density index used to estimate the phenotypic variability of leaf pilosity.

DNA extraction and genotyping

Each sample (10–12 mg of dried tissue) was placed in a 1.2-mL collection microtube, immersed in liquid nitrogen, and disrupted/homogenized by bead beating using 3-mm tungsten carbide beads in a TissueLyser II equipped with 2 × 96-well plate adapter set (Qiagen, Hilden, Germany). Total DNA was extracted using the DNeasy 96 Plant Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were quantified using a Qubit 4 fluorometer (Invitrogen, Carlsbad CA, USA) and a BioDrop μLITE spectrophotometer (Biochrom Ltd, Cambridge, UK). DNA solutions were then adjusted to 15–75 ng μL^{-1} in a minimum volume of 35 μL. Samples were genotyped using the DArTseq complexity reduction approach at the Diversity Arrays Technology Pty Ltd (DArT) laboratory at the University of Canberra, Australia (Sansaloni *et al.* 2011). PstI/MseI enzymes were used for digestion of DNA solutions (Kilian *et al.* 2012). Each DNA fragment obtained had a maximum of 69 base pairs and the average reading depth was 10×. Molecular markers (single nucleotide polymorphisms; SNPs) were obtained following DArTseq's proprietary bioinformatics procedure, and high-density sequencing steps were performed using the Illumina NovaSeq 6000 platform.

To retain the most informative SNPs, a series of filters was applied with the DARTR library v.2.0.4 (Gruber *et al.* 2018) in R v.4.1.2 (R Core Team 2021). The DArTseq analytical procedure includes a quality control step involving the re-sequencing of ~25% of samples, providing a reproducibility value for each locus. SNPs with reproducibility < 0.99 were eliminated. We also applied a series of filters to: (i) remove monomorphic SNPs and those with a minor allele frequency < 1%; (ii) retain only loci with < 10% missing data; and (iii) retain only individuals with < 5% missing data. The filtration steps were performed independently for each of the following datasets: (i) the six *S. chlorolepis* colonies on the margins of the Mount Albert plateau, (i) the colonies and the grid, (iii) both transects and the grid, (iv) T_{NORTH} alone, (v) T_{SOUTH} alone, and (vi) the grid alone (Table 1).

Genetic structure and diversity

The fixation index (F_{ST}) was used to estimate pairwise genetic differences among the six *S. chlorolepis* colonies as well as between the two species. F_{ST} was calculated according to the method of Weir and Cockerham (1984) using the gl.fst.pop function in the DARTR library and the 95% confidence interval was estimated using 1000 bootstrap replicates. For each colony and the grid, allelic richness (Ar), inbreeding index (F_{IS}) and genetic diversity indices [observed heterozygosity (H_O) and expected heterozygosity (H_E)] were obtained using the R library HIERFSTAT v.0.5-11 (Goudet 2005) whereas mean nucleotide diversity across loci (π), Tajima's D values were computed in VCFtools (Danecek *et al.* 2011). Mean pairwise relatedness

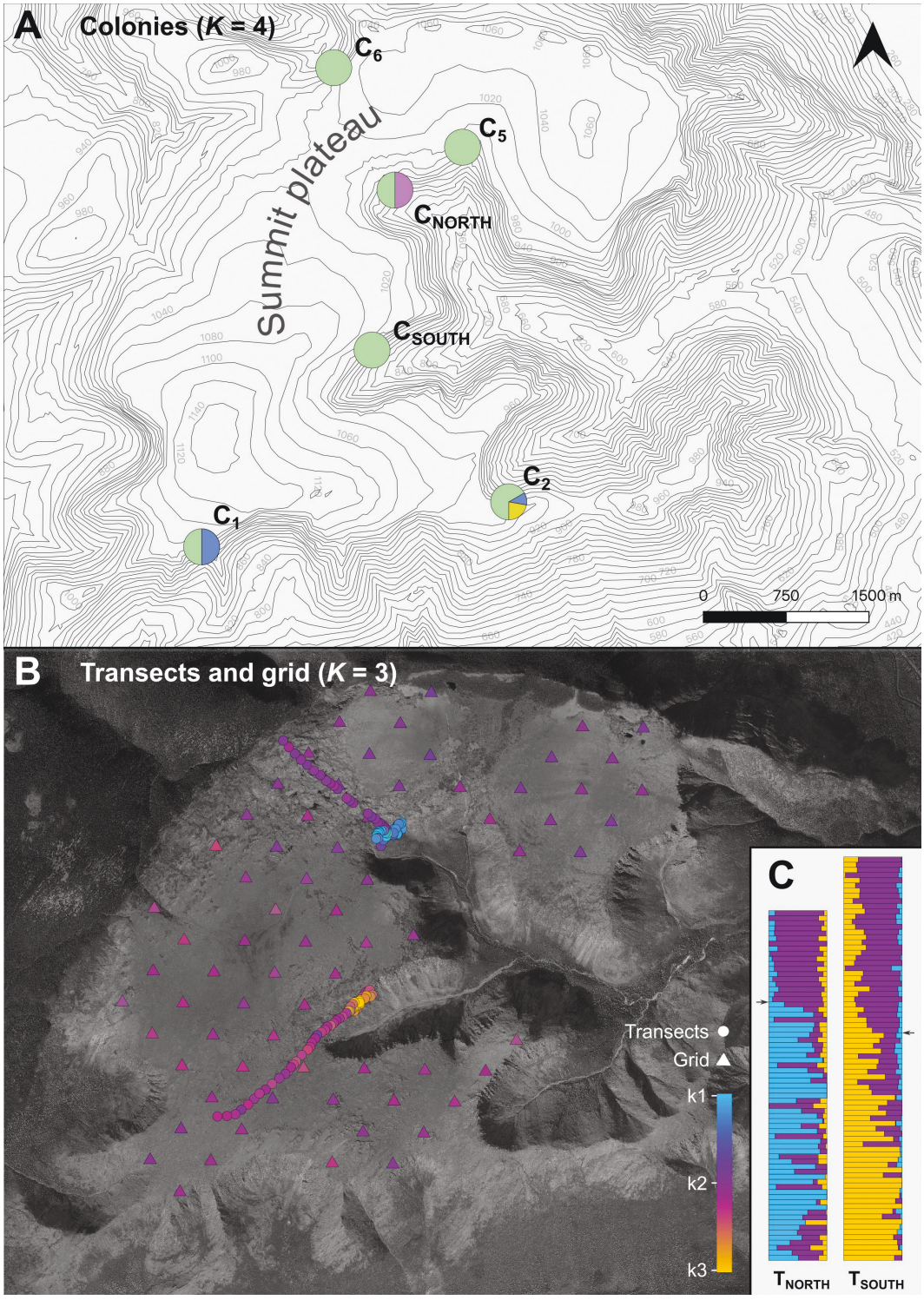

Figure 2. Sampling sites at Mount Albert, Canada, and genetic structure of *Salix chlorolepis* and *Salix brachycarpa*. A, Pie charts show the proportion of genetic assignment to each group ($K_{colonies} = 4$) for six colonies of *S. chlorolepis*. B, Individuals sampled on transects (circles) and orthogonal grid (triangles) coloured with an CMYK colour gradient whereby the proportion of the three blended colours represents each individual's assignment to the three genetic groups ($K_{transects\ \&\ grid} = 3$). Ancestry coefficient (Q) values of k1, k2, and k3 were converted into the percentages of cyan (hex#02befe), magenta (hex#9901cb), and yellow (hex#fecc00), respectively using the *rgb* function in R. C, Assignment of each individual sampled in north and south transects (T_{NORTH} and T_{SOUTH}) to the three genetic groups. Individuals are ordered by their distance from the centre of the *S. chlorolepis* colony (bottom) to the end of the transects (top). The arrow indicates the limit of sympatric zones.

(ϕ) was calculated using the relatedness2 option in VCFtools, where a negative value indicates genetic dissimilarity, a value near 0 signifies no genetic relationship, and a value close to 0.5 denotes high genetic similarity (Manichaikul *et al.* 2010). Hardy–Weinberg equilibrium was tested with the PEGAS v.1.1 library (Paradis 2010). Partitioning of genetic diversity between

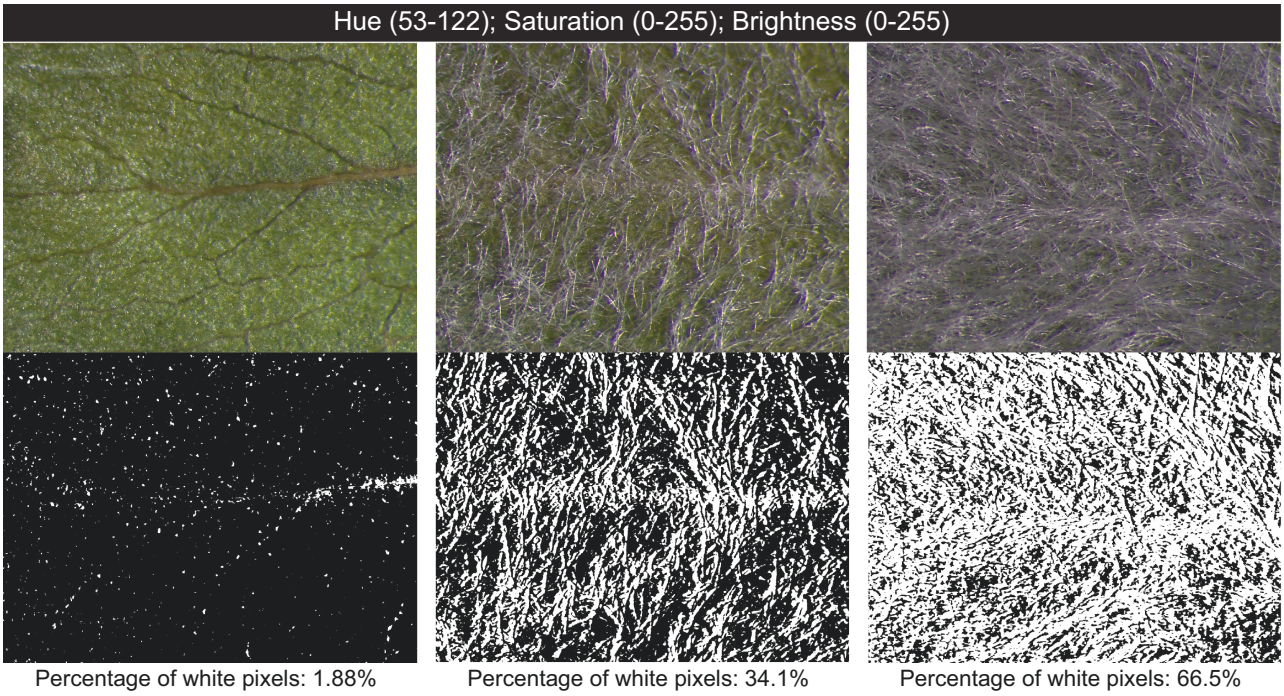

Figure 3. Raw images (top) converted to matrices of black and white pixels (bottom) for three distinct leaf hair density phenotypes and their corresponding percentage of white pixels.

S. chlorolepis (colonies) and *S. brachycarpa* (grid), among *S. chlorolepis* colonies, and within the colonies or the grid was estimated with an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) in the POPPR library (Kamvar *et al.* 2014, 2015). Significance tests were based on 1000 permutations. To estimate the number of ancestral lineages, we used the sparse nonnegative matrix factorization algorithm (sNMF; Frichot *et al.* 2014) implemented in the R library LEA v.3.8.0 (*snmf* function; Frichot and François 2015). Datasets comprising (i) all six *S. chlorolepis* colonies, (ii) the colonies and the grid, (iii) both transects and the grid, (iv) T_{NORTH} alone, (v) T_{SOUTH} alone, and (vi) the grid alone were analysed independently. The number of genetic groups (K) tested was adjusted from $K = 1$ to $K = 10$ and 10 replicates were applied for each K value. The choice of K value was established by the cross-entropy criterion (Frichot *et al.* 2014) and a visual inspection of the spatial clustering of the K genetic groups. Because *S. chlorolepis* individuals from colonies C_{NORTH} and C_{SOUTH} belong to two distinct genetic groups (see Results), transects T_{NORTH} and T_{SOUTH} were analysed independently for all subsequent analyses.

Multilocus associations with the environment and the phenotype (T_{NORTH}, T_{SOUTH}, grid)

Partial Mantel tests were performed for T_{NORTH}, T_{SOUTH}, and the grid using the R library VEGAN v.2.6-2 (Oksanen *et al.* 2022) to assess correlations between environment, leaf pilosity, and multilocus genotype of individuals, conditional on geographical distance. After imputing missing data (R library MISSMDA; Josse and Husson 2016), all environmental variables were reduced to the first axis of a principal component analysis (PC1). Matrices of Euclidean distance for the environment (PC1), leaf hair density, and geographical position were calculated with the dist function in VEGAN, while genetic distances were calculated with the gl.dist.ind function in the DARTR library. Spatial autocorrelation between environmental, phenotypic, and genotypic variables was checked. Mantel tests were run using 999 random permutations per test. Both Pearson and Spearman correlation coefficients are reported for comparison.

We performed generalized dissimilarity models (GDMs; Ferrier *et al.* 2007, Fitzpatrick and Keller 2015) to address the individual influence of each environmental variable, phenotype, and geographic position on the multilocus genotypes from T_{NORTH}, T_{SOUTH}, and the grid. Genetic distance matrices calculated for partial Mantel tests were also used for GDMs. The environmental variables, leaf pilosity, and geographical position of individuals, as well as the response variable (i.e. genetic distances), were integrated into a GDM model using the R library GDM v.1.5.0-3 (Fitzpatrick *et al.* 2021, Mokany *et al.* 2022). Two models (one with and the other without the effect of geographical position) were tested for each dataset with 500 permutations. This GDM procedure provided the relative importance of each retained variable with its probability (P value), as well as the deviance explained by each model assayed.

For T_{NORTH} and T_{SOUTH}, the proportion of genetic admixture that belongs to *S. chlorolepis* and *S. brachycarpa* was estimated using ancestry coefficient values (Q) with $K = 2$ ancestral populations (*snmf* function). For each transect, the ancestry coefficients, which indicate the probability that each individual belongs to either of the $K = 2$ groups, were plotted against the distance from the centre of the *S. chlorolepis* colony. This analysis was used to assess whether sampled individuals identified a priori on a morphological basis are indeed assigned a posteriori to two distinct genetic groups corresponding to the two taxa, with introgressed individuals having mixed ancestry. Given the

high variability of leaf pilosity observed in the field, the relationship between leaf hair density index and ancestry coefficients was also assessed. The R library SEGMENTED v.1.6-0 (Muggeo 2003, 2008) was used to fit a segmented regression model for each transect and extract the breakpoint as well as the probability (P) associated with each linear segment.

Genotype–phenotype associations and outlier loci analysis

To shed some light on the genetic diversity associated with leaf pilosity in the two taxa, we carried out genome-wide genotype±phenotype association tests and F_{ST}-outlier analyses to identify candidate loci putatively under selection (or tightly linked to such genes; hereafter designated as 'candidate loci'). On the one hand, association tests between the genotype and phenotypic traits are used to flag candidate loci whose allele frequencies are correlated with variable phenotypes (here: leaf hair density) between individuals (Coop *et al.* 2010, Frichot *et al.* 2013, Rellstab *et al.* 2015). On the other hand, F_{ST}-outlier analyses detect candidate loci that stand out from the genomic background thanks to extreme level of genetic differentiation between the two taxa above what is expected from neutrality, hence putatively involved in divergent selection and speciation (Whitlock and Lotterhos 2015, Luu *et al.* 2017). Because all such screening analyses involve multiple hypotheses testing, they are sensitive to Type I errors (false positives). To overcome this potential bias, several complementary methods with different assumptions were used, as recommended (Rellstab *et al.* 2015, Vaux *et al.* 2023). Each analysis detailed next was carried out independently on T_{NORTH}, T_{SOUTH}, and the grid. The latent factor mixed model (LFMM, Frichot *et al.* 2013) and partial redundancy analysis (pRDA, Capblancq and Forester 2021) approaches were employed as genotype–phenotype association methods, while PCAdapt (Luu *et al.* 2017) and OutFLANK (Whitlock and Lotterhos 2015) approaches were used to scan the genome for F_{ST}-outliers.

The LFMM is a univariate linear approach implemented in the lfmm2 function of the R library LEA v.3.8.0 (Caye *et al.* 2019). This approach uses a correction factor (latent factor), generally attributed to the genetic structure of the population, to correct potential confounding effects when identifying candidate loci (Frichot *et al.* 2013, 2014, Frichot and François 2015). Missing data were imputed with the *impute* function from the same library and the latent correction factor (K) was extracted at the genetic structure analysis stage (snmf function). Candidate loci associated with leaf pilosity (i.e. leaf hair density index) were retained when their probability (P) was below the 1% threshold.

The second approach used to detect candidate loci associated with leaf pilosity phenotype is pRDA (Forester *et al.* 2018). Using the genome as the response variable and the environment or phenotype as the predictor, the constrained ordination axes of the RDA represent loci that co-vary with the multivariate environment and/or phenotype (Rellstab *et al.* 2015, Capblancq and Forester 2021). To avoid false positives related to the neutral genetic structure of populations (Excoffier *et al.* 2009), pRDA was performed with the R library VEGAN v.2.6-2. This approach involves the addition of conditioning matrices consisting of (i) geographic coordinates and (ii) ancestry coefficients (Q) from sNMF analysis. We considered candidate loci with standard deviation scores ≥ 2.5 ($P \leq .012$) on axes selected using a scree plot as being significantly associated with leaf pilosity (Forester *et al.* 2018).

To target candidate 'speciation' loci regardless of phenotype, we generated principal component analyses using the R library PCADAPT v.4.3.3 (Luu *et al.* 2017). This multivariate approach predicts that SNPs with excessive association with the genetic structure of the two taxa analysed might be considered under putative divergent selection. The pcadapt function was run a first time with $K = 20$ and the appropriate number of components was chosen using Cattell's rule (Cattell 1966) to interpret the scree plot. PCAdapt was performed a second time with the adjusted number of components and the false discovery rate was set at a threshold of 0.05. Candidate loci below this threshold were identified as markers 'impermeable' to introgression, indicative of genomic regions potentially experiencing divergent selection that maintains species differentiation despite ongoing gene exchange between taxa.

The OutFLANK approach, implemented in R library OutFLANK, relies on detecting deviations from a neutral chi-square F_{ST} distribution across populations, inferred for loci free of spatially heterogeneous selection or homogeneous balancing selection (Whitlock and Lotterhos 2015). This approach, modified from Lewontin and Krakauer (1973), predicts that loci under divergent selection exhibit F_{ST} values higher than the overall genomic background and are thus located at the extreme right tail of F_{ST} distribution. Subsets of 'purebred' individuals for each of the two species were independently selected in T_{NORTH} and T_{SOUTH} based on ancestry coefficients ($Q > 0.85$ as 'pure' *S. brachycarpa* and $Q < 0.15$ as 'pure' *S. chlorolepis*). OutFLANK version 0.2 was run with left and right trim fractions of 6% and 5%, respectively, and a minimum heterozygosity threshold of 0.1. To obtain a minimum number of candidate loci potentially under divergent selection (i.e. maintaining interspecific differences despite introgression), we retained 1% of the loci in the distribution with the lowest probabilities (P). The level of interspecific differentiation at these candidate loci is thus exceptionally high compared to the structure estimated across the whole genome.

Highly validated candidate loci that were flagged at least three times on one or both transects and which rarely expressed a phenotype beyond 20% leaf hair density for the homozygous genotype of the minor allele were retained for further analysis. To annotate these highly validated candidate loci, we identified genic regions of the *Salix purpurea* 5.1 reference genome (Phytozome genome ID: 519, NCBI taxonomy ID: 77065; Zhou *et al.* 2020) overlapping candidate loci using Phytozome V13, the Comparative Plant Genomics portal of the US Department of Energy Joint Genome Institute (phytozome.jgi.doe.gov; Goodstein *et al.* 2012), then retrieved gene ontology (GO) terms associated with the coding regions.

RESULTS

Genomic data, diversity, and genetic structure

The DArTseq genotyping technology produced a dataset totalling 99 736 SNPs for the 258 sequenced individuals. After all filtration steps, the final datasets include: the six *S. chlorolepis* colonies (14 046 SNPs and 51 individuals), T_{NORTH} (13 263 SNPs and 69 individuals), T_{SOUTH} (13 203 SNPs and 78 individuals),

and the grid (14 109 SNPs and 72 individuals) (Table 1). Within the six *S. chlorolepis* colonies, allele richness (Ar) values ranged from 1.210 to 1.234, observed heterozygosity (H_O) from 0.180 to 0.223, expected heterozygosity (H_E) from 0.212 to 0.235, and nucleotide diversity (π) from 0.216 to 0.241 (Table 2). All colonies have weakly positive values of Tajima's *D* between 0.265 and 0.397, indicating no recent selective sweep or population expansion. Relatedness (ϕ) values between −0.181 and −0.045 indicate overall genetic dissimilarity among individuals within colonies. All colonies are in Hardy–Weinberg equilibrium, which provides support for the delimitation into various population units over the species range. Overall, colony C_1 (south-west of the plateau) has the lowest genetic diversity values, while colony C_2 (south-east of the plateau) has the highest. Inbreeding index (F_{IS}) values were all positive and significant, ranging from 0.041 to 0.145, whereas pairwise F_{ST} values ranged from 0.021 to 0.067 (Table 2). On average, colonies C_1 and C_{NORTH} are the most differentiated (highest F_{ST} values), while colonies C_2 and C_{SOUTH} are the least differentiated (lowest F_{ST} values), with respect to all other colonies. Within the grid, the allelic richness of *S. brachycarpa* reached 1.229, the H_O and H_E values were 0.187 and 0.229, respectively, nucleotide diversity is 0.231. Tajima's *D* is slightly higher than in the colonies (0.929). F_{IS} was positive and significant (0.180) although relatedness is negative (−0.234). The sampling grid deviated from Hardy–Weinberg equilibrium and shows significant heterozygote deficiency. Pairwise F_{ST} values between *S. brachycarpa* and colonies of *S. chlorolepis* range from 0.08 to 0.035 and are thus generally lower than those estimated across *S. chlorolepis* colonies. This result is supported by AMOVA indicating that the share of genetic variation partitioned between *S. chlorolepis* from the colonies and *S. brachycarpa* from the grid is not significant. By contrast, differentiation across *S. chlorolepis* colonies accounted for 6% of the total genetic variation (Supplementary Information, Table S2).

The cross-entropy criterion obtained by sNMF analysis suggests the existence of three ancestral genetic lineages. Nevertheless, a visual inspection of the hierarchical clustering suggests it might be preferable to retain a layout with $K = 4$ groups, given its consistency with the geographical structure among the six *S. chlorolepis* colonies distributed at the plateau margin (Fig. 2A, Supporting Information, Figure S3). One genetic group is common to all colonies (green lineage; Fig. 2A). Colonies C_{NORTH} and C_1 stand out from the rest of the colonies due to the coexistence of two distinct genetic groups (green and blue at C_1 and green and purple at C_{NORTH}; Fig. 2A), which supports the relatively higher F_{ST} values found in these colonies. Colony C_2, which is genetically close to C_1, stands out from the other colonies located further north (C_{SOUTH}, C_{NORTH}, C_5, C_6). Some individuals in this colony are assigned to the same two groups as C_1 (green and blue; Fig. 2A), while others are assigned to the fourth genetic group (yellow, Fig. 2A). When considering all *S. chlorolepis* colonies together with *S. brachycarpa* from the sampling grid, sNMF grouped all *S. brachycarpa* individuals with the most common *S. chlorolepis* genetic group (green lineage; Supporting Information, Figure S4).

When considered all together by sNMF, the individuals genotyped in T_{NORTH}, T_{SOUTH}, and the grid fall into two genetic groups according to the cross-entropy criterion. However, the configuration of individuals with $K = 3$ is clearly spatially structured

Table 2. Genetic diversity and differentiation of six colonies of *Salix chlorolepis* and *S. brachycarpa* on Mount Albert, Canada.

Colonies/Grid	*n*	Ar	H_O	H_E	π	T *D*	ϕ	F_{IS}	HWE	Pairwise F_{ST}					
										C_1	C_2	C_{SOUTH}	C_{NORTH}	C_5	C_6
S. chlorolepis															
C_1	6	1.210^a	0.186^{ab}	0.212^a	0.216^a	0.364	-0.056^d	0.091^b	E	-	0.038	0.045	0.067	0.057	0.061
C_2	9	1.234^e	0.223^c	0.235^e	0.241^c	0.387	-0.045^d	0.041^a	E	0.038	-	0.021	0.045	0.033	0.036
C_{SOUTH}	10	1.224^{cd}	0.186^{ab}	0.226^{cd}	0.232^b	0.284	-0.181^b	0.145^e	E	0.045	0.021	-	0.035	0.025	0.028
C_{NORTH}	10	1.218^b	0.184^{ab}	0.220^b	0.225^b	0.397	-0.144^{bc}	0.130^{cd}	E	0.067	0.045	0.035	-	0.044	0.048
C_5	10	1.218^{cd}	0.180^a	0.220^{bc}	0.226^b	0.366	-0.180^b	0.144^{de}	E	0.057	0.033	0.025	0.044	-	0.036
C_6	6	1.221^{bc}	0.188^b	0.224^{bcd}	0.228^b	0.265	-0.102^{cd}	0.116^c	E	0.061	0.036	0.028	0.048	0.036	-
Mean pairwise F_{ST}										0.054	0.034	0.031	0.048	0.039	0.042
S. brachycarpa															
Grid	72	1.229^{de}	0.187^b	0.229^d	0.231^b	0.929	-0.234^a	0.180^f	HD	0.035	0.015	0.008	0.030	0.017	0.017

Notes: *n*: number of individuals; Ar: allelic richness; *TD*: Tajima's *D*; ϕ: relatedness; HWE: Hardy–Weinberg equilibrium (E: equilibrium, HD: heterozygote deficiency). Superscript letters associated with diversity indices indicate significant differences.

(Fig. 2B), whereby two of the three genetic groups are strictly limited to the geographical locations of *S. chlorolepis* colonies C_{NORTH} and C_{SOUTH}, and the third group includes individuals identified as *S. brachycarpa* spread over the summit plateau (sampling grid) and allopatric zones of the transects. Several individuals from the sympatric zone of the transects, located within *S. chlorolepis* colonies, show some level of genetic admixture (Fig. 2B, C). When analysed individually by sNMF, T_{NORTH}, and T_{SOUTH} each comprise two genetic lineages spatially corresponding to the transition from *S. chlorolepis* (i.e. sympatric zones) to *S. brachycarpa* (i.e. allopatric zones) (Table 1). Analysis of the sampling grid by sNMF indicates a single genetic lineage on the summit plateau (Table 1).

Genotype–phenotype–environment associations

Along both the T_{NORTH} and T_{SOUTH} transects, partial Mantel tests indicate significant correlations between genetic distance and leaf pilosity, between leaf pilosity and environmental distance, as well as between genetic and environmental distances, after removing the conditional effect of geographical distance (Table 3). However, no significant correlation was detected for *S. brachycarpa* in the grid. The deviance (i.e. allelic variation) explained by the GDMs controlling for the effect of spatial autocorrelation is 13.2% for T_{NORTH}, 9.8% for T_{SOUTH}, and 7.3% for the grid (Table 4). Unlike the grid GDM, which has no statistical support ($P = .112$), the models for both transects are statistically significant ($P < .0001$). Leaf hair density is the most important indicator in the T_{NORTH} and T_{SOUTH} models, which include the transition from *S. chlorolepis* colonies to *S. brachycarpa*. In comparison, leaf hair density has little importance in the grid GDM, which includes only *S. brachycarpa* individuals. The GDMs that take geographical coordinates into account are all statistically significant (Supporting Information, Table S5), corroborating the spatial autocorrelation detected by the Mantel tests. These models show slightly higher deviations than previous models. Geographical distance and leaf hair density are important indicators in T_{NORTH} and T_{SOUTH}, whereas environmental variables have generally low relative importance. Leaf hair density is the least important indicator retained in the sampling grid (Supporting Information, Table S5).

Individuals identified a priori as *S. chlorolepis* based on their morphology in the field (transects) have significantly lower values of leaf hair density than those identified as *S. brachycarpa* ($F_{2,216} = 70.05$, $P < .001$; Fig. 4A), whereas putative hybrids are not significantly different from *S. chlorolepis*. Correspondingly, individuals identified a priori as *S. chlorolepis* and S. *brachycarpa* were assigned a posteriori to different ancestry coefficients ($F_{2,144} = 60.06$, $P < .001$; Fig. 4B), despite some misidentifications. However, ancestry coefficients of putative hybrids do not differ significantly from those of individuals sampled as *S. chlorolepis*. In both transects (T_{NORTH} and T_{SOUTH}), ancestry coefficients (Q) vary between 0 and 1 only for individuals located within 145 m of the centre of each *S. chlorolepis* colony, which corresponds to the limit of the two sympatric zones (Fig. 5; T_{NORTH}: F-test = 17.2, $P < .0001$; T_{SOUTH}: F-test = 9.10, $P < .0001$). Beyond this threshold geographical distance, Q values are high (> 0.75) and far less variable (this pattern is duplicated in both transects but exacerbated in T_{NORTH}). In both transects, the relationship between leaf hair density index and ancestry coefficient is clearly segmented into two distinct linear relationships (Fig. 6; T_{NORTH}: F-test = 0.015, $P < .0001$; T_{SOUTH}: F-test = 0.050, $P < .0001$). Leaf hair density is constantly low up to a breakpoint indicating a threshold value of genetic admixture between ancestral lines that corresponds to $Q = 0.61$ at T_{NORTH} and $Q = 0.42$ at T_{SOUTH}, i.e. an average of $Q = 0.5$ between the two transects (T_{NORTH}: $F_{1,35} = 0.07$, $P = .79$; T_{SOUTH}: $F_{1,28} = 1.53$, $P = .22$). Before the breakpoint, individual leaf hair density does not exceed 20%. Beyond the Q threshold, leaf pilosity is more variable and increases linearly with ancestry coefficient (T_{NORTH}: $F_{1,28} = 12.41$, $P = .0015$; T_{SOUTH}: $F_{1,46} = 11.37$; $P = .0015$).

Table 3. Correlation coefficients from partial Mantel tests between matrices of phenotypic, environmental, and genetic distances (conditional on geographic distance). Top rows report Pearson's r and bottom rows report Spearman's ρ. Values in bold are significant and P values are in brackets.

Partial Mantel test	T_{NORTH}	T_{SOUTH}	Sampling grid
LHD × PC1-env	**0.360** (.001)	**0.155** (.004)	0.043 (.261)
	0.327 (.001)	**0.091** (.018)	0.031 (.265)
SNPs × LHD	**0.144** (.001)	**0.174** (.001)	0.036 (.289)
	0.151 (.004)	**0.145** (.002)	0.029 (.294)
SNPs × PC1-env	**0.132** (.005)	**0.127** (.002)	0.091 (.091)
	0.127 (.019)	**0.079** (.028)	0.083 (.092)

Notes: LHD: leaf hair density (phenotypic distance); PC1-env: environmental distance; SNPs: single nucleotide polymorphisms (genetic distance).

Table 4. Properties of GDMs and relative importance of phenotypic and environmental variables. Spatial autocorrelation was controlled.

	T_{NORTH}	T_{SOUTH}	Sampling grid
deviance	3.239	2.126	0.909
percent deviance explained	13.160	9.821	7.272
model P value	<.0001	<.0001	.112
	Importance	Importance	Importance
leaf hair density	12.215	14.911	4.396
organic layer thickness	0.680	2.312	
slope inclination	7.734	2.175	
slope orientation	3.551		
lichen cover	0.412	2.976	5.928
moss cover	0.381	0.612	10.097
herbaceous cover	4.435	14.990	21.601
shrub cover	2.453	1.635	24.793
bare ground cover	0.364	-	-
canopy height	0.751	2.184	3.963
elevation	11.392	6.930	3.736

Candidate loci

A total of 1075 candidate loci were identified by at least one of the four methods employed to scan the genome. Of these loci, 107

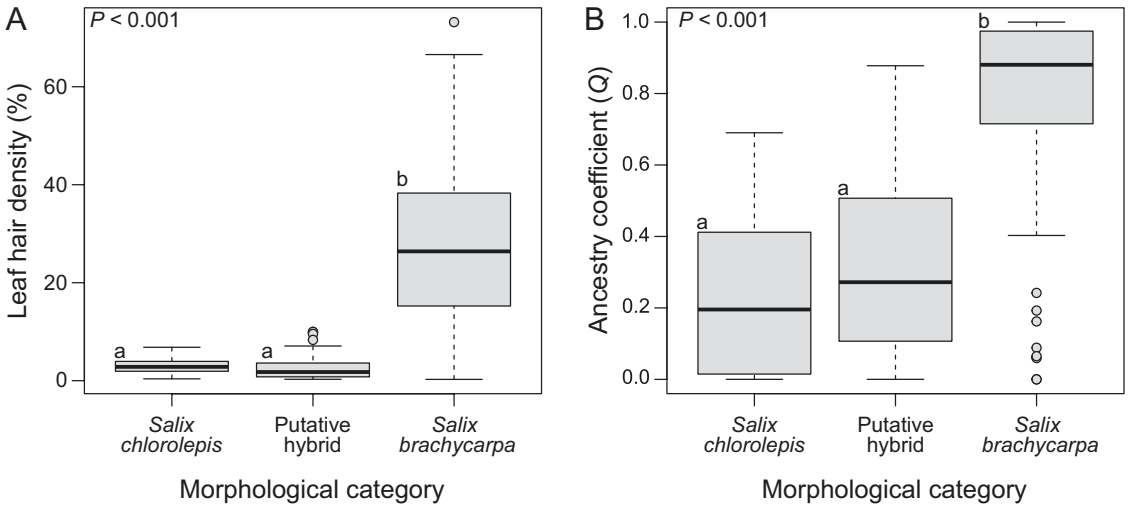

Figure 4. Comparison of the three morphological categories identified a priori on transects T_{NORTH} and T_{SOUTH} based on their leaf hair density (A) and ancestry coefficient (Q) values (B). Boxplots indicate lower quartile, median and upper quartile, and whisker length is 1.5× interquartile range. Different letters indicate statistically significant differences after a Tukey's HSD test.

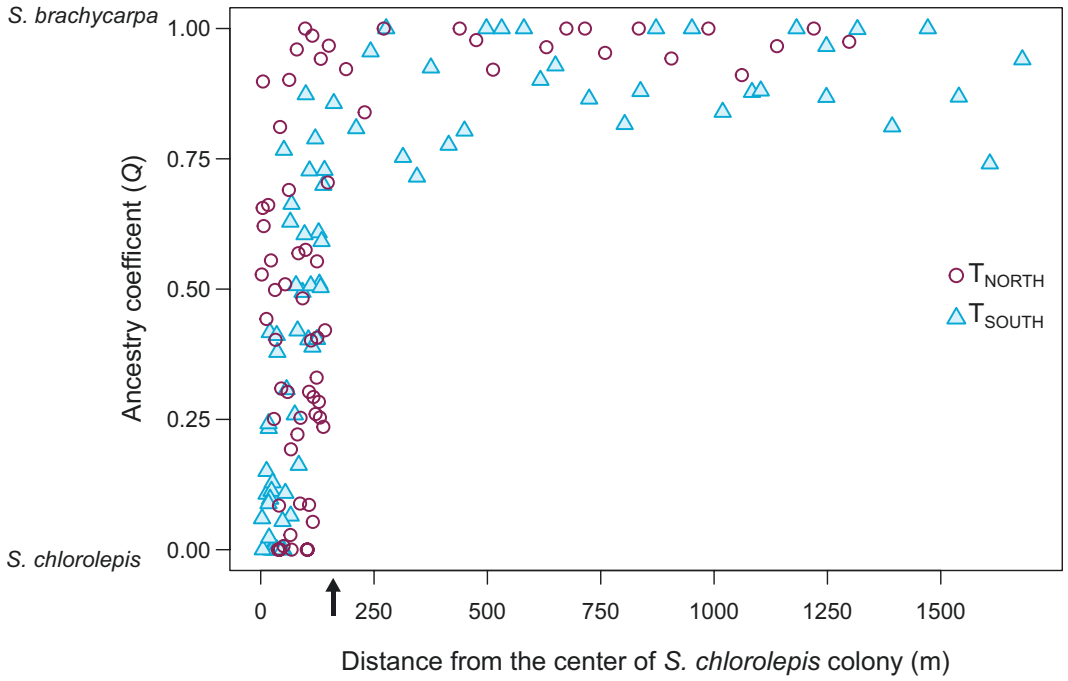

Figure 5. Variation in ancestry coefficient (Q) values obtained independently for transects T_{NORTH} and T_{SOUTH} using sNMF ($K = 2$) as a function of distance from the centre of the corresponding *Salix chlorolepis* colony (C_{NORTH} and C_{SOUTH}). The arrow indicates the limit of the two sympatric zones (145 m). $Q = 0$: *Salix chlorolepis* and $Q = 1$: *S. brachycarpa*.

SNPs coincided for a minimum of two approaches (mean 2.3; CV 0.31), without necessarily being replicated on both transects or the grid (Fig. 7). LFMM analysis retained 134, 137, and 165 SNP loci associated with leaf hair density for T_{NORTH}, T_{SOUTH}, and the sampling grid, respectively. pRDA tests identified 34, 181, and 20 loci associated with leaf hair density for T_{NORTH}, T_{SOUTH}. and the grid, respectively. PCAdapt flagged 296, 99, and 2 SNP loci with excessive association with genetic structure for T_{NORTH}, T_{SOUTH}, and the grid, respectively. OutFLANK retained 119 and 122 loci exhibiting outlier F_{ST} values relative to the interspecific difference observed in the overall genomic background between *S. brachycarpa* and *S. chlorolepis* for T_{NORTH} and T_{SOUTH}, respectively (Fig. 7). Finally, 15 highly validated candidate SNP loci were identified at least three times on one or both transects, and nine of these seldom expressed a phenotype beyond 20% leaf hair density for the homozygous genotype of the minor allele (Figs 7, 8; Table 5). By contrast, individuals homozygous for the other allele at these nine highly validated candidate loci can express the full range of observed leaf pilosity phenotypes (Fig. 8). Mapping these nine candidate loci to the *Salix purpurea* reference genome resulted in the identification of seven genic regions with GO annotations indicating involvement in a variety of

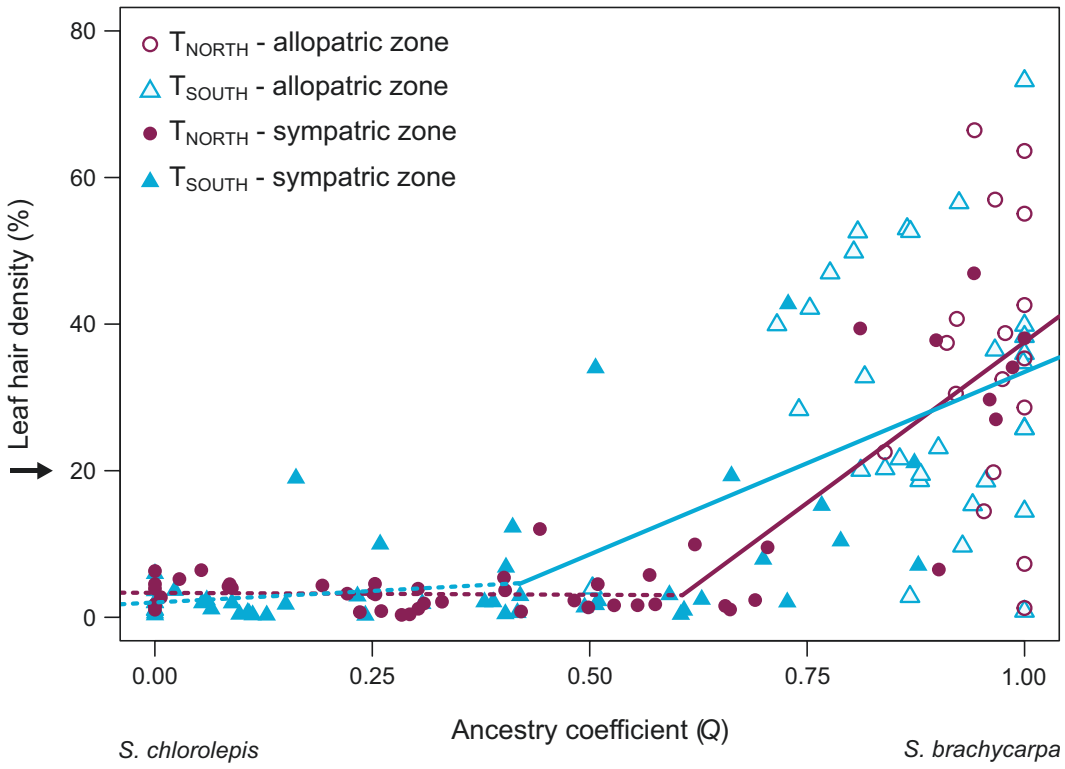

Figure 6. Segmented regressions of variation in leaf hair density as a function of ancestry coefficient (Q) obtained separately for transects T_{NORTH} and T_{SOUTH} using sNMF ($K = 2$). The arrow indicates the maximum leaf hair density reached by individuals with $Q \leq 0.5$ (20%). Linear segments that reflect no significant variation before the breakpoints are shown by dashed lines. $Q = 0$: *Salix chlorolepis* and $Q = 1$: *S. brachycarpa*.

biological processes, none of which previously known to be directly connected to leaf hair pathways (Supporting Information, Table S6).

DISCUSSION

Genetic structure and diversity of *S. chlorolepis*

The same genetic group is shared to various extents by all *S. chlorolepis* colonies, suggesting a common genetic ancestry (green lineage; Fig. 2A). This could reflect a formerly less fragmented distribution of *S. chlorolepis*, with wider coverage of the margin and perhaps even on the plateau of the serpentinized Mount Albert massif (Finger *et al.* 2023). A gradual retreat of *S. chlorolepis* towards a few late-melting snowbeds could have occurred in response to Holocene climate change, as reported in the Alps, where several taxa were forced to move to track their ecological niches (Tinner and Kaltenrieder 2005, Wagner *et al.* 2021). Yet, genetic connectivity can also be maintained by gene flow through pollen and/or seeds dispersal among spatially isolated colonies (Alsos *et al.* 2007, Jabis *et al.* 2011, Finger *et al.* 2014, 2023, González-Robles *et al.* 2021, Hernández-Leal *et al.* 2022).

Although a common genetic ancestry persists within *S. chlorolepis*, a significant fine-scale spatial genetic structuring across the colonies reflects its currently fragmented geographical distribution, despite the small size of the entire species range (7.9 km²; COSEWIC 2020). This genetic differentiation, combined with rather low levels of genetic diversity within colonies, might reflect the impact of genetic drift on these very small colonies isolated from one another. The snowbed habitat of *S. chlorolepis* could also exacerbate isolation across colonies by causing a colony-specific phenological lag in flowering that depends on the rate of snowmelt (Cooper *et al.* 2011, Kudo 2020). Delayed melting time entails a physical barrier that impedes pollination by flowering individuals from neighbouring colonies that have already been cleared of snow. For instance, the prevailing north-westerly winds in winter (Lemieux 2007) combined with the relatively weak slope at the C_{NORTH} colony result in snow accumulation that persists later into spring and summer compared to neighbouring colonies (C_{SOUTH}, C_5, C_6). As illustrated in the C_{NORTH} colony, which exhibits increased genetic difference, the persistence of a fine-scale genetic structure across *S. chlorolepis* colonies could be partly attributed to flowering asynchrony caused by variable snowmelt dates, a process previously reported in other alpine-snowbed plants (Yamagishi *et al.* 2005, Hirao and Kudo 2008, Shimono *et al.* 2009).

Rare and/or endemic plants often suffer from a loss of genetic diversity in fragmented habitats (Ellstrand and Elam 1993, Hedrick and Kalinowski 2000). Indeed, *S. chlorolepis* shows a rather low level of genetic diversity as well as significant inbreeding throughout its entire range, despite an overall genetic dissimilarity (negative relatedness) among individuals. A comparable diversity pattern is also found for *S. brachycarpa*, which is locally more abundant and globally more widespread. These results testify to the geographically isolated and ecologically restricted environment in which the two species evolve, which

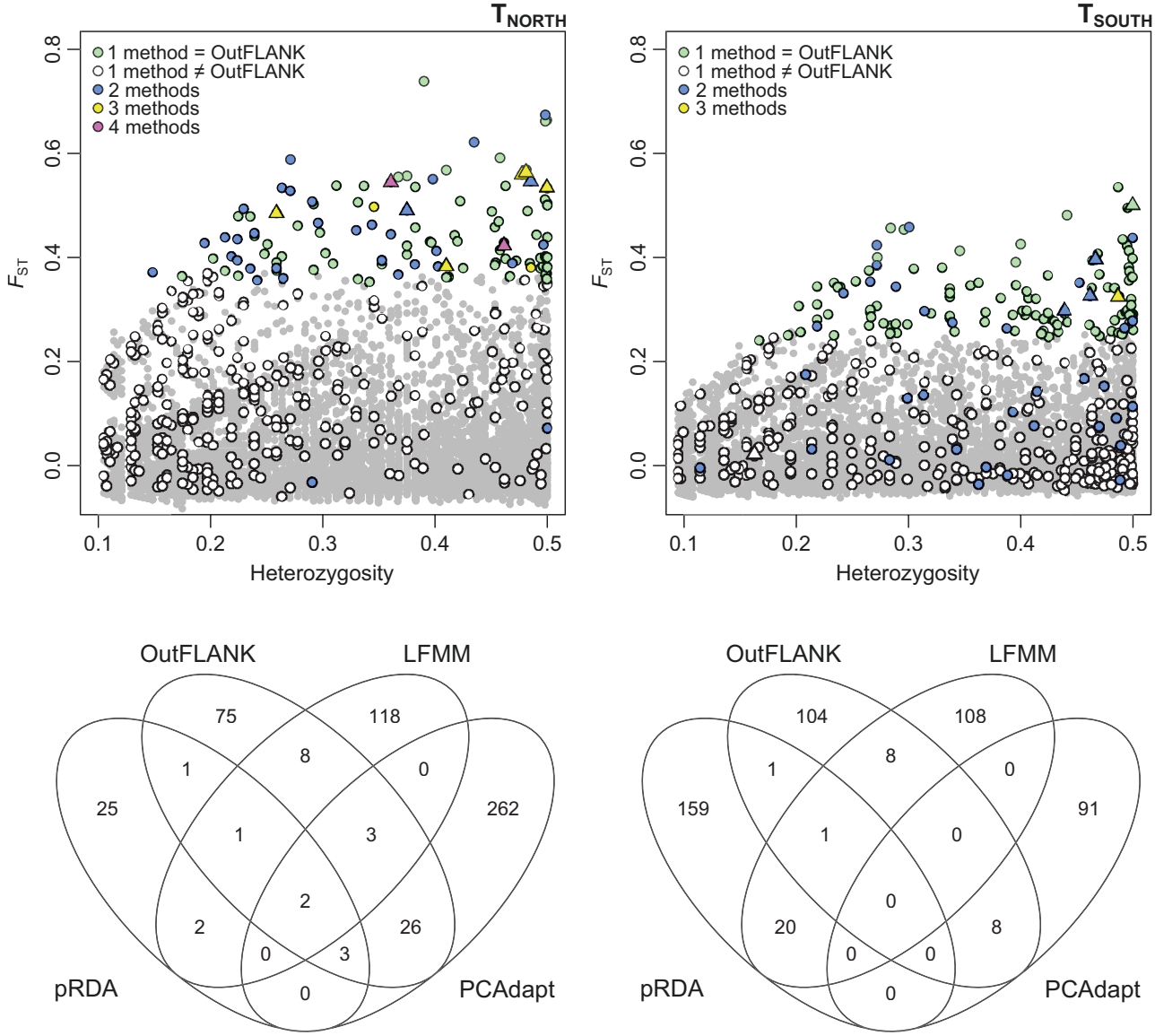

Figure 7. Candidate loci identified using four population genomics methods (OutFLANK, LFMM, pRDA, PCAdapt) for transects T_{NORTH} and T_{SOUTH}. Top panels: SNPs flagged by at least one of the four methods. Triangles indicate the nine highly validated candidate SNP loci that rarely express a phenotype beyond 20% leaf hair density for the homozygous genotype of the minor allele. Bottom panels: Venn diagrams of the number of candidate loci identified by the four methods.

is conducive to genetic drift throughout the system studied. In addition to the low population size (total population of *S. chlorolepis* estimated to <300 individuals; COSEWIC 2020), dioecious reproductive strategy and biased sex ratio in willows (Ueno *et al.* 2007) might contribute to explain low level of genetic diversity. Because low genetic diversity greatly increases the risk of extinction (Spielman *et al.* 2004), the loss of a single *S. chlorolepis* colony could substantially reduce the total genetic diversity within the species, making it even more vulnerable. This threat is exacerbated by the existence of a genetic structure within the species that implies genetic differences across colonies, emphasizing their uniqueness. In fact, pairwise F_{ST} values indicate higher genetic differences between *S. chlorolepis* colonies than between colonies and *S. brachycarpa*, highlighting the genetic peculiarity of each colony. Thus, conservation efforts should aim at maintaining the genetic integrity of individual colonies. To this end, a regular monitoring of the demographic trajectory of each colony would inform whether genetic restoration measures may eventually become necessary (Frankham 2015, Finger *et al.* 2023).

Introgressive hybridization and gene flow

Molecular analyses corroborate the century-old hypothesis of introgressive hybridization between *S. chlorolepis* and *S. brachycarpa* (Schneider 1918). Morphological criteria used to identify parental species are generally reliable despite a few misidentifications, but telling apart *S. chlorolepis* from hybrids remains a challenge in the field (Fig. 4). The high variability in ancestry coefficients, clearly circumscribed within *S. chlorolepis* colonies, indicate interspecific gene flow within the sympatric zones between *S. chlorolepis* and *S. brachycarpa* (Fig. 5). Despite ongoing hybridization with locally abundant *S. brachycarpa*,

Figure 8. Leaf pilosity according to major allele homozygous (0), heterozygous (1), and minor allele homozygous (2) genotype for nine highly validated candidate SNP loci, flagged at least three times on one or both transects and rarely expressing a phenotype beyond 20% leaf hair density for the homozygous genotype of the minor allele. Red triangles identify *Salix chlorolepis* individuals ($Q \leq 0.1$), green circles indicate introgressed hybrids ($0.1 < Q < 0.9$) and yellow diamonds represent *S. brachycarpa* ($Q \geq 0.9$). Different letters indicate significantly different levels of leaf hair density between genotypes.

pure *S. chlorolepis* individuals were found within the colonies and backcrossing towards *S. chlorolepis* is not uncommon (Fig. 2C), which suggest that rare and threatened *S. chlorolepis* maintain its genetic integrity and is recalcitrant to genetic swamping by a more common congener. Yet, the introgression process remains restricted to sympatric zones, with no evidence of first-generation hybridization outside *S. chlorolepis* colonies. Nevertheless, we do not exclude that some gene flow from *S. chlorolepis* may eventually reach *S. brachycarpa* individuals on the summit plateau, since willows are capable of long-distance dispersal (Alsos *et al.* 2007), and are both wind- and insect-pollinated (Sacchi and Price 1988, Peeters and Totland 1999, Tamura and Kudo 2000). Indeed, pairwise F_{ST} values, AMOVA, and sNMF all indicate smaller genetic difference between the two species than among *S. chlorolepis* colonies, which might reflect increased interspecific gene flow compared with gene flow among colonies (Table 2; Supporting Information, Table S2, Fig. S4). For instance, *S. brachycarpa* individuals on the summit plateau exhibit some genetic admixture (Fig. 2B, C), suggesting interspecific gene flow, especially from the C_{SOUTH} colony. This is also indicated by ancestry coefficients showing slight introgression by *S. chlorolepis* ($0.75 < Q < 1$) in some *S. brachycarpa* individuals in the allopatric zone of T_{SOUTH} (Fig. 5).

Leaf pilosity and candidate loci

Using multilocus approaches (partial Mantel tests, GDMs), we tested whether environmental factors, independent of hybridization, could influence the expression of leaf pilosity in *S. brachycarpa*. Partial Mantel tests indicate significant associations between phenotype, environment, and genetics only when the analysed datasets include both *S. chlorolepis* and *S. brachycarpa* (T_{SOUTH} and T_{NORTH}). However, no association is detected when only *S. brachycarpa* is included (sampling grid). Associations between phenotype, environment, and genetics therefore appear

Table 5. The nine highly validated candidate SNP loci flagged at least three times on one or both transects and rarely expressing a phenotype beyond 20% leaf hair density for the homozygous genotype of the minor allele. For each SNP, diagnostic approaches are indicated by 'T_{NORTH}' and/or 'T_{SOUTH}' according to the transect where the SNP was retained by the genome scan.

SNP loci	LFMM	pRDA	PCAdapt	OutFLANK
62676131-35-C/T	T_{SOUTH} T_{NORTH}	T_{NORTH}	T_{NORTH}	T_{SOUTH} T_{NORTH}
14574545-20-T/A	T_{NORTH}	T_{NORTH}	T_{NORTH}	T_{SOUTH} T_{NORTH}
3318520-66-A/G		T_{SOUTH} T_{NORTH}	T_{NORTH}	T_{NORTH}
53604372-62-A/G	T_{SOUTH} T_{NORTH}		T_{NORTH}	T_{SOUTH} T_{NORTH}
3331702-47-T/A		T_{NORTH}	T_{NORTH}	T_{NORTH}
14575867-19-C/T		T_{SOUTH}	T_{NORTH}	T_{NORTH}
3315228-25-A/G	T_{NORTH}		T_{NORTH}	T_{NORTH}
14574952-30-A/G		T_{NORTH}	T_{NORTH}	T_{NORTH}
3311384-38-C/T	T_{SOUTH} T_{NORTH}			T_{SOUTH} T_{NORTH}

to be effectively related to introgression, since no association is observed when considering only one species, even though this species exhibits the full phenotypic spectrum and experiences a wider environmental range. In GDMs, leaf pilosity phenotype stands out from the other environmental predictors in both T_{SOUTH} and T_{NORTH} transects, whereas pilosity has no statistical support in *S. brachycarpa* across the grid. On the one hand, these results indicate that the environment has no significant effect on pilosity in *S. brachycarpa* outside the zone of sympatry with *S. chlorolepis*. On the other hand, the results suggest leaf pilosity is a key factor associated with allelic variation, independently of the environment, when both species are considered. These results thus justify excluding environmental factors from further analyses to assess in greater detail the interplay between leaf pilosity and the genomics of both species, at the single-locus level.

Multiple factors can influence the performance of the various methods employed in population genomics, including the choice of experimental design, biogeographic and demographic contexts, and sample size (Lotterhos and Whitlock 2015). The limitations inherent to each of these different methods are well documented (Strasburg *et al.* 2012, de Villemereuil *et al.* 2014, Bragg *et al.* 2015, Rellstab *et al.* 2015, Vaux *et al.* 2023). To identify candidate loci associated with variation in leaf pilosity and potentially experiencing divergent selection between the two taxa, we opted for a robust consensus approach whereby each of the four methods employed relies on independent statistical assumptions as well as its own model to control for neutral genetic structure (De Mita *et al.* 2013, Rellstab *et al.* 2015), and was replicated along the two transects. The results reveal that, between *S. chlorolepis* and *S. brachycarpa*, there is a genomic imprint coherent with selection for both the pilose foliage phenotype (LFMM, pRDA) and speciation (PCAdapt, OutFLANK) for 15 candidate loci identified at least three times on one or both transects. For nine of these highly validated candidate loci, the homozygous genotype of the minor allele almost invariably corresponds to a glabrous foliage (< 20% leaf hair density), which is the main morphological criterion for identifying the species *S. chlorolepis* (Figs. 6, 8). This *S. chlorolepis*-specific phenotype is also diagnostic on a multilocus scale. Indeed, ancestry coefficient values show that leaf hair density increases above the 20% threshold only when individuals are predominantly related to *S. brachycarpa* ($Q > 0.5$). In fact, strongly pilose phenotypes are mainly found outside the sympatric zone, in the absence of *S. chlorolepis* (Figs 2, 6). Hence, the genetic imprints are highly consistent with the established morphological criteria and are thus diagnostic of both species. Still, the existence of a few almost glabrous *S. brachycarpa* individuals in the allopatric zone ($Q > 0.9$; leaf hair density < 20%) illustrates the wide spectrum of phenotypic expression for this trait in *S. brachycarpa* (but not in *S. chlorolepis*).

The same nine highly validated candidate loci seem to be jointly involved both in the expression (or lack thereof) of leaf hair density, the main diagnostic trait of these taxa, and in maintaining the integrity of the parental species in the face of introgression (Table 5, Figs 7, 8). Despite ongoing introgressive hybridization influencing the genomic background, we posit that alleles at these nine candidate loci, putatively under divergent selection, may ensure a maintenance of the interspecific barrier as well as a clear phenotypic distinction between the two species (Shaw and Mullen 2011, Seehausen *et al.* 2014). Given that interspecific boundaries are generally semi-permeable across the genome (Wu 2001, Strasburg *et al.* 2012), such genomic heterogeneity could therefore indicate the existence of differential introgression at the genomic level (Harrison and Larson 2014, 2016). This mechanism, well documented in hybrid zones, implies that some regions of the genome introgress more readily than others that are virtually 'impermeable' to introgression (Hamilton *et al.* 2013, Larson *et al.* 2013, de Lafontaine *et al.* 2015, Rifkin *et al.* 2019, Mostert-O'Neill *et al.* 2021). Such genomic heterogeneity in the face of introgression could mitigate the risks of genetic assimilation of the rare species by the more common species (Ellstrand and Elam 1993, Levin *et al.* 1996, Burgess *et al.* 2005).

Yet, introgression necessarily involves the interspecific exchange of alleles elsewhere in the genome. *S. brachycarpa* is a generalist species that colonizes a wide spectrum of ecological conditions, from open subalpine forests to alpine habitats and from wet to mesic environments. In contrast, *S. chlorolepis* is restricted to a few late-melting snowbeds on the serpentine soils of Mount Albert, which suggests that this species has a narrower environmental tolerance spectrum. It is possible that the persistence of *S. brachycarpa* in the stressful conditions induced by serpentine soils is enabled by the transfer of adaptations inherited from introgressive hybridization events

with locally adapted *S. chlorolepis*. Retention of favourable alleles through interspecific genetic admixture (adaptive introgression) has been recorded in other plant species (Rieseberg *et al.* 2003, Arnold *et al.* 2012) and has recently been suggested for the European willows *S. waldsteiniana* and *S. foetida* (Marinček *et al.* 2023). To our knowledge, on serpentine soils, *S. brachycarpa* grows only in the presence of *S. chlorolepis*, i.e. only at Mount Albert, suggesting its otherwise low affinity for this edaphic environment.

CONCLUSION

Our study provided a first assessment of the genetic status of one of the most threatened plant species in Canada. We flagged key genetic markers that suggest the existence of some genomic regions probably resistant to interspecific gene flow, which ensures that *S. chlorolepis* maintains genetic integrity despite ongoing introgression with *S. brachycarpa*. These same candidate loci testify that such genomic regions 'impermeable' to introgression might also be involved directly or indirectly in the segregation of the main diagnostic phenotype, leaf pilosity, between the two species. These highly validated loci mapped to seven known genic regions that may represent interesting candidate genes for investigation in further functional genomics studies. The consistency of our results, which combine geographical, phenotypic, environmental, and genomic data, finally corroborates field observations made over a century ago (Fernald 1905, Schneider 1918).

SUPPLEMENTARY DATA

Supplementary data is available at *Botanical Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

The authors thank R. J. Petit and F. Dufresne for their help on a previous draft; F. Coursol for providing key geographic information about the colonies; as well as M. Allard, C. Boulerice, K. Chagnon-Taillon, L. Allard, M. Couture, M. Deslauriers, S. Harimanana, J. Mas, I. Pecl, and C. Pelletier for their assistance in the field or the laboratory.

CONFLICT OF INTEREST

None declared.

FUNDING

This research was funded and supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant Program (RGPIN-2018-06586) and Canada Research Chairs Program (CRC-2017-00112, CRC-2022-00518) awarded to G.d.L. A.A. received a Master's training scholarship from the Fonds de recherche du Québec – Nature et technologies (FRQNT).

DATA AVAILABILITY

The data used in this manuscript are available on figshare data repository (Atikessé *et al.* 2025; https://doi.org/10.6084/m9.figshare.28789502).

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