

Genomic differentiation of endangered polyploid *Braya* (Brassicaceae) populations in the limestone barrens ecosystem at risk support separate management units

Maria Esther Nieto-Blazquez

menietoblazquez@gmail.com

Senckenberg Biodiversity and Climate Research Centre

Nathan MacNeil

Memorial University of Newfoundland

Lourdes Peña-Castillo

Memorial University of Newfoundland

Luise Hermanutz

Memorial University of Newfoundland

Julissa Roncal

Memorial University of Newfoundland

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Abstract

Endangered species recovery benefits from robust genetic insights. *Braya longii* and *B. fernaldii* are morphologically distinct but closely related octoploid self-fertilizing species, restricted to the island of Newfoundland's globally rare limestone barrens. Despite distinct morphology, they are currently managed under a unified recovery plan due to ecological similarity. We assessed genetic diversity, inbreeding, population structure, management units (MUs), evolutionary significant units (ESUs), and demographic history to inform long-term conservation. Using genotyping-by-sequencing, we obtained 92,671 SNPs from 85 individuals across eight populations. Results revealed variable genetic diversity, low to moderate population differentiation within and between species, and support that *B. longii* is distinct and evolved from *B. fernaldii*. Phylogenetic and structure analyses showed strong population structuring and relationship patterns that do not conform with geographic distance. Despite bioinformatic filtering of homologous variants with GBS-SNP-CROP, parameter estimation in polyploids remained uncertain, with elevated heterozygosity and biased effective population sizes, highlighting the need for improved analytical tools for polyploid genomics. Our analyses support one ESU encompassing both species and seven MUs, critical for guiding augmentation, reintroduction, and translocation strategies. This study demonstrates that narrow endemic polyploids can show complex genetic patterns shaped by demographic history and anthropogenic disturbance. These results provide essential genomic guidance for the recovery of *Braya* species and restoration of the limestone barrens ecosystem.

Introduction

Ecosystem restoration and species recovery benefit from genetic knowledge of populations and species (Thomas et al. 2014; Maschinski and Albrecht 2017; Gann et al. 2019; Rossetto et al. 2023). Genetic tools have aided the estimation of population-level parameters and processes such as effective population size, bottlenecks, gene flow, hybridization, and genetic isolation (Willi et al. 2022). There is compelling evidence that inbreeding depression and loss of genetic diversity can decrease fitness and increase extinction risk (Frankham 2005; Markert et al. 2010; Agrawal and Whitlock 2012; Ralls et al. 2020; but see Teixeira and Huber 2021). Altogether, these genetic parameters must be taken into account when implementing species recovery strategies including genetic rescue, maladaptation prediction, and assisted gene flow (Aitken et al. 2024). Moreover, restoration strategies normally seek to achieve genetically diverse populations since they are known to be more fit (Reed and Frankham 2003; Dostálek et al. 2010; Pizza et al. 2021) and resilient to environmental change (Schueler et al. 2013; Thomas et al. 2014). However, in cases where there is evidence of strong local adaptation, localized sources of plant propagules for restoration may be warranted (Thornton et al. 2008; Gann et al. 2019).

One of the applications of genetic knowledge in rare species recovery is the careful selection of plant propagule sources for population (re)introductions or augmentations within or outside historic range (McKay et al. 2005; Breed et al. 2013; Tardy and Godefroid 2024). Sources of plant propagule can be determined by the delineation of conservation units. Genetically defined conservation units can effectively safeguard hotspots of genetic diversity, private and adaptive alleles of a species and should be the focus of the management actions described above (Moritz

1999; Palsbøll et al. 2007; Gauthier et al. 2010). Evolutionary Significant Units (ESU) and Management Units (MU) are two of the most frequently used conservation units and have a variety of definitions (Funk et al. 2012 and references therein). In this work, we use the ESU definition of separately managed population groups because they are ecologically and genetically distinct (Moritz 1994; Crandall et al. 2000; Allendorf and Luikart 2006), while MUs are demographically independent populations whose dynamics do not depend on immigration (Moritz 1994). ESUs may contain one or many MUs (Yorke et al. 2011; Funk et al. 2012). ESUs are valuable as they can capture different evolutionary and potentially adaptive trajectories (Moritz 1999; Willi et al. 2022).

Polyploidism is defined as a heritable increase in genome copy number, and is an important speciation mechanism in plants with an estimated frequency of occurrence of up to one third in angiosperms (Wood et al. 2009; Heslop-Harrison et al. 2023). Polyploidization renders greater adaptability for lineages, leading to their successful colonization of novel niches and extreme environments (López-Jurado et al. 2019; Edgeloe et al. 2022). It also may lead to increased niche breadth and geographic range of the newly formed polyploid species (Grant 1981; McIntyre 2012; Wefferling et al. 2024), and as such are less likely to be species of conservation concern (Pandit et al. 2011). However, cases of endangered polyploids with specialized niches and narrow distributions exist (e.g. Lopez and Barreiro 2013) and are key to our understanding of polyploid genetics and niche evolution.

In Canada, 37% of the total number of species listed under the Canadian Species at Risk Act are plants and lichens (McCune and Morrison 2020), yet very few studies have used genetic information to manage these species, of which only two have delineated conservation units based on genetic differentiation (Yorke et al. 2011; Lesica et al. 2016). Most of these genetic studies have addressed the question of whether range edge populations are genetically distinct and diverse to warrant separate management and conservation, or identified what genetic factor (e.g. inbreeding, bottleneck) poses a threat to species survival (e.g. Gauthier et al. 2010; Fine et al. 2013; Nowell et al. 2022; Lait et al. 2025). This paper aims to add empirical knowledge on the understudied field of conservation genomics of rare polyploids using a Canadian case study.

Braya longii Fernald and *Braya fernaldii* Abbe (Brassicaceae) are two herbaceous, calciphile, octoploid plant species with allopolyploid origins (Warwick et al. 2004). Based on Internal Transcribed Spacer (ITS) sequencing, it is hypothesized that the two species could have originated from an arctic/subarctic North American or Eurasian *B. glabella* ancestor with hybrid origin. *B. longii* and *B. fernaldii* are very similar morphologically, have similar life histories, primarily self-fertilize and only disperse seeds a short distance (within 50 cm of the adult plant, Tilley 2003; Parsons and Hermanutz 2006). They are listed as endangered under the Canadian Species at Risk Act, and the Newfoundland and Labrador Provincial Endangered Species Act, due to inhabiting a limited range on the limestone barrens of the Canadian Island of Newfoundland (Fig. 1), experiencing habitat loss and degradation, invasive pests, and pathogens (Hermanutz et al. 2002; Squires et al. 2009; Environment and Climate Change Canada 2020; de la Bastide et al. 2022). *B. longii* differs from *B. fernaldii* in having larger petals, smaller sepals, and pubescent siliques (Meades 1997a, b; Parsons 2002); however these are highly variable. Due to their similar

life histories and habitat affinities, these plant species are managed under the same recovery action plan (Environment and Climate Change Canada 2020). Critical habitat (i.e. the habitat necessary for the survival or recovery of a listed wildlife species) of both *Braya* species consists of a substrate of exposed calcareous bedrock outcrops, thin layers of frost-shattered calcareous gravel and shallow calcareous soils; vegetation height less than 10 cm; and vegetation cover rarely exceeding 50% (Environment and Climate Change Canada 2020).

While limestone is globally distributed, the total area of treeless landscapes with exposed limestone in temperate to boreal regions is very limited, making the limestone barrens a globally rare habitat (Limestone Barrens Species at Risk Recovery Team 2021). The limestone barrens on the Great Northern Peninsula of Newfoundland are a hotspot of plant diversity housing almost 50% of the island's rare plants (Hermanutz et al. 2002) and make up less than 1% of the total island's area (Limestone Barrens Species at Risk Recovery Team 2021). This harsh habitat represents a niche for Arctic-alpine plants that require and/or tolerate high calcium and magnesium levels, and are adapted to nutrient deficient substrates, low temperatures, strong winds, and a relatively short growing season (Limestone Barrens Species at Risk Recovery Team 2021). The limestone barren degradation in Newfoundland is due to road and building construction, housing development, quarrying, and the use of motorized vehicles (Limestone Barrens Species at Risk Recovery Team 2021). A recovery plan has been established to mitigate further vegetation and substrate disturbance, to restore damaged limestone barrens, and to ensure the recovery of species at risk such as *Braya*. Within this recovery plan, at least three actions would benefit from a population genetic study of *Braya*: i) improve species identification and delineation of individual genotypes, ii) usage of population augmentation, re-introductions and translocation with genetically adequate propagule source, and iii) maintenance of an *ex-situ* live plant and/or seed bank collection at Memorial University's Botanical Garden (Limestone Barrens Species at Risk Recovery Team 2021).

The objectives of this study were (1) to estimate levels of population genetic diversity, inbreeding, and structure for both *Braya* species, (2) to recommend MUs and ESUs for both *Braya* species, (3) to elucidate their demographic history, and (4) to recommend strategies for long-term *in-situ* and *ex-situ* conservation that will not only secure the persistence of these two endemic rare species but also the restoration of the limestone barrens ecosystem. Due to *Braya*'s life history traits of selfing, low-range seed dispersal (Parsons and Hermanutz 2006), and small population sizes, we hypothesize that *B. longii* and *B. fernaldii* should exhibit low intrapopulation genetic diversity and high interpopulation genetic differentiation (Hamrick and Godt 1996; Reutemann et al. 2024). Another hypothesis is that every population will be genetically distinct, and therefore each population should constitute a separate MU, which needs to be preserved.

Materials and Methods

Study site, sample collection and DNA extraction

In 2017, under appropriate permits, we collected leaf samples from 39 *B. longii* and 46 *B. fernaldii* individuals across eight populations (Table 1), which span across both species' distributions in the

limestone barrens of the Great Northern Peninsula in Newfoundland, Canada, for a total of 85 *Braya* samples (Fig. 1). Leaves were selected from healthy-looking (i.e., no obvious signs of pest or pathogen damage, e.g. de la Bastide et al. 2022) adult individuals from which no more than 20% of the plant's biomass was extracted. Leaf samples were dried and stored in silica gel. The low number of sampled *Braya* populations and individuals reflects the low total number of populations left in the wild

Table 1

Genetic diversity statistics for each *Braya* population in the Limestone Barrens of Newfoundland generated from Hierfstat's basic.stats function. HO = observed heterozygosity, HS = observed gene diversity, DST = gene diversity among populations, and FIS = inbreeding coefficient averaged over loci.

Population per species (acronym)	#samples	HO	HS	DST	FIS
<i>Braya longii</i>					
Sandy Cove 1 (SC1)	10	0.5234	0.2912	0.0397	-0.7974
Sandy Cove 2 (SC2)	5	0.5368	0.2714	0.0595	-0.9778
Yankee Point (YP)	14	0.5424	0.2729	0.0580	-0.9872
Shoal Cove Disturbed (ShCD)	10	0.5415	0.2731	0.0578	-0.9828
<i>Braya fernaldii</i>					
Green Island Brook (GIB)	15	0.5649	0.3031	0.0278	-0.8639
Anchor Point (AP)	8	0.5554	0.2969	0.0340	-0.8707
Wild Bight (WB)	6	0.5399	0.2724	0.0585	-0.9818
Cape Norman (CN)	6	0.5821	0.3466	-0.0157	-0.6796
Bellburns (BB)	6	0.5817	0.3278	0.0031	-0.7746
Total	80	0.5515	0.2944	0.0366	-0.8736

We ground at least 20 mg of dried leaf tissue using a TissueLyser LT machine (Qiagen). We isolated DNA using the DNeasy Plant Mini Kit from Qiagen with the following modifications to the original manufacturer's protocol. We added 600 µL of Buffer AP1 to the ground plant material. The incubation time for cell lysis was 15 mins at 65 °C. We added 195 µL of

Buffer P3 to the lysate. We used 50 µL of Qiagen Buffer AE for the final DNA elution. We diluted total DNA extractions to 20 ng/µl in Qiagen Buffer EB.

Genotyping-by-sequencing (GBS) and SNP discovery

The Institut de Biologie Intégrative et de Systèmes of the University of Laval in Canada conducted a two-enzyme GBS (Poland et al. 2012) on the DNA extracted. We followed Abed et al. (2019) for all steps of DNA digestion with SbfI and MspI restriction enzymes, design and ligation of unique barcodes 10–12 base pairs (bp) long and Illumina TruSeq HT adaptor, PCR amplification and

genomic library preparation. Genome Quebec performed the DNA sequencing on the Illumina HiSeq 4000 PE150. We inspected data quality with FastQC v0.11.9 (Andrews 2010) and discarded reads with an average Phred quality score lower than 20. We processed data using the GBS-SNP-Calling Reference Optional Pipeline (GBS-SNP-CROP) v4.1 (Melo and Hale 2019). We chose this pipeline because it can handle polyploid individuals by providing ploidy-dependent filtering parameters and a Z-score metric used to filter homologous variants (McKinney et al. 2017). It allows filtering variants from various non-diploid scenarios by identifying the proportion of observed allelic counts for each variant and comparing them to the expected allele counts. The Z-score is the deviation from this expected value. GBS-SNP-CROP uses Trimmomatic v0.39 (Bolger et al. 2014) to remove low-quality reads and adapter sequences, and PEAR v0.9.11 (Zhang et al. 2014) to merge the paired-end reads into single reads.

The sample with the highest number of reads (*B.fernaldii*-BB-617) was selected to create a reference genome using VSEARCH v2.15.1 (Rognes et al. 2016). This strategy was used because it had been shown to produce the highest number of SNPs when compared to other strategies (Melo et al. 2016). Reads were aligned using BWA aligner v0.7.12 (Li and Durbin 2009), and sorted and indexed using SAMTools v1.7 (Li et al. 2009). We used the suggested initial parameter values as in (Melo et al. 2016), however, since this approach rendered an insufficient number of SNPs for downstream analysis, we used a different set of parameters in GBS-SNP-CROP (Table S1, all others were default values). To account for the polyploid nature of the two *Braya* species, the genotype matrix was subsequently filtered for paralogs using the Z-score provided by GBS-SNP-CROP using the threshold of McKinney et al. (2017) of $|Z_i| < 7$.

Genetic diversity and structure of *Braya* populations in the limestone barrens

We estimated genetic diversity statistics at the population level using the R package Hierfstat v0.5-11 (Goudet 2005). The basic.stats function in Hierfstat provided the observed heterozygosity (HO), observed gene diversity (HS), gene diversity among populations (DST), and inbreeding coefficient (FIS). The pairwise.neifst function provided pairwise population *FST* statistics using Nei's minimum genetic distance (Nei 1987). We also conducted a Mantel test to detect isolation by distance (IBD). We used the R package ade4 v1.7-19 (Chessel et al. 2004) to measure the correlation between the matrix of pairwise *FST* values among the eight *Braya* populations sampled and their geographic distances. We calculated pairwise geographic distances in kilometers with the Geographic Distance Matrix Generator (Ersts 2015). The Mantel test used the Pearson coefficient, 999 replicates, and the default alpha value of 0.05.

To investigate the genetic structure among *Braya* individuals we used three approaches. First, we applied a popular Bayesian, model-based clustering method, STRUCTURE v2.3.4. (Pritchard et al. 2000). We ran STRUCTURE on the two *Braya* species separately but also combined since both species are morphologically similar, are sister in a phylogenetic

study of the genus *Braya* (Warwick et al. 2004), and are managed under the same action plan. We determined the number of genetic clusters (*K*) with default parameter settings, correlated allele frequencies, and without a priori population information. The linkage model proposed by Falush et al. (2003) was not designed to handle linkage disequilibrium between markers that are very

tightly linked (Porrás-Hurtado et al. 2013), which is the case of SNPs, thus we used the admixture model in every analysis, which assumes that each individual draws some genetic information from each of the K genetic clusters. All analyses consisted of 20 iterations for each value of K , and of 400,000 Markov Chain Monte Carlo (MCMC) generations after a 400,000-generation burn-in for each iteration. We allowed K to vary from one (no population structure) to 10 genetic clusters. We then used STRUCTURE HARVESTER v0.6.94 (Earl and vonHoldt 2012) to obtain a suggested number of genetic clusters for each iteration using the ΔK test (Evanno et al. 2005). We aligned genetic clusters across iterations with CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007). The results from CLUMPP were then processed using DISTRUCT v1.1 (Rosenberg 2004) to produce bar plots representing the membership coefficient of each individual to genetic clusters.

Second, we performed a Discriminant Analysis of Principal Components (DAPC) using the R package adegenet v2.1.5 (Jombart 2008). We tested the hypothesis that every collection site comprises a separate genetic group (K of nine for the two species together). To optimize the number of principal components to retain, we conducted a cross-validation test of 1,000 replicates with the xvalDapc function of adegenet, which calculates the number of principal components attaining the lowest mean squared error. Lastly, we conducted an analysis of molecular variance (AMOVA) implemented in GenoDive v3.06 (Meirmans 2020) to test the distribution of genetic variation using the ploidy independent infinite allele model, and testing its significance with 999 permutations. Missing dosage information for polyploids was replaced with randomly drawn alleles based on estimated allele frequencies assuming Hardy-Weinberg equilibrium. Three stratifications were used - among species, among populations within species, and within populations.

Phylogenetic and demographic history analysis

To infer the phylogenetic relationships among populations, we performed a maximum-likelihood (ML) analysis using a concatenated SNP matrix, including all individuals from each population. The ML analysis was performed using RAxML v8.2.12 (Stamatakis 2014), applying the GTR + Γ nucleotide substitution model and a rapid bootstrap (BS) algorithm with 100 replicates. An outgroup species was not available for DNA sequencing, thus we present an unrooted tree.

We conducted a demographic modeling analysis to investigate population bifurcation events and determine the ancestral relationships between the two *Braya* species. Based on the observed genetic structure and phylogenetic results, we defined four populations for the historical demographic analysis: (1) Green Island Brook-Wild Bight-Bellburns (GIB/WB/BB), (2) Cape Norman-Anchor Point (CN/AP), (3) Yankee Point (YP), and (4) Sandy Cove 1-Sandy Cove 2-Shoal Cove Disturbed (SC/ShCD). For this analysis, we performed coalescent simulations using the multidimensional site-frequency spectrum (SFS) as a summary statistic. The observed SFS for the SNP dataset was generated using easySFS (Gutenkunst et al. 2009; <https://github.com/isaacovercast/easySFS>) with the -a flag, which ensures that all SNPs present in the VCF file are included.

We designed six competing demographic models to explore the evolutionary history of the two species. Model 1 and Model 2 depict alternate population bifurcations between species with *B.fernaldii* and *B.longii*, respectively. Model 3 and 4 depict *B.fernaldii* nested within the ancestral *B.longii*, and *B.longii* nested within the ancestral *B.fernaldii*, respectively. Model 5 depicts *B.longii* nested within the ancestral *B.fernaldii* (since preliminary analyses favoured this bifurcation pattern) with current gene flow within species, and Model 6 depicts *B.longii* nested within the ancestral *B.fernaldii* with current gene flow within and between species (Fig. S1). We estimated demographic parameters under a ML approach using the observed SFS. For each model, we performed 1,000 simulations with fastsimcoal2 v2.6.0.3 (Excoffier et al. 2013) to generate the expected SFS under a given set of parameters and to compute the likelihood of each model. A mutation rate of 1×10^{-8} was applied and we assumed a generation time of five years for *Braya* species (Limestone Barrens Species at Risk Recovery Team 2021) and set the first split in each model between the most recent common ancestor (MRCA) and Newfoundland's *Braya* species to be 1,000 generations (5,000 years ago/5 years generation time). We used 5,000 years ago as the estimated time of colonization of the MRCA, given that the Laurentide Ice Sheet of the last glaciation fully retreated from Newfoundland between 13,000 and 9,000 years BP (Bryson et al. 1969; Shaw et al. 2006), suggesting that the current flora of Newfoundland was established after this time. To identify the best-fit model, we compared the estimated maximum likelihoods (MaxEstLhood) using the Bayesian information criterion (BIC) and assuming 2 parameters (k), sample size and time of demographic events.

Results

Genotyping-by-sequencing, filtering, and SNP discovery

The paired-end GBS yielded 40.3 GB of raw data containing over 366.7 million reads between 85 and 97 bp in length. Demultiplexing gave us an average of 2.4 million reads per sample, ranging from < 1 to 15 million reads per sample. We removed five *B. fernaldii* individuals from Cape Norman with less than 1 million reads from further analyses as low read count impacts coverage and can lead to excessive missing data and unreliable genotype calls. The final dataset included 80 *Braya* individuals. Read quality inspection in FastQC reported a Phred score average of 39, and GC content of 46 to 47%. Without filtering, GBS-SNP-CROP yielded 92,671 SNPs. After filtering for high confidence variants, we retrieved 2,387 SNPs when homologous variants were not filtered using the Z-score method, and 1,449 SNPs when removing homologous variants. All downstream analyses used this data matrix of 1,449 SNPs with 17.8% missing data and is available in Zenodo (doi: 10.5281/zenodo.17249348).

Genetic diversity of *Braya* populations

Population genetic statistics of HO and HS showed that *B. fernaldii* had higher genetic diversity than *B. longii* (Table 1). Within *B. fernaldii*, the population of Cape Norman displayed the highest HO (0.5821) and HS (0.3466; Table 1). One of the most northerly *B. fernaldii* populations, Wild Bight, had the lowest HO (0.5399) and HS (0.2724). Cape Norman and Wild Bight are the northernmost populations and are geographically close, yet they showed the highest (CN) and

lowest (WB) genetic diversity values, respectively. For *B. longii*, Yankee Point had the highest HO (0.5424), and Sandy Cove 1 had the highest HS (0.2912). For all *Braya* populations, *B. longii* at Sandy Cove 2 had the highest DST (0.0595), and *B. fernaldii* at Cape Norman had the lowest (-0.0157). All *FIS* were negative (Table 1), suggesting an excess in heterozygotes in all *Braya* populations in relation to what was expected under random mating.

FST values showed low to moderate differentiation among collecting sites suggesting high to moderate gene flow among them, although the effects of genome duplication in these polyploid species may also contribute to this pattern. Genetic differentiation between *B. longii* sites was lower than the differentiation between *B. fernaldii* sites. Within *B. longii*, the highest pairwise *FST* values were for Yankee Point and Shoal Cove Disturbed (0.0483) and the lowest between Sandy Cove and Shoal Cove Disturbed (0.0146; Table 2). Within *B. fernaldii*, the highest pairwise *FST* values were for Anchor Point and Bellburns (0.0945), and the lowest between Anchor Point and Cape Norman (0.0454; Table 2). On average, pairwise *FST* values between interspecific populations were higher (0.08) than between intraspecific populations (0.071 for *B. fernaldii* and 0.034 for *B. longii*). The Mantel test did not show a significant correlation between population genetic differentiation and geographic distances ($R = -0.015$, $p\text{-value} = 0.533$), suggesting lack of isolation by geographic distance.

Table 2

Above the diagonal are pairwise *FST* values amongst eight *Braya* populations in the limestone barrens. Below the diagonal are distances in kilometers amongst the *Braya* populations obtained from Geographic Distance Matrix Generator (Ersts 2011). Population acronyms as in Table 1. Bold *FST* values correspond to the highest and lowest estimates within species as discussed in the text.

		<i>B. longii</i>			<i>B. fernaldii</i>				
		SC	YP	ShCD	GIB	AP	WB	CN	BB
<i>B. longii</i>	SC	-	0.0403	0.0146	0.0600	0.0668	0.0584	0.0708	0.0638
	YP	6.67	-	0.0483	0.0877	0.0784	0.0858	0.0994	0.0981
	ShCD	1.93	4.74	-	0.0799	0.0832	0.0803	0.0992	0.0872
<i>B. fernaldii</i>	GIB	8.31	14.84	10.21	-	0.0754	0.0531	0.0728	0.0628
	AP	17.42	11.29	15.64	24.75	-	0.0818	0.0454	0.0945
	WB	59.05	65.56	60.96	50.75	74.47	-	0.0873	0.0677
	CN	57.49	64.04	59.40	49.20	73.14	2.48	-	0.0699
	BB	122.89	116.63	121.12	129.79	105.48	175.61	174.85	-

Genetic structure of *Braya* populations

STRUCTURE analysis of the two species combined at a *K* of 8, which was the best-fit model, yielded the following genetic groups within *B. longii*: 1) Shoal Cove Disturbed and Sandy Cove 1, 2) Yankee Point, and 3) Sandy Cove 2; and the following genetic groups within *B. fernaldii*: 4) Wild Bight and Bellburns (the most geographically distant collection sites), 5) Anchor Point and half of Cape Norman individuals, and 6) Green Island Brook (Fig. 2a). A few admixed individuals

from Bellburns, Cape Norman, Green Island Brook, and Sandy Cove representing both species formed their own genetic group. Individuals from Shoal Cove Disturbed and Anchor Point showed the least admixture, while individuals from all other collection sites showed different admixture levels indicating evidence of past or current gene flow between populations and species (Fig. 2a). When STRUCTURE was run separately for each species, the genetic groups observed were identical to the combined analysis, with individuals from each species forming four genetic clusters, respectively, and a best K of 4 according to the Evanno test (Fig. 2b-c). A higher admixture was revealed in the combined analysis for Yankee Point, Sandy Cove 2, and Wild Bight.

For the DAPC analysis of the two species combined, the suggested number of principal components to retain was 13 since they achieved the lowest mean squared error. The first, second and third discriminant functions accounted for 74% of the total variation. Individuals clustered into seven genetic groups (Fig. 3a-b), which were in concordance with the STRUCTURE results. As in STRUCTURE, *B. fernaldii* individuals from Cape Norman and Anchor Point clustered together. *Braya longii* individuals from Shoal Cove Disturbed and Sandy Cove 1 also clustered (Fig. 3a-b).

The third discriminant function separated Green Island Brook from *B. longii* individuals. Individuals from all other collection sites formed their own genetic cluster. *Braya fernaldii* from Wild Bight was distinguishable from Bellburns, which was not observed in the STRUCTURE barplots (Fig. 2). Analysis of molecular variance revealed that the variation among collecting sites within species accounted for 46.8%, while the variation within collecting sites and between species represented 35.3% and 17.9%, of the total variation, respectively (Table 3).

Table 3

Analysis of molecular variance for population differentiation in two *Braya* species using 1,449 SNPs. SSD = sum of square deviations, d.f.= degrees of freedom, MS = mean square deviations

Source of variation	SSD	d.f	MS	% variance	F-stat	F-value	P-value
Within collecting sites	2649.920	71	37.323	0.353	Rho_st	0.647	--
Among collecting sites within species	3179.625	7	454.232	0.468	Rho_sc	0.570	0.001
Between species	1325.472	1	1325.472	0.179	Rho_ct	0.179	0.009

Phylogenetic analysis

RAxML analysis for the 80 *Braya* individuals showed an evolutionary split between the two species supporting their current morphology-based taxonomic recognition (Fig. 4a). Individuals from every *B. fernaldii* collecting site formed a distinct clade with bootstrap (BS) values of 70% to 99%, except for Cape Norman which was paraphyletic with a clade of Anchor Point individuals nested within [CN,AP; BS of 77%]. This result was concordant with the STRUCTURE and DAPC analyses. The two most geographically distant collecting sites of Bellburns and Wild Bight formed two sister clades with high BS support of 98% and 99%, respectively. The separation of these two sites was achieved in the DAPC analysis, but not in the STRUCTURE barplots. This clade [BB,WB] was sister to a clade of Green Island Brook individuals with 88% BS support. This latter clade

(GIB[WB,BB]) appeared sister to the clade of [CN,AP] with 91% BS support. For *B. longii*, we observed three highly supported clades - individuals from Yankee Point formed a clade with 89% BS support, Shoal Cove Disturbed and Sandy Cove 1 with BS of 95%, and Sandy Cove 2 with a BS of 100%. The phylogenetic results for *B. longii* were in agreement with those from STRUCTURE and the DAPC.

Demographic history of *Braya* in Newfoundland

The results from the fastsimcoal2 analyses indicated similar MaxEstLhood values across all demographic models (Table S2). However, statistical comparisons using the BIC identified model five as the best-fitting scenario (MaxEstLhood = -34,326.79, BIC = 68668.15; Fig. 4b and Table S2). This model supports a hypothesis where *B. fernaldii* is the ancestral species of the *Braya* populations in Newfoundland and proposes a divergence pattern beginning with *B. fernaldii* CN/AP population, followed by *B. fernaldii* GIB/WB/BB, *B. longii* SC/ShCD, and *B. longii* YP and current gene flow within species (Fig. 4c and Table S2). Demographic parameters estimated and their BIC values under the six demographic models are presented in Table S2. Assuming a generation time of five years (Limestone Barrens Species at Risk Recovery Team 2021), the divergence between the most recent common ancestor (MRCA) and *B. fernaldii* (CN/AP) occurred approximately 937 generations ago (~ 4,685 years). This was followed by the split between *B. fernaldii* (CN/AP) and *B. fernaldii* (GIB/WB/BB) around 692 generations ago (~ 3,460 years). Subsequently, *B. longii* (SC/ShCD) diverged from *B. fernaldii* (GIB/WB/BB) approximately 417 generations ago (~ 2,150 years). The most recent divergence occurred between *B. longii* populations SC/ShCD and YP, 117 generations ago (~ 558 years). Estimated effective population sizes (N_e) ranged from 45,863 (*B. fernaldii* GIB/WB/BB) to 69,811 (*B. longii* YP).

Discussion

Our study provides evidence for low to moderate genetic differentiation and varying levels of genetic diversity across populations of the endangered and polyploid *B. longii* and *B. fernaldii* species. Leveraging genomic data from GBS, we propose one ESU and seven MUs that reflect the genetic architecture of these rare Newfoundland endemics under a unified recovery plan as already implemented due to their similar life histories and habitat affinities. These insights are crucial for guiding conservation efforts, informing decisions on genetically appropriate source populations for restoration initiatives, and facilitating the strategic curation of *ex-situ* collections. Ultimately, this research offers a foundational genetic understanding vital for the long-term survival and effective conservation management of these endangered plant species.

Genetic diversity and structure of *Braya* populations

The contrasting genetic diversity levels observed among *Braya* populations mirror patterns found in other North American boreal generalists. For instance, trembling aspen (*Populus tremuloides* Michx.) exhibits subtle genetic structure across northwestern North America, with limited neutral differentiation and unexpectedly high allelic richness in some regions despite postglacial recolonization (Latutrie et al. 2016). Similarly, black spruce (*Picea mariana* Britton, Sterns & Poggenb.) populations show lineage-specific adaptive variation, with genetic diversity shaped by

historical factors and climate-driven selection (Napier et al. 2020). These examples suggest that demographic history, gene flow, selfing and dispersal limitation can create fine-scale diversity landscapes, and could explain why Cape Norman and Wild Bight (*B. fernaldii*) have contrasting genetic diversities despite their close geographical proximity (Volis et al. 2016).

Many calcicolous (limestone-loving) endemics often display consistently low within-population diversity and strong among-population differentiation due to their narrow habitat specialization. For instance, studies on the alpine species *Primula farinosa* L. have revealed significant genetic differentiation among isolated populations with limited gene flow, a pattern often attributed to historical fragmentation (Madec 2014; Gajewski et al. 2018). Similarly, glacial relict populations of the rock plant *Draba aizoides* L. are genetically less variable and strongly differentiated from their core populations, a consequence of long-term isolation (Widmer and Baltisberger 1999; Vogler and Reisch 2013). In contrast to these examples, *Braya* showed high levels of within population diversity (expected heterozygosity) and low to moderate among population differentiation. This can be due to the octoploid nature of the genome or historical gene flow.

All collection sites formed their own genetic group in all genetic structure and phylogenetic analyses except for three cases. 1) Individuals at Sandy Cove 1 and Shoal Cove Disturbed belonged to the same genetic group. Anthropogenic movement of substrate may have contributed to seed migration across the landscape—a hypothesis supported by anecdotal accounts from quarrying activity at Sandy Cove and Shoal Cove. 2) Individuals from Sandy Cove 2 formed their own genetic group. Sandy Cove 1 and 2 are separated by a road. A lack of soil disturbance or a localized founder event at Sandy Cove 2 might explain this unique genetic cluster different from Sandy Cove 1. 3) Individuals from Anchor Point and Cape Norman formed a single genetic group. This result cannot be explained by the gravel movement hypothesis since there are no accounts of disturbance at these sites; instead, historical demography or gene flow via long-distance seed dispersal by strong winds could explain this pattern.

Conjectures on gravel exchange between Yankee Point and other *B. longii* sites were not supported by our results since this population formed a distinct group in all our analyses. Yankee Point may represent a relict of the natural population that was mostly extirpated during land clearing in the early 1970s. Furthermore, the presence of admixture in a few individuals from Sandy Cove 1, Green Island Brook, Cape Norman and Bellburns suggests historic or ongoing gene flow between species, or an ancestral genetic pool retained from the hybrid ancestor. A lack of IBD, confirmed by the non-significant Mantel test, further supports the hypothesis that gene flow in *Braya* is not strictly dictated by geography but possibly influenced by habitat fragmentation, human-mediated disturbances, historical demography and complex postglacial dynamics, especially in the central area of the Northern Peninsula.

The AMOVA results corroborated the population structure analyses, with the majority of genetic variation (47%) occurring between populations and only 35% within populations. This result contrasts those from other North American plant species like *Helianthemum canadense* (L.) Michx. where most variation is found within populations (Yorke et al. 2011). The percentage of total genetic variation found among *Braya* populations likely results from their primarily selfing reproductive system and limited seed dispersal capabilities (Hamrick and Godt 1996; Parsons and

Hermanutz 2006), which act to reduce gene flow between populations. A majority genetic variation found among *Braya* populations is a quintessential pattern of selfing species, where reduced genetic exchange within a population leads to homozygosity and the accumulation of genetic differences among isolated populations. This is consistent with observations in other predominantly selfing plants, such as *Lobelia inflata* L., where strong genetic structure occurs between distinct homozygous lineages co-occurring in the same population (Busch et al. 2022). In contrast, widespread, outcrossing species with effective gene flow, such as the boreal conifer white spruce (*Picea glauca* (Moench) Voss), show very little differentiation between populations and retain most of their genetic diversity within populations (Rajora et al. 2023).

Phylogenetic relationships and demographic history of *Braya* populations

Phylogenetic analysis supported the morphological species boundaries between *B. fernaldii* and *B. longii*, revealing well-supported evolutionary clades that aligned with the STRUCTURE and DAPC results. *Braya* individuals formed clades matching the collection sites with high BS support and supporting the hypothesis that every population is genetically distinct except for Sandy Cove, Shoal Cove Disturbed, Cape Norman and Anchor Point. The phylogenetic relationships of all *Braya* coastal limestone populations do not show a bifurcation topology determined by geographic proximity (e.g. a north to south bifurcation order). Instead, we found geographically distant populations (like Bellburns and Wild Bight) as sisters, which can be explained by shared ancestry or historical connectivity facilitated by seed dispersal.

Demographic modeling further clarified the evolutionary history of these populations and provided a temporal context for the observed genetic divergences. Gene flow within, but not between, *Braya* species inferred with the best-fit demographic model supports the taxonomic recognition of the two *Braya* species. The best-supported model also indicated that *B. fernaldii* is the ancestral lineage, with subsequent divergence events unfolding in a stepwise pattern over the last ~ 4,700 years, and the last population diverging 558 years ago (Table S2). Postglacial colonization dynamics, which included multiple sea level inundations and retreats, especially in the lower coastal areas and the tip of the Northern Peninsula, followed by reproductive isolation and localized gene flow within populations, most likely shaped the current *Braya* population structure. Additionally, N_e is of special relevance in conservation genetics because it reflects the rate at which genetic diversity is lost due to drift and inbreeding, thereby influencing a population's evolutionary potential and extinction risk (Fedorca et al. 2024). The high N_e values we observed in *Braya* indicate substantial historical genetic diversity, which may result from large ancestral population sizes, polyploidy, or demographic processes prior to recent habitat fragmentation and anthropogenic pressures.

Polyploidism implications in data analysis

Although polyploidy plays a central role in plant evolution and genomic data are now more widely available, our understanding of polyploid population genetics remains limited (Dufresne et al. 2014). This is largely due to the inherent complexity of analyzing polyploid data. These complications include segregation patterns (disomic and polysomic) that might vary at the loci-

level, double reduction, which increases homozygosity at certain loci, and mixed ploidy levels (Meirmans et al. 2018). These complications cause the allele dosage (i.e., copy number of each allele) difficult to estimate, as it might differ at individual loci (Dufresne et al. 2014; Meirmans et al. 2018). Most analytical methods use allele dosage assumptions corresponding to diploid data (Dufresne et al. 2014), with few exceptions focusing on autopolyploid genetic data (Meirmans 2023; Stoeckel et al. 2024). However, we are not aware of analytical methods specific for allopolyploid genetic data. Allopolyploid organisms, such as the two *Braya* species studied here, tend to have disomic inheritance that leads to fixed heterozygosity. In this case, expected heterozygosity, as calculated by most analytical methods, would be overestimated, and thus the fixation index *FST* would be underestimated (Dufresne et al. 2014). Furthermore, strict disomy results in negative values of *FIS* (Meirmans and Van Tienderen 2013) as the ones we observed in Table 1. Under these considerations, the negative *FIS* values we obtained might be interpreted as an indication of strict disomic inheritance in both *Braya* species instead of evidence of lack of inbreeding, which would contradict *Braya*'s life history traits of selfing. This pattern of high heterozygosity alongside negative inbreeding coefficients in *Braya* are reminiscent of those seen in other allopolyploid taxa such as *Senna glutinosa* subsp. *glutinosa*, where reproductive strategies like apomixis or selfing, coupled with complex genome structure, result in unexpectedly high genetic diversity and low apparent inbreeding (Delnevo et al. 2024).

Given the lack of tools specific for allopolyploid genetic data, we attempted to choose methods that would be more robust to the effects of polyploid data. For example, paralog filtering in GBS-SNP-CROP (Melo and Hale 2019) mitigates the over-counting of SNPs resulting from duplication. Similarly, STRUCTURE was found to be the most robust method to the presence of multiple ploidy levels for the inference of population genetic structure (Stift et al. 2019). However, when estimating demographic history with coalescent-based simulators like fastsimcoal2, it is important to acknowledge that these tools are typically designed for diploid genetic data. We therefore acknowledge that applying fastsimcoal2 to polyploid populations without accounting for complexities such as inheritance mode or allele dosage can lead to biased demographic inferences (Blischak et al. 2023). For example, accurately estimating *Ne* in polyploid species is challenging because multiple chromosome sets complicate assumptions about allele inheritance, mutation rates, and population structure, often violating the standard diploid models used in most demographic inference methods (Waples 2025). Moreover, factors such as population size, selfing rate, and whether the species is allo- or autopolyploid can further bias *Ne* estimation (Jighly et al. 2018).

Recommended strategies for long-term in-situ and ex-situ conservation

For *B. longii* and *B. fernaldii*, our genomic analyses provide critical insights for delineating two types of conservation units. Our results support the taxonomic distinction of the two *Braya* species, however, since they have similar critical habitats and ecology, and taking into account the definition of ESU (i.e. separately managed population groups because they are ecologically and genetically distinct), we propose both species should be treated as the same ESU. This supports the current approach of the Limestone Barrens Recovery Plan (Limestone Barrens Species at Risk Recovery Team 2021).

Within each species, our genomic findings also inform the designation of seven genetically distinct MUs. For *B. longii*, these include Shoal Cove Disturbed/Sandy Cove 1, Yankee Point, and Sandy Cove 2; and for *B. fernaldii*, Anchor Point/Cape Norman, Wild Bight, Bellburns, and Green Island Brook. Therefore, future translocations, augmentations or re-introductions should use seeds sourced from the corresponding MU. *Ex-situ* conservation of seeds representing the seven MUs described above is a useful propagule source and a vital strategy to mitigate population genetic and demographic loss, and to prevent species extinction (Werden et al. 2020). Our results support the ongoing *ex-situ* conservation program established in 2015 at Memorial University's Botanical Garden, which following the recommendations from the Center for Plant Conservation (2019), harvests and stores seeds by keeping maternal lines, populations and species separate. This *ex-situ* seed collection represents all *Braya* populations, and seeds are collected every 2 or 3 years to replenish the seed bank as needed.

To date one large scale *B. longii* augmentation has been conducted at Sandy Cove restoration site starting in 2017 using seeds collected from this same site (Sandy Cove 1), and while successful establishment and seed production has been achieved, herbivory and fungal pathogens (de la Bastide et al. 2022) causing mortality are continuing threats to long-term persistence (Hermanutz, pers comm). There have been three other smaller augmentations and translocations of *B. longii* using ~ 200 seeds each that have not persisted for a variety of reasons such as human disturbance (Pelley 2011). There has been a single translocation of *B. fernaldii* undertaken that has persisted since 2006 and is self-sustaining. Seeds from the geographically closest population were used in all these past translocations. To add resilience to *Braya* species, the Limestone Barrens Species at Risk Recovery Team (2021) has high priority activities to investigate how and where to carry out habitat restorations inclusive of augmentations, reintroductions, and translocations to historically unoccupied limestone barrens to ensure long-term persistence on the landscape in the face of environment and climate change. Genetic diversity of the source population is not the only factor considered when choosing a propagule source, careful consideration of the reproductive health and ecological similarity of source populations to the restoration site is paramount (Florentine et al. 2023; Gibson-Roy 2023). By using these genetically informed MUs, conservation practitioners can enhance the success of augmentation, reintroduction and translocation efforts, minimize risks of outbreeding depression, and facilitate the long-term adaptation and persistence of *Braya* species in Newfoundland.

Conclusions and future directions

Future research should investigate how these different MUs will respond to changing climate, which include warmer winters, greater growing degrees days and changing precipitation patterns, to assess their potential for persistence and adaptive capacity under these future climate change scenarios. This will inform future augmentations and translocations. Efforts should be expanded to include populations of *Braya fernaldii* not sampled in this study (such as Port aux Choix, Watts Point) to try to understand how to best increase connectivity from the sparser southern populations to the more contiguous northern end of the distribution. Additionally, the development of analytical methods and tools tailored specifically for polyploid species would help address persistent uncertainties in parameter estimation like an elevated heterozygosity, low

inbreeding, and biased effective population size despite the use of an analytical bioinformatic pipeline that filters homologous variants from SNP datasets. Narrow endemic polyploid species can show complex genetic diversity and relationship patterns that do not conform with geographic distance and that could be explained by demographic history and anthropogenic disturbance. Ultimately, translating these genomic insights into conservation action will require close collaboration between geneticists, ecologists, land managers, and policymakers to ensure the persistence of *Braya* species in the face of ongoing habitat loss and climate change.

Statements and Declarations

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Data availability: The dataset generated and analyzed during the current study are available in the Zenodo repository (doi: 10.5281/zenodo.17249348).

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Figures

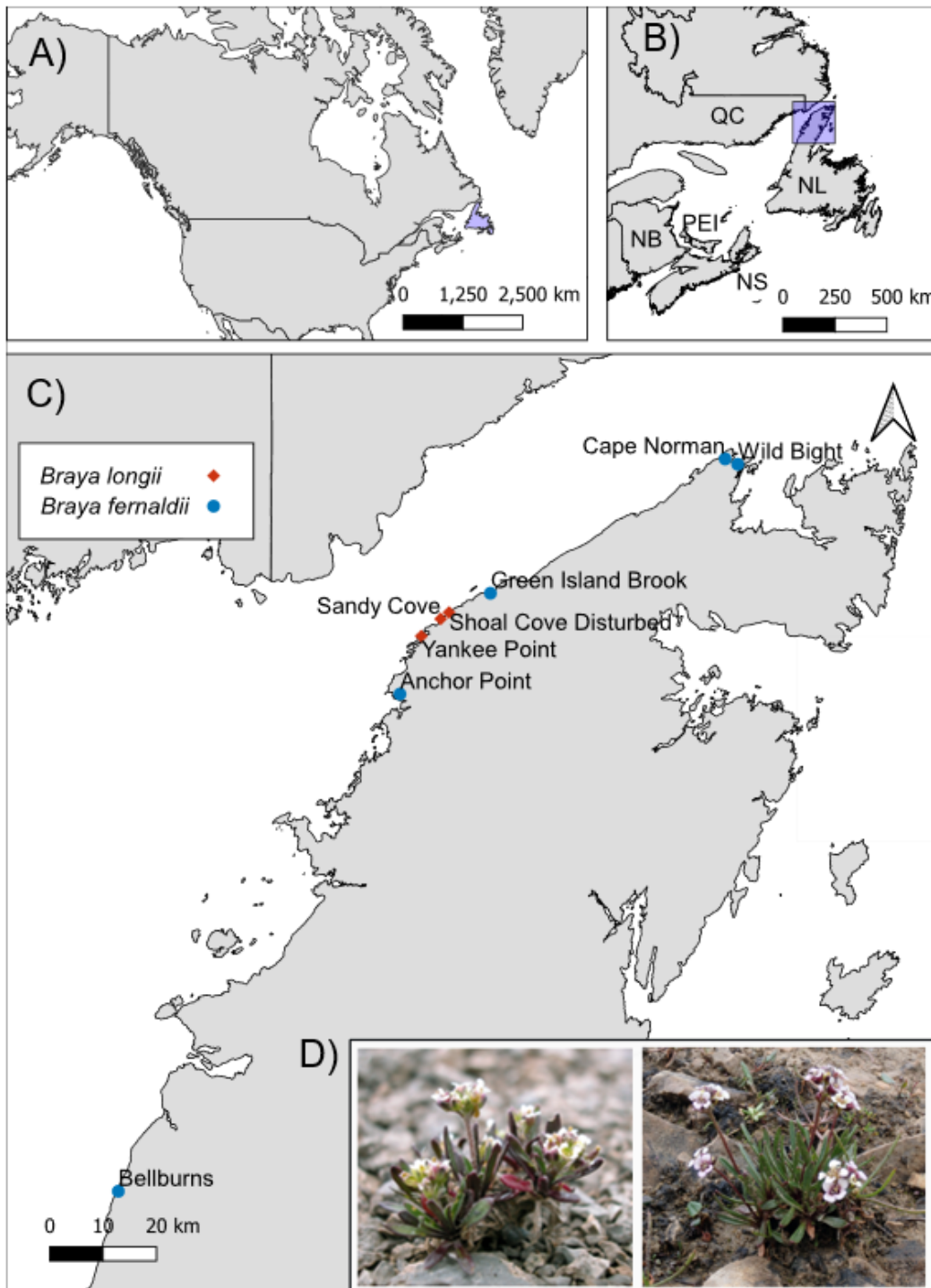


Figure 1

A) Map of North America highlighting the position of the island of Newfoundland. B) Map of Canadian Atlantic provinces (NL, QC, PEI, NB, NS) highlighting the study site location. C) Map of *Braya fernaldii* and *B. longii* sampled localities in the limestone barrens ecosystem at risk of Newfoundland, Canada. D) *B. fernaldii* (left) and *B. longii* (right). Photos by Susan Meades.

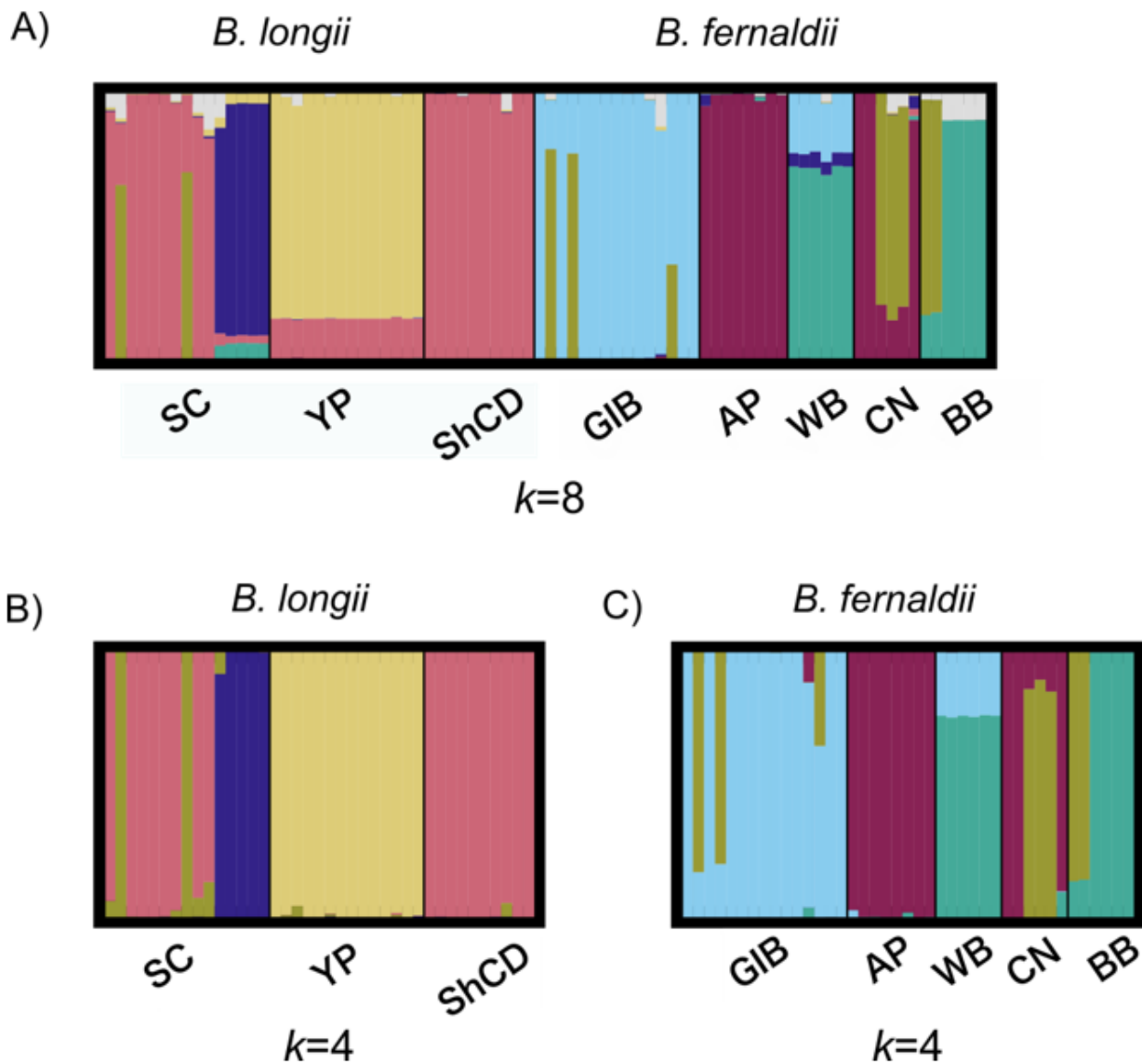


Figure 2

Bar plots showing the genetic structure of A) both *Braya* species combined for K of 8, B) *B. longii* populations only for K of 4, and C) *B. fernaldii* populations only for K of 4. Acronyms of sampled localities are indicated at the bottom of the bar plots.

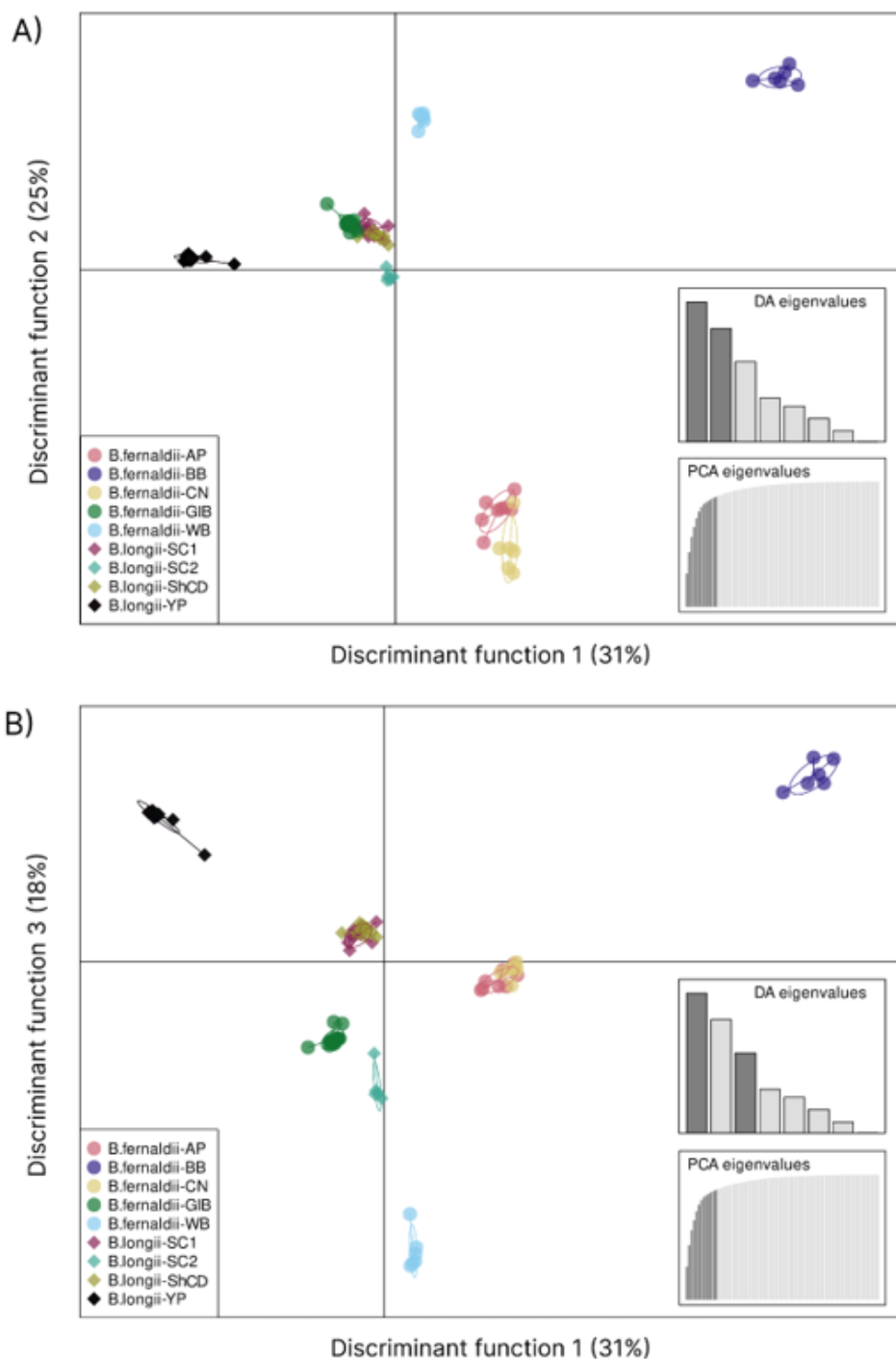


Figure 3

Visualization of the discriminant analysis of principal components(DAPC) using genotyping-by-sequencing data. Inset figures show DA and PCA eigenvalues. Sampling localities appear in different colours. Diamond markers correspond to *B. longii* individuals, and circles correspond to *B. fernaldii* individuals.

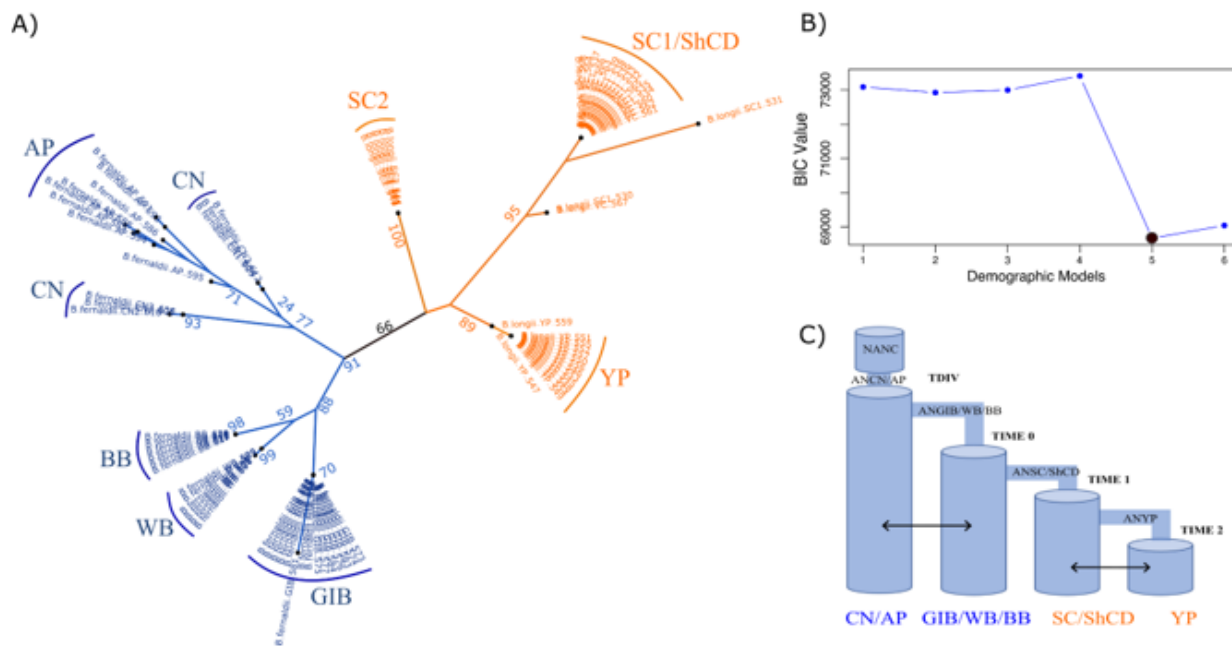


Figure 4

A) Maximum likelihood tree reconstructed in RAxML of the concatenated SNPs for 80 *B. longii* (red) and *B. fernaldii* (blue) individuals sampled in the limestone barrens of Newfoundland, Canada. SNP dataset (1,449 SNPs) filtered to account for polyploidization. Groups represent individuals from the species and populations as listed in Table 1 with their respective acronyms. Numbers along branches are bootstrap support values, B) Line plot indicating BIC values for the six models analyzed in fastsimcoal2, and C) depiction of the best-fitting model (model 5) recovered in fastsimcoal2. NANC refers to the MRCA effective population size of *Braya* species in Newfoundland and AN refers to the ancestral effective population size of each population. TIME refers to the different divergence times (specified as historical events) in the analysis.

Supplementary Files

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