



ARTICLE

Genetic Diversity of Native Red Mulberry at Its Northwestern Boundary Suggests Postglacial Founder Effects

Spencer J. Schreier¹ and Madhav P. Nepal^{2,*} 

¹Department of Agronomy, Horticulture and Plant Science, South Dakota State University, Brookings, SD, USA

²Department of Biology and Microbiology, South Dakota State University, Brookings, SD, USA

*Corresponding Author: Madhav P. Nepal. Email: madhav.nepal@sdstate.edu

Received: 11 July 2026; Accepted: 17 September 2026; Published: 19 October 2026

ABSTRACT: *Morus rubra* is native to the eastern United States, with its range extending into the Upper Midwest and southern Ontario, Canada. Its present distribution suggests that past glacial events in North America may have influenced the genetic structure of populations at the species' northwestern range boundary. This study assessed genetic variation among six *Morus rubra* (*M. rubra*) populations that likely experienced postglacial colonization using published nuclear microsatellite markers and sequence variation from the chloroplast *trnL-trnF* region. Five nuclear microsatellite markers previously developed for *Morus alba* were successfully transferred to *M. rubra*, and the chloroplast *trnL-trnF* region provided an additional maternally inherited marker for evaluating chlorotype diversity. Nuclear microsatellite diversity was higher in southern unglaciated populations than in northern glaciated populations, a pattern broadly mirrored by chloroplast haplotype variation. Although based on a limited number of markers, these results are consistent with expectations of postglacial founder effects and leading-edge colonization following glacial retreat. Alternative demographic processes, including recent population fragmentation and restricted gene flow, cannot be excluded. Nonetheless, the findings suggest that postglacial colonization contributed to shaping contemporary genetic structure in *M. rubra* at its northwestern range boundary. Because introgressive hybridization between *M. rubra* and the naturalized invasive *M. alba* has been documented previously, reduced genetic diversity in marginal populations may increase vulnerability to genetic swamping, highlighting the importance of continued monitoring and conservation of these populations.

KEYWORDS: Red mulberry phylogeography; postglacial colonization; founder effects; leading edge expansion; microsatellite transferability; chlorotype diversity; population genetics; conservation biology

1 Introduction

Morus rubra L. (Moraceae), commonly known as Red Mulberry, is one of the 13 recognized species in the genus *Morus* and is native to the eastern United States, with its distribution extending into the Upper Midwest and southern Ontario, Canada [1–3]. As an ecologically important native tree, *M. rubra* supports wildlife, has a long history of nutritional and traditional medicinal use by Native Americans [4], and has become an increasing conservation concern because of habitat fragmentation and hybridization with the introduced *M. alba* [5].

Evidence from numerous plant species suggests that glacial cycles during the Pleistocene profoundly influenced population structure throughout the Northern Hemisphere [6–11]. The present distribution of *M. rubra* has therefore raised the question of whether the Wisconsin glaciation and subsequent recession

of the Laurentide Ice Sheet shaped the genetic structure of populations at the species' northwestern range boundary. During the Pleistocene, cyclic glaciation and the subsequent recession of the Laurentide Ice Sheet periodically eliminated and opened habitats across North America, providing new niches for surviving plant populations to recolonize [12–16]. Such recession may have provided new niches for surviving populations of *M. rubra* to colonize. This colonization may have shaped the genetic structure of the current *M. rubra* populations in the Upper Midwest. Evidence suggests that when biotic or abiotic factors influence the movement of individuals into open niches far away from their source population, allelic frequencies may fluctuate across the expanded population range [12,13]. During leading-edge range expansion, newly established populations may carry only a subset of the genetic variation present in source populations, potentially producing serial founder effects and reduced diversity toward the expanding range margin [7,17–19]. Recent genomic studies of leading-edge plant populations further demonstrate that range expansion can be accompanied by reduced effective population size and increased isolation, while contemporary ecological processes may also contribute to these patterns [20]. However, reduced genetic diversity at range margins is not uniquely indicative of historical colonization. Similar patterns can arise through alternative evolutionary and demographic processes, including genetic drift, population isolation, habitat fragmentation, historical bottlenecks, and recent population decline [21]. Thus, contemporary genetic patterns must be interpreted cautiously when evaluating hypotheses of postglacial range expansion.

The evolutionary history and population structure of a species can be investigated using nuclear and chloroplast DNA markers [21]. Because chloroplast DNA is maternally inherited, highly conserved, and evolves slowly [22,23], it is particularly useful for reconstructing historical migration routes and postglacial recolonization [10,24–26]. In contrast, biparentally inherited nuclear markers provide greater resolution of contemporary population structure and gene flow [27]. Together, chloroplast and nuclear markers provide complementary perspectives on historical colonization and present-day genetic diversity. A recent synthesis of population genetic studies in long-lived forest trees has demonstrated that reductions in genetic diversity with increasing distance from glacial refugia can persist in contemporary populations [15]. Despite the ecological and conservation importance of *M. rubra*, the genetic structure and demographic history of populations at its extreme northwestern range boundary remain largely unknown. While comprehensive genomic resources for *M. rubra* are still in development, combining transferable nuclear simple sequence repeat (SSR) markers with chloroplast DNA sequences offers a cost-effective, exploratory toolkit to establish baseline population genetics. Because chloroplast DNA is maternally inherited and evolves slowly, it is useful for detecting historical maternal lineages [23]. In contrast, biparentally inherited nuclear SSRs provide resolution for more recent population structure and gene flow [27]. Together, this combined marker set provides an initial framework to assess whether contemporary genetic patterns at the northwestern boundary align with historical postglacial expansion, or if they reflect more recent demographic isolation.

Applying these complementary molecular markers to *M. rubra* provides an opportunity to evaluate whether contemporary patterns of genetic diversity retain signatures consistent with postglacial range expansion. Because the present study includes a limited number of populations and loci, the resulting data are best suited to evaluating the founder-effect scenario as a working demographic hypothesis and establishing baseline information, rather than reconstructing demographic history conclusively. *Morus rubra* is currently considered endangered in Canada [5,28,29] and in Connecticut and Massachusetts, and threatened in Michigan and Vermont in the United States [30]. In addition, *M. rubra* hybridizes readily with the invasive *M. alba*, introduced in North America from Asia during the colonial era [31], increasing the risk of genetic swamping in small or isolated populations [5]. We hypothesized that, if repeated leading-edge colonization accompanied recession of the Laurentide Ice Sheet, populations near the northwestern range

boundary of *M. rubra* would retain lower nuclear and chloroplast genetic diversity than populations located farther south. To evaluate whether the observed genetic patterns are consistent with this hypothesis, we assessed the interspecific transferability of 12 nuclear microsatellite markers, retaining five for population genetic analysis, and characterized the nuclear and chloroplast genetic diversity of six *M. rubra* populations in the Upper Midwest.

2 Materials and Methods

2.1 DNA Extraction and PCR Screening

Fresh leaf samples were collected from 8–25 *Morus rubra* individuals in each of six populations from Kansas, Iowa, Wisconsin, and Nebraska (Table 1). To minimize the inclusion of hybrids, individuals were identified as *M. rubra* (Fig. 1) prior to sampling using a suite of diagnostic morphological characters and ITS sequences evaluated in our previous studies [1,32]. Because *M. rubra* has suffered severe habitat loss and is characterized by a sparse, patchy distribution at its extreme northwestern range boundary, locating large, contiguous populations is highly challenging. While we recognize that these six populations and uneven sample sizes represent a limited spatial sampling, they reflect the accessible remnant stands of *M. rubra* in this region and serve as a necessary preliminary baseline for this range margin. Leaves were dried in silica gel, and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Corp., Valencia, CA) following the manufacturer's protocol. A total of 12 nuclear microsatellite markers previously developed for *M. indica* (= *M. alba*) and *M. boninensis* were screened for cross-species amplification and transferability to *M. rubra* [33,34]. PCR conditions were optimized separately for each marker set. The MulSTR markers were amplified in 15- μ L reactions containing 25 ng genomic DNA, 3 μ L of 5 $\times$ GoTaq Flexi buffer, 1.5 μ L of 2.5 mM MgCl₂, 1.2 μ L of 10 mM dNTP mixture, 0.38 μ L each of 10 μ M forward and reverse primers, and 1 U of GoTaq DNA polymerase. Amplification consisted of an initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 45 s, 51°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. Five nuclear microsatellite markers produced clear and reproducible amplification and were retained for genotyping and population genetic analyses, whereas the remaining markers did not amplify consistently in *M. rubra*. The chloroplast *trnL*–*trnF* intergenic spacer was amplified following Nepal and Ferguson [2]. PCR was performed in 15- μ L reactions containing 25 ng genomic DNA, 5 μ L of 5 $\times$ buffer, 1 μ L of dNTPs, 2.5 μ L of 2.5 mM MgCl₂, 2.5 μ L of 10 μ M primers, and 2 U of GoTaq DNA polymerase. Cycling conditions consisted of an initial denaturation at 94°C for 5 min; 27 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. Nuclear and chloroplast PCR products were verified by electrophoresis on 1.2% agarose gels stained with ethidium bromide and visualized under ultraviolet light. Thus, the final dataset included six molecular markers comprising five nuclear microsatellite loci and the chloroplast *trnL*–*trnF* region (Table 2).

2.2 Microsatellite Genotyping

The PCR conditions described above were used for microsatellite genotyping, with an M13-tailed forward primer and FAM-labeled universal primer incorporated for fluorescent fragment analysis. Genotyping was performed at the Iowa State University DNA Facility using an ABI 3730xl DNA Analyzer (Applied Biosystems). Chloroplast *trnL*–*trnF* sequences were obtained for 96 *Morus rubra* individuals. Following chloroplast sequencing, nine individuals had insufficient remaining DNA for microsatellite genotyping, leaving 87 individuals for nuclear microsatellite evaluation. Electropherograms were visually inspected to verify allele calls, and nine additional individuals exhibited multiallelic profiles, defined as more than two reproducible allelic peaks at one or more microsatellite loci. Because the downstream population genetic analyses assumed diploid inheritance,

these multiallelic individuals were excluded rather than recoded or reduced to two alleles. The final nuclear microsatellite dataset therefore comprised 78 individuals genotyped at five transferable MulSTR loci. Allele sizes for retained individuals were scored using GeneMarker v2.4.0 (SoftGenetics).

2.3 Chloroplast Sequencing

The amplified *trnL-trnF* region was purified using the QIAquick PCR Purification Kit (Qiagen Corp., Valencia, CA) and sequenced at the Iowa State University DNA Facility. Chloroplast sequences for all 96 individuals, including the nine individuals excluded from nuclear microsatellite analyses because of multiallelic profiles, were successfully obtained. Sequences were edited in Sequencher v5.0 (Gene Codes Corporation) and aligned in ClustalX [35] to identify sequence polymorphisms and define chlorotypes. The 96 *trnL-trnF* sequences generated in this study are available in NCBI GenBank under accession numbers HM747181.1, KF672605.1, and KF672609.1–KF672702.1.

Table 1: Sampling locations, population abbreviations, geographic coordinates, and inferred glacial histories of six *Morus rubra* populations from the Upper Midwest United States. Populations were classified as originating from either glaciated or unglaciated regions based on their location relative to the southern extent of Pleistocene glaciation. Sample sizes for each population are given in parentheses following the collection site name. Latitude and longitude coordinates are provided for each sampling location.

Sample Collection Site (Sample Size)	Population Abbreviation	Latitude N/Longitude W	Inferred Glacial History
Waubonsie State Park, Iowa (17)	WAUB	40°40'20"/95°41'18"	Glaciated
Indian Cave State Park, Nebraska (15)	INDI	40°15'10"/95°33'16"	Glaciated
Wyalusing State Park, Wisconsin (18)	WYAL	42°58'47"/91°6'31"	Glaciated
Pottawatomie Lake #2, Kansas (8)	POTT	39°13'43.068"/96°31'51.132"	Glaciated
Konza Prairie, Kansas (17)	KONZ	39°6'16.056"/96°35'42.396"	Unglaciated
Alma, Kansas (12)	ALMA	39°0'1.9794"/96°11'22.92"	Unglaciated

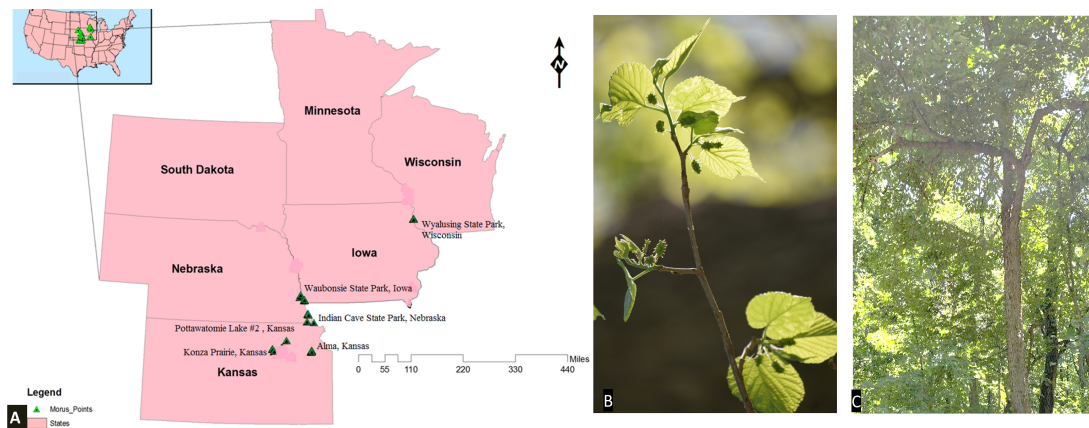


Figure 1: Sampling sites and representative photographs of a young fruiting twig and mature tree of *Morus rubra*. (A) Locations of the six populations sampled near the northwestern range boundary of *M. rubra* in the Upper Midwest: Waubonsie State Park, Iowa (WAUB); Indian Cave State Park, Nebraska (INDI); Wyalusing State Park, Wisconsin (WYAL); Pottawatomie Lake #2, Kansas (POTT); Konza Prairie, Kansas (KONZ); and Alma, Kansas (ALMA). WAUB, INDI, WYAL, and POTT were classified as occurring within formerly glaciated regions, whereas KONZ and ALMA were classified as unglaciated. The inset shows the sampling region within the United States. (B) Representative young fruiting twig of *M. rubra*, showing foliage and developing cylindrical infructescences. (C) Representative mature *M. rubra* tree illustrating adult growth form with widely spaced planar branches.

Table 2: Characteristics of five nuclear microsatellite loci and the chloroplast *trnL-trnF* region retained for genetic analyses of *Morus rubra*. Primer sequences are shown in the 5′–3′ direction. Ta, annealing temperature; bp, base pairs; SSR, simple sequence repeat.

Marker	Primer Sequence (5′–3′)	Repeat Motif	T _a (°C)	Size (bp)	NCBI Accession
MulSTR1 nuclear SSR	F GCCGTGTACCAGTGGAGTTTGCA R TGACCGTTTCTTCCACTTACCTAATG	(GTT) ₆ + (GTT) ₄	51	157–163	AY326440
MulSTR2 nuclear SSR	F CGTGGGGCTTAGGCTGAGTAGAGG R CACCACCACTACTTCTCTTCTCCAG	(GTT) ₁₁	51	157–178	AY326441
MulSTR4 nuclear SSR	F GGTC AAGCGCTCCAGAGAAAAG R GGTGCAGAGGATGAAAGATGAGGT	(GAA) ₆	51	124–142	AY326443
MulSTR5 nuclear SSR	F CCCCTGCAATGCCCTCTTTC R TGGGCGAGGCAGGGAAGATTC	(CCA) ₈	51	157–172	AY326444
MulSTR6 nuclear SSR	F TCCTTAGGTTTTGGGGTCTGTTTACAT R CCTCATTCTCCTTTCATTATGTGTG	(GT) ₁₅	51	158–162	AY326445
<i>trnL-trnF</i> chloroplast	F GCAATCGCCAAGTTTAAAGC R TCCTGGTGGTGAATTTCTTGA	(T) ₁₀	55	781–791	HM747181.1

2.4 Genetic Diversity

Nuclear genetic diversity analyses were conducted using the 78 individuals retained after exclusion of nine individuals exhibiting multiallelic microsatellite profiles. Results from GeneMarker V2.4.0 (Softgenetics) were examined for genetic diversity. Evaluation of linkage disequilibrium and deviation from Hardy-Weinberg Equilibrium (HWE) for all loci was performed in Arlequin V3.1 [36]. Given the limited number of loci evaluated in this dataset (five microsatellite markers) and the exploratory nature of this study, uncorrected *p*-values were retained, and sequential Bonferroni corrections were not applied. Genetic diversity of each locus was estimated by its polymorphism (P), allele count, and heterozygosity using Arlequin V3.1 [36]. Within each population, the microsatellites expressed varied allelic richness (A) and heterozygosity levels (H_o ; H_e). These values were averaged to determine the genetic diversity indices for each population, namely, allele count and heterozygosity. Within-population fixation coefficients (F_{IS} values) were estimated using FSTAT [37] to quantify heterozygote deficiency relative to Hardy-Weinberg expectations. Because dedicated null-allele screening was not performed, F_{IS} values were interpreted as measures of heterozygote deficiency rather than direct evidence of inbreeding, as null alleles, allele dropout, population subdivision, or other marker-related effects could also contribute to departures from HWE.

2.5 Population Genetic Structure

Population structure was analyzed for the segregation of population genetic structures. Nei's genetic diversity indexes [38] were calculated to determine gene differentiation (G_{st}). Total gene diversity (H_t), gene diversity within populations (H_s), and gene diversity among populations (D_{st}) were determined using the statistical program FSTAT [37]. An AMOVA was performed using the Fst analysis of Arlequin V3.1 [36] to identify differentiation within populations and among populations (F_{st}). F_{st} and G_{st} values were compared because these measures were expected to be closely correlated. A Bayesian model replica was performed to infer population allele composition and theoretical ancestry based on individual genotypes at multiple loci using STRUCTURE [39]. The analysis was run using the admixture model with correlated allele frequencies. Allele ancestry and frequency within $K = 1-9$ populations were examined with a burn-in period of 10,000 and 50,000 Markov chain Monte Carlo (MCMC) iterations after each burn-in. Twenty independent repetitions were completed for each value of K to ensure replicate consistency. The optimal number of clusters (K) was determined to be 4 based on the method [40], implemented via STRUCTURE HARVESTER [41].

2.6 Chlorotype Analysis

Analysis of the chloroplast DNA focused on the number of chlorotypes produced by cpDNA sequence polymorphism. Sequenced cpDNA results were re-examined using Sequencher version 5.0 (Gene Codes Corporation) to edit unclear sequence results. Sequencher editing fidelity was verified by comparison with ClustalX [42] results prior to analysis of potential chlorotypes among individuals. ClustalX [42] displayed the amplified sequences in parallel and their SNPs. Sites with SNPs were cross-referenced by examining nucleotide indels, transversions, and transitions. With these SNPs identified, a number of chlorotypes were identified and compared to chlorotype diversity by geographic location of each population, to observe correlation between latitude and chlorotype diversity. The data matrix was analyzed using the programs DnaSP [43] and Network 4.1.0.9 (<https://www.fluxus-engineering.com>). The software DnaSP [43] evaluated the haplotype diversity of the amplified chloroplast region and the nucleotide diversity (π).

3 Results

3.1 Marker Transferability and Locus-Level Genetic Diversity

Of the 12 nuclear microsatellite markers screened, five MulSTR loci produced clear and reproducible genotypes in *M. rubra*, and were retained for nuclear population genetic analyses. Chloroplast sequences were available for 96 individuals; nine had insufficient remaining DNA for microsatellite genotyping, leaving 87 individuals for nuclear evaluation. Of these, nine displayed multiallelic profiles and were excluded from downstream nuclear analyses, resulting in a final microsatellite dataset of 78 individuals. All five loci were polymorphic across the sampled *M. rubra* populations. The number of alleles per locus ranged from 5 to 6, observed heterozygosity (H_O) from 0.21 to 0.50, and expected heterozygosity (H_E) from 0.52 to 0.76 (Table 3). Total gene diversity (H_t) ranged from 0.65 to 0.84, with a mean of 0.76, whereas mean within-population diversity (H_s) ranged from 0.54 to 0.78, with an overall mean of 0.66. Gene diversity among populations (D_{st}) ranged from 0.06 to 0.12, and G_{st} ranged from 0.07 to 0.17, averaging 0.13. H_O was lower than H_E at every locus, and each locus deviated from Hardy–Weinberg expectations in at least two populations at nominal $p < 0.05$; MulSTR1 and MulSTR6 deviated in all six populations (Table 3).

Table 3: Locus-level nuclear microsatellite diversity and population differentiation in *Morus rubra*. A, mean number of alleles for each locus; H_O , observed heterozygosity; H_E , expected heterozygosity; H_t , total gene diversity; H_s , mean within-population gene diversity; D_{st} , gene diversity among populations; G_{st} , coefficient of genetic differentiation. The final column lists populations with departures from Hardy–Weinberg equilibrium at nominal $p < 0.05$.

Locus	A	H_O	H_E	H_t	H_s	D_{st}	G_{st}	Populations with HWE Deviations
MulSTR1	5	0.21	0.52	0.65	0.54	0.11	0.17	WAUB, INDI, WYAL, POTT, KONZ, ALMA
MulSTR2	6	0.28	0.59	0.70	0.61	0.10	0.14	WAUB, WYAL, KONZ, ALMA
MulSTR4	6	0.48	0.65	0.84	0.78	0.06	0.07	WAUB, INDI
MulSTR5	5	0.22	0.71	0.77	0.66	0.11	0.15	WAUB, INDI, WYAL
MulSTR6	6	0.50	0.76	0.84	0.72	0.12	0.14	WAUB, INDI, WYAL, POTT, KONZ, ALMA
Mean	5.6	0.34	0.65	0.76	0.66	0.10	0.13	

3.2 Population Genetic Diversity and Structure

Descriptive contrasts in nuclear and chloroplast diversity were observed between populations from formerly glaciated and unglaciated areas (Table 4). In the four glaciated populations, mean nuclear allele number ranged from 2 to 4 per locus, H_O from 0.11 to 0.25, and H_E from 0.47 to 0.58. The two unglaciated populations had 9–10 alleles per locus, H_O of 0.57–0.63, and H_E of 0.89–0.90. Averaged across populations,

the glaciated group had lower mean allele number (3.0 vs. 9.5), H_O (0.208 vs. 0.600), and H_E (0.523 vs. 0.895). Chloroplast diversity showed the same directional group-level pattern but greater among-population heterogeneity: SNP chlorotype richness averaged 2.75 in glaciated versus 3.5 in unglaciated populations, all-site variant richness averaged 4.75 versus 7.0, and all-site H_d averaged 0.548 versus 0.685. All populations showed positive F_{IS} values, ranging from 0.307 in ALMA to 0.800 in WAUB; the mean F_{IS} was 0.616 for glaciated populations and 0.341 for unglaciated populations. Because only four glaciated and two unglaciated populations were sampled, these group contrasts are descriptive rather than formal tests of glacial history.

Table 4: Population-level nuclear and chloroplast diversity in six *Morus rubra* populations. Population history indicates whether each sampling location occurred within a formerly glaciated (G) or unglaciated (U) region during the last glacial maximum. N_{SSR} = number of individuals included in nuclear microsatellite analyses; A = mean number of nuclear alleles per locus within each population; P = percentage of polymorphic nuclear loci; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = within-population fixation coefficient; N_{cp} = number of chloroplast *trnL-trnF* sequences analyzed; SNP chlorotype richness = number of chlorotypes defined by nucleotide-substitution polymorphisms; all-site variant richness = number of unique chloroplast sequence variants defined using both nucleotide substitutions and insertion/deletion polymorphisms; H_d = haplotype diversity calculated from all variable sites.

Population	History	N_{SSR} (Total 78)	A	P (%)	H_O	H_E	F_{IS}	N_{cp} (Total 96)	SNP Chlorotype Richness	All-Site Variant Richness	All-Site H_d
WAUB	G	17	3	80	0.11	0.52	0.800	17	4	7	0.713
INDI	G	11	3	80	0.24	0.52	0.541	14	1	5	0.659
WYAL	G	18	4	100	0.25	0.58	0.585	12	5	6	0.818
POTT	G	8	2	100	0.23	0.47	0.537	8	1	1	0.000
KONZ	U	12	9	100	0.57	0.89	0.374	25	5	10	0.870
ALMA	U	12	10	100	0.63	0.90	0.307	20	2	4	0.500

AMOVA attributed 83.6% of the nuclear microsatellite variation to differences within populations and 16.4% to differences among populations, producing an overall F_{ST} of 0.1649 (Table 5). The ΔK analysis identified $K = 4$ as the strongest clustering solution among the values evaluated (see Supplemental File S2). At $K = 4$, KONZ and ALMA displayed broadly similar ancestry profiles, whereas the four formerly glaciated populations differed in the relative contributions of the inferred clusters. INDI and WYAL showed broadly similar but heterogeneous ancestry composition, POTT was dominated by one ancestry component, and WAUB contained multiple ancestry components also represented in other populations (Fig. 2). Given the five-locus marker set, these clustering patterns are interpreted as descriptive population structure rather than definitive ancestry assignments.

Table 5: Analysis of molecular variance for six *Morus rubra* populations based on five nuclear microsatellite loci. d.f. = degrees of freedom; F_{ST} = fixation index measuring differentiation among populations.

Source of Variation	d.f.	Sum of Squares	Variance Component	Variation (%)	F_{ST}
Among populations	5	48.31	0.31	16.4	0.1649
Within populations	150	239.15	1.59	83.6	—
Total	155	287.46	1.91	100.0	—

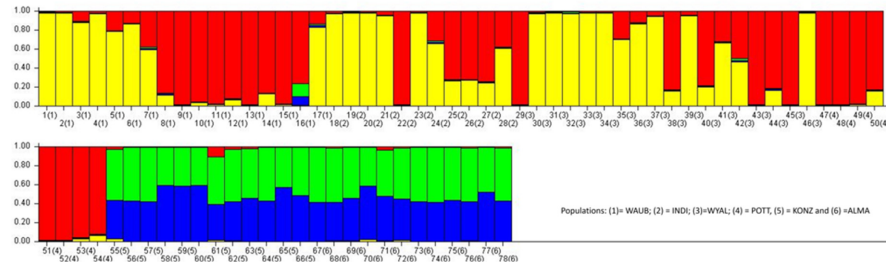


Figure 2: Bayesian population structure of six *Morus rubra* populations at $K = 4$, the strongest ΔK -supported solution among the values evaluated. Each vertical bar represents an individual, and colored segments indicate estimated membership coefficients in four genetic clusters. Individuals are grouped by population in the order WAUB (1), INDI (2), WYAL (3), POTT (4), KONZ (5), and ALMA (6).

3.3 Chloroplast DNA Diversity

The final chloroplast dataset comprised 96 *Morus rubra* *trnL-trnF* sequences representing the six sampled populations, together with one *Trophis involucrata* sequence used as the outgroup. The 945-bp alignment contained 13 nucleotide-substitution polymorphisms within *M. rubra*, resolving 11 SNP-defined chlorotypes (C1–C11; Table 6). C1 was predominant, occurring in 71 of 96 individuals (74.0%) and in all six populations; overall SNP chlorotype diversity was $H_d = 0.446$. When all 32 positions variable within *M. rubra* were considered, including 13 nucleotide substitutions and 19 gap/indel-variable positions, 25 all-site sequence variants were resolved and overall haplotype diversity increased to $H_d = 0.875$. Population-specific SNP chlorotype richness ranged from one to five, whereas all-site variant richness ranged from one to ten (Table 4). Across the complete 97-sequence alignment, 19 substitution-variable positions and 41 total variable positions were recorded when outgroup-specific differences were included (Table 6; Supplementary File S1). The maximum-likelihood phylogeny showed shallow differentiation among many *M. rubra* sequences, with bootstrap support concentrated on a limited number of branches, consistent with the predominance of C1 and limited substitutional divergence in this chloroplast region (Fig. 3).

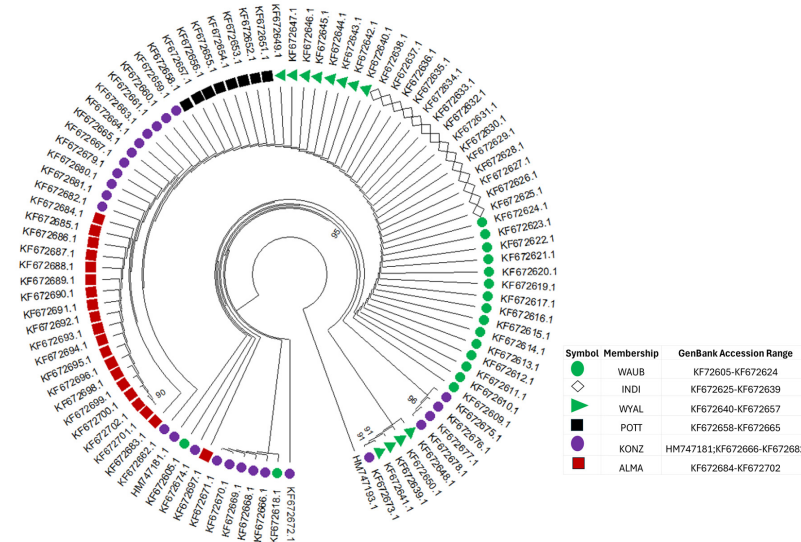


Figure 3: Maximum-likelihood phylogeny of chloroplast *trnL-trnF* sequences from 96 *Morus rubra* individuals representing six populations. Population membership is indicated by colored symbols, and *Trophis involucrata* (HM747193.1) was used as the outgroup. Numbers at major nodes indicate bootstrap support values.

Table 6: Distribution and nucleotide profiles of chloroplast *trnL*–*trnF* chlorotypes in *Morus rubra*.

A. Chlorotype frequencies among the six sampled populations.												
Chlorotype		WAUB	INDI	WYAL	POTT	KONZ	ALMA	Total	Representative Accession			
C1		14	14	8	8	13	14	71	HM747181.1			
C2		1	0	0	0	6	0	7	KF672618.1			
C3		0	0	0	0	0	6	6	KF672697.1			
C4		0	0	0	0	4	0	4	KF672675.1			
C5		0	0	1	0	1	0	2	KF672639.1			
C6		0	0	1	0	0	0	1	KF672648.1			
C7		0	0	1	0	0	0	1	KF672650.1			
C8		0	0	1	0	0	0	1	KF672641.1			
C9		1	0	0	0	0	0	1	KF672605.1			
C10		0	0	0	0	1	0	1	KF672674.1			
C11		1	0	0	0	0	0	1	KF672610.1			
Total		17	14	12	8	25	20	96	—			
B. Nucleotide states at the 19 substitution-variable positions in the complete 97-sequence alignment.												
Positions 5–854												
Chlorotype	N	Representative Accession	5	94	197	220	235	611	837	839	843	854
C1	71	HM747181.1	A	A	C	T	C	C	-	C	C	-
C2	7	KF672618.1	A	A	C	C	C	T	-	C	C	-
C3	6	KF672697.1	A	A	C	T	C	G	-	C	C	-
C4	4	KF672675.1	A	A	C	T	A	C	-	C	C	-
C5	2	KF672639.1	A	A	C	T	C	C	-	C	C	-
C6	1	KF672648.1	A	A	C	T	C	C	-	C	C	-
C7	1	KF672650.1	A	A	C	T	C	C	-	C	C	-
C8	1	KF672641.1	A	A	C	T	C	C	T	C	C	T
C9	1	KF672605.1	A	A	T	T	C	C	-	C	C	-
C10	1	KF672674.1	A	G	C	T	C	C	T	C	C	-
C11	1	KF672610.1	C	A	C	T	C	C	-	C	C	-
OUTGROUP	1	HM747193.1	A	A	C	C	C	C	C	A	T	C
Positions 871–945												
Chlorotype	N	Representative Accession	871	912	913	927	929	931	937	942	945	
C1	71	HM747181.1	G	T	G	C	G	-	G	G	G	
C2	7	KF672618.1	G	T	G	C	G	-	G	G	G	
C3	6	KF672697.1	G	T	G	C	G	-	G	G	G	
C4	4	KF672675.1	G	T	G	C	G	-	G	G	G	
C5	2	KF672639.1	G	T	G	C	G	-	A	A	G	
C6	1	KF672648.1	G	T	G	C	G	-	A	G	G	
C7	1	KF672650.1	G	T	G	C	G	-	G	A	G	
C8	1	KF672641.1	G	A	T	T	A	C	A	A	A	
C9	1	KF672605.1	G	T	G	C	G	-	G	G	G	
C10	1	KF672674.1	G	T	G	C	G	-	G	G	G	
C11	1	KF672610.1	G	T	G	C	G	-	G	G	G	
OUTGROUP	1	HM747193.1	A	T	G	C	G	T	G	G	G	

Notes: Chlorotypes C1–C11 were defined from the 96 *M. rubra* sequences; the *Trophis involucrata* sequence (HM747193.1) is shown as OUT and was not included in population frequencies. Population sample sizes are WAUB = 17, INDI = 14, WYAL = 12, POTT = 8, KONZ = 25, and ALMA = 20 (total *M. rubra* *n* = 96). A dash (–) denotes an alignment gap. The 19 positions are substitution-variable in the complete 97-sequence alignment; 13 are nucleotide-substitution polymorphisms within *M. rubra*. Positions 837, 854, and 931 include gap/base variation within *M. rubra*, and positions 839, 843, and 871 distinguish the *Trophis* outgroup.

4 Discussion

The nuclear and chloroplast datasets provide complementary, broadly concordant evidence at the glaciated-versus-unglaciaded group level. Nuclear diversity was markedly lower in formerly glaciaded

populations, and mean SNP chlorotype richness, all-site variant richness, and all-site haplotype diversity were also lower in the glaciated group. Chloroplast diversity was more heterogeneous among individual populations, however, and the data did not show a simple south-to-north gradient. These patterns are consistent with expectations under postglacial colonization and leading-edge founder effects but do not uniquely identify that process. Given the six sampled populations, five nuclear loci, and a single chloroplast region, the results are best treated as baseline evidence; recent habitat fragmentation, restricted gene flow, genetic drift, and small effective population size could generate similar spatial patterns and must also be considered.

4.1 Microsatellite Transferability and Heterozygote Deficiency

Cross-species amplification of microsatellite loci is a cost-effective alternative to developing species-specific markers and has been widely applied in plant genetic studies [44]. Five of the 12 nuclear markers screened here amplified consistently in *M. rubra*, demonstrating the transferability of loci developed for other *Morus* species. Transferability most directly reflects conservation of primer-binding regions and should not, by itself, be interpreted as evidence of low genome-wide differentiation among *Morus* species. The five selected loci nevertheless provided a preliminary view of population-level variation.

Observed heterozygosity was lower than expected heterozygosity across the retained microsatellite loci in every population. The H_E values of 0.52–0.76 were also lower than the 0.85–0.90 values reported for these markers in other *Morus* material [33]. These heterozygote deficits may reflect biological processes such as inbreeding or population subdivision within fragmented range-edge stands, but the available data do not distinguish among possible causes. Morphological and ITS screening was used to target *M. rubra* individuals, but this screening does not exclude cryptic introgression or other processes that could influence multilocus genotype patterns. However, because these microsatellite markers were developed for other *Morus* species, methodological factors such as null alleles, allele dropout, and duplicated loci [45,46] cannot be excluded as contributors to the observed deficiency. Because dedicated null-allele screening was not performed for the present dataset, F_{IS} values are interpreted as measures of heterozygote deficiency rather than direct evidence of inbreeding. Recent phylogenomic studies have documented reticulation, incomplete lineage sorting, and cytonuclear discordance across *Morus*, reinforcing the need to evaluate possible hybrids with species-diagnostic nuclear SNPs or genome-scale data [47,48].

4.2 Patterns Consistent with Postglacial Founder Effects

The clearest result was the pronounced reduction in nuclear diversity in the glaciated populations, accompanied by lower chloroplast diversity when populations were summarized by glacial history. Mean nuclear allele number was 3.0 in glaciated populations compared with 9.5 in unglaciated populations, and mean H_E was 0.523 compared with 0.895. Mean SNP chlorotype richness was 2.75 compared with 3.5, mean all-site variant richness was 4.75 compared to 7.0, and mean all-site H_d was 0.548 compared with 0.685 in glaciated and unglaciated populations, respectively. The chloroplast contrast was therefore directionally consistent with the nuclear pattern at the group level, although it was more heterogeneous among individual populations. These group-level patterns are consistent with leading-edge expansion, in which repeated colonization by subsets of a source population reduces allelic and haplotypic diversity toward the range margin [7–10,17]. A recent synthesis of nuclear microsatellite studies in European beech similarly recovered decreasing genetic diversity with increasing distance from glacial refugia, illustrating the persistence of postglacial demographic signatures in long-lived forest trees [15].

The STRUCTURE analysis also differentiated the ancestry profiles of the two unglaciated Kansas populations from most formerly glaciated samples, while AMOVA detected moderate among-population differentiation ($F_{ST} = 0.1649$) despite most variation occurring within populations (83.6%). The substantially higher nuclear diversity of KONZ and ALMA, together with their broadly similar STRUCTURE ancestry profiles at $K = 4$, is compatible with a source-like pattern expected under northward range expansion. However, the present limited sampling and molecular dataset cannot establish KONZ or ALMA as direct historical source populations of WAUB, INDI, WYAL, and POTT. Because formal demographic analyses such as bottleneck tests, Approximate Bayesian Computation (ABC), or coalescent simulations, were not performed due to the limited marker set, our results cannot distinguish historical postglacial expansion from more recent evolutionary and demographic processes. Reduced diversity at range margins can also result from contemporary demographic processes, including severe habitat fragmentation, genetic drift, historical bottlenecks, isolation by distance, and small effective population sizes. A broad analysis of North American plants emphasized that both glacial history and central-marginal demographic processes must be jointly considered when interpreting reduced diversity at northern margins [16].

4.3 Concordance and Differences between Nuclear and Chloroplast Patterns

The nuclear and chloroplast markers were broadly consistent in distinguishing unglaciated populations from the glaciated populations, but they differed in the strength and population-level distribution of diversity. Nuclear diversity showed a pronounced contrast between the two geographic groups, whereas chloroplast diversity varied substantially among populations. For example, the glaciated WYAL population contained five SNP-defined chlorotypes and six all-site variants, whereas unglaciated ALMA contained two SNP-defined chlorotypes and four all-site variants. Similarly, all-site haplotype diversity was 0.818 in WYAL but 0.500 in ALMA. These departures from a simple geographic gradient may reflect contemporary gene flow, persistence of maternal lineages, lateral colonization from unsampled populations, or disturbance-related movement of propagules.

Maternal introgression from *M. alba* could also alter the geographic distribution of chlorotypes because hybrid fitness depends partly on the direction of the cross [49]. Anthropogenic disturbance has been shown to complicate simple relationships between geography and genetic diversity in recently colonized landscapes [50]. Thus, the heterogeneous chloroplast distribution and absence of a strict latitudinal decline indicate that a simple postglacial founder-effect model cannot fully explain the observed variation. Historical colonization, contemporary gene flow, habitat fragmentation, local demographic processes, and potential hybridization may have contributed jointly to the present genetic structure. Although substitutional divergence in the *trnL-trnF* region was shallow, chloroplast sequence variation can retain useful historical information because plant chloroplast DNA generally evolves slowly and is highly conserved [23,51,52]. The SNP-defined chlorotype C1 occurred in 71 of 96 individuals and in all six populations, supporting its interpretation as a widespread maternal lineage across the sampled range. However, a single chloroplast region cannot establish the ancestral status of C1 or determine its specific role in postglacial colonization. The limited resolution among many individuals in the maximum-likelihood phylogeny is likewise consistent with shallow substitutional differentiation within the *trnL-trnF* region. A recent whole-plastome analysis of *M. rubra* across a broader geographic sample also recovered 12 haplotypes and identified multiple variable intergenic regions suitable for marker development [53]. That genomic evidence supports the value of chloroplast variation while indicating that whole-plastome or multilocus chloroplast data will provide greater resolution than *trnL-trnF* alone.

4.4 Conservation Implications and Study Limitations

Positive F_{IS} values occurred in all populations, and WAUB combined the highest F_{IS} (0.800) with the lowest H_O (0.11). Because heterozygote deficits can also arise from null alleles, allele dropout, or population subdivision, these values should not be interpreted as direct estimates of mating among close relatives. Nevertheless, the combination of low nuclear allelic diversity, high heterozygote deficiency, and geographic marginality identifies WAUB and other formerly glaciated populations as priorities for further genetic assessment. More broadly, population genetic diversity is shaped jointly by life history, climate, and biogeographic context, and range-edge populations may retain unique or conservation-relevant variation despite reduced average diversity [54]. These results indicate that multiple populations warrant further conservation-genetic assessment rather than prioritizing sites solely on the basis of current diversity estimates, because northern populations such as WAUB and WYAL also contain low-frequency chloroplast variants and genetic combinations that may represent regionally informative variation.

A critical limitation of this study is the inability to directly evaluate introgressive hybridization with the naturalized invasive congener *M. alba*. Because no *M. alba* samples, confirmed hybrid reference individuals, or species-diagnostic genomic markers were included in our molecular dataset, any inferences regarding current introgression within these populations remain speculative. Therefore, hybridization is presented here not as a process demonstrated by our present dataset, but as a well-documented ecological implication that exacerbates the vulnerability of these marginal populations. Prior studies demonstrate that interspecific hybridization can reduce the persistence of rare species [55], accelerate displacement by invasive congeners [56], and alter population size and genetic composition through recurrent gene flow [57]. When fertile hybrids backcross predominantly with the rare species, genetic swamping and assimilation may follow [58,59]. This risk is well documented for *M. rubra* and *M. alba* [5], and the ecological loss of *M. rubra* may affect associated native forest species [60]. Accordingly, future conservation assessment should combine broader genomic characterization with verification of species identity and monitoring of hybridization before population-specific management units are designated.

Furthermore, the phylogeographic and demographic inferences are constrained by six sampled populations, five transferable nuclear loci, a single chloroplast region with shallow substitutional divergence, uneven sample sizes, and the rare and patchy distribution of *M. rubra*. The low statistical power provided by this limited set of markers restricts our ability to detect hidden admixture, estimate population differentiation with high precision, resolve fine-scale population structure, or reconstruct demographic history conclusively. Genome-wide nuclear markers are therefore needed to improve estimates of population structure and demographic history and to evaluate the extent and directionality of introgression. Recent work from our laboratory has generated complete chloroplast [53] and mitochondrial [61] genomes, from which hundreds of candidate organellar markers are undergoing validation. Low-coverage Illumina genome-skimming data are also available, and development of a high-quality nuclear reference genome is underway using PacBio long-read and Hi-C scaffolding data. Integrating these resources with expanded range-wide sampling of *M. rubra*, *M. alba*, and putative hybrids will improve estimates of gene flow, evaluate whether distinct conservation units can be supported, and strengthen strategies for the restoration and long-term management of native *M. rubra* populations.

5 Conclusions

Nuclear microsatellite genotype and chloroplast sequence variation showed consistent reductions in genetic diversity in populations from formerly glaciated regions compared with the two southern unglaciated populations. Glaciated populations averaged 3.0 nuclear alleles per locus, H_O of 0.208, and H_E

of 0.523 whereas unglaciated populations averaged 9.5 alleles, H_O of 0.600, and H_E of 0.895. Mean SNP chlorotype richness, all-site variant richness, and all-site haplotype diversity were 2.75, 4.75, and 0.548 in glaciated populations compared with 3.5, 7.0, and 0.685 in unglaciated populations, respectively.

Together with the STRUCTURE and AMOVA results, these patterns are consistent with a postglacial leading-edge expansion scenario but do not distinguish historical founder effects from contemporary range-edge processes. All populations exhibited heterozygote deficiencies, with the highest F_{IS} observed in WAUB; however, these values should not be interpreted as direct evidence of inbreeding. Hybridization with *M. alba* was not evaluated directly in this study. The observed population-level differences therefore identify priorities for further conservation-genetic assessment rather than definitive conservation units. Broader geographic sampling, genome-wide nuclear markers, expanded chloroplast data, species-diagnostic markers, and formal demographic modeling will be necessary to evaluate historical colonization, contemporary gene flow, and introgression and to inform future population-specific conservation actions.

Acknowledgement: S. J. Schreier received the Excellence in Research Award from the Van D. and Barbara B. Fishback Honors College, South Dakota State University. This work was conducted as part of Schreier's participation in BIOL 498 (Undergraduate Research and Scholarship) under the mentorship of M. P. Nepal in the Department of Biology and Microbiology, South Dakota State University. We thank Bibek Adhikari, Jeff Carstens, and Sanam Parajuli for their assistance in the field.

Funding Statement: This research was supported by the South Dakota Agricultural Experiment Station Hatch Project (Award No. SD00H800-23) awarded to M. P. Nepal.

Author Contributions: Madhav P. Nepal conceived and designed the experiments; Spencer J. Schreier performed the experiments; Madhav P. Nepal and Spencer J. Schreier analyzed the data; Madhav P. Nepal and Spencer J. Schreier wrote and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The authors confirm that all data supporting the findings of this study are available within the article and its Supplementary Materials. The 96 *trnL-trnF* sequences generated and analyzed in this study have been deposited in the NCBI GenBank database and are accessible under accession numbers HM747181.1, KF672605.1, and KF672609.1-KF672702.1. Supplementary Files S1 and S2 associated with this study are publicly available in Supplementary Materials.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: The supplementary material is available online at <https://www.techscience.com/doi/10.32604/phyton.2026.088912/s1>. Supplementary File S1. Variable sites in the chloroplast *trnL-trnF* alignment of *Morus rubra*, showing 25 sequence variants, corresponding SNP chlorotypes, counts across six populations, and representative accession numbers. Numbered columns indicate alignment positions; “–” denotes alignment gaps, and OUT denotes the outgroup. Supplementary File S2. Bayesian population structure of six *Morus rubra* populations. Each vertical bar represents an individual, and colored segments indicate estimated membership coefficients in the two inferred genetic clusters. Individuals are grouped by sampling population: WAUB (1), INDI (2), WYAL (3), POTT (4), KONZ (5), and ALMA (6). The dominant population structure corresponds broadly to the geographic contrast between the four northern populations from historically glaciated regions (WAUB, INDI, WYAL, and POTT) and the two southern populations from unglaciated regions (KONZ and ALMA).

References

1. Nepal MP. Systematics and reproductive biology of the genus *Morus* L. (Moraceae). Manhattan, KS, USA: Kansas State University; 2008. p. 191.

2. Nepal MP, Ferguson CJ. Phylogenetics of *Morus* (Moraceae) inferred from ITS and *trnL-trnF* sequence data. *Syst Bot.* 2012;37(2):442–50. [[CrossRef](#)].
3. Nepal MP, Purintun JM. Systematics of the genus *Morus* L. (Moraceae): Taxonomy, phylogeny and potential responses to climate change. In: *Mulberry: Genetic improvement in context of climate change*. Boca Raton, FL, USA: CRC Press; 2021. p. 2–20. [[CrossRef](#)].
4. Moerman DE. Medicinal plants of native America, Vols. 1 and 2. Ann Arbor, MI, USA: University of Michigan Press/Museum of Anthropological Archaeology; 1987. [[CrossRef](#)].
5. Burgess KS, Morgan M, Deverno L, Husband BC. Asymmetrical introgression between two *Morus* species (*M. alba*, *M. rubra*) that differ in abundance. *Mol Ecol.* 2005;14(11):3471–83. [[CrossRef](#)].
6. Pielou EC. After the ice age: The return of life to glaciated North America. Chicago, IL, USA: University of Chicago Press; 1991. [[CrossRef](#)].
7. Broyles SB. Postglacial migration and the loss of allozyme variation in northern populations of *Asclepias exaltata* (Asclepiadaceae). *Am J Bot.* 1998;85(8):1091–7. [[CrossRef](#)].
8. Gugerli F, Sperisen C, Büchler U, Magni F, Geburek T, Jeandroz S, et al. Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. *Mol Ecol.* 2001;10(5):1255–63. [[CrossRef](#)].
9. Palmé AE. Chloroplast DNA variation, postglacial recolonization and hybridization in hazel, *Corylus avellana*. *Mol Ecol.* 2002;11(9):1769–79. [[CrossRef](#)].
10. Gamache I, Jaramillo-Correa JP, Payette S, Bousquet J. Diverging patterns of mitochondrial and nuclear DNA diversity in subarctic black spruce: Imprint of a founder effect associated with postglacial colonization. *Mol Ecol.* 2003;12(4):891–901. [[CrossRef](#)].
11. Skrede I, Eidesen PB, Portela RP, Brochmann C. Refugia, differentiation and postglacial migration in Arctic-alpine Eurasia, exemplified by the mountain avens (*Dryas octopetala* L.). *Mol Ecol.* 2006;15(7):1827–40. [[CrossRef](#)].
12. Broecker WS, van Donk J. Insolation changes, ice volumes, and the O¹⁸ record in deep-sea cores. *Rev Geophys.* 1970;8(1):169–98. [[CrossRef](#)].
13. Davis MB. Quaternary history of deciduous forests of eastern North America and Europe. *Ann Mo Bot Gard.* 1983;70(3):550. [[CrossRef](#)].
14. Ingólfsson Ó. The Quaternary Period. In: *Encyclopedia of the arctic*. Reykjavik, Iceland: University of Iceland; 2008.
15. Stefanini C, Csilléry K, Ulaszewski B, Burczyk J, Schaepman ME, Schuman MC. A novel synthesis of two decades of microsatellite studies on European beech reveals decreasing genetic diversity from glacial refugia. *Tree Genet Genomes.* 2022;19(1):3. [[CrossRef](#)].
16. López-Delgado J, Meirmans PG. History or demography? Determining the drivers of genetic variation in North American plants. *Mol Ecol.* 2022;31(7):1951–62. [[CrossRef](#)].
17. Hewitt G. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol J Linn Soc.* 1996;58(3):247–76. [[CrossRef](#)].
18. Raven PH, Johnson GB. *Biology*. 9th ed. Dubuque, IA, USA: McGraw-Hill; 2010.
19. Bergstrom CT, Dugatkin LA. *Evolution*. 1st ed. New York, NY, USA: W. W. Norton & Company, Inc.; 2012.
20. Cisternas-Fuentes A, Koski MH. Drivers of strong isolation and small effective population size at a leading range edge of a widespread plant. *Heredity.* 2023;130(6):347–57. [[CrossRef](#)].
21. Hewitt GM. Genetic consequences of climatic oscillations in the Quaternary. *Philos Trans R Soc Lond Ser B Biol Sci.* 2004;359(1442):183–95. [[CrossRef](#)].
22. Hartl DL, Clark AG. *Principles of population genetics*. 4th ed. Sunderland, MA, USA: Sinauer Associates, Inc.; 2007.
23. Wolfe KH, Li WH, Sharp PM. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc Natl Acad Sci U S A.* 1987;84(24):9054–8. [[CrossRef](#)].
24. Heuertz M, Fineschi S, Anzidei M, Pastorelli R, Salvini D, Paule L, et al. Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe. *Mol Ecol.* 2004;13(11):3437–52. [[CrossRef](#)].
25. Dumolin S, Demesure B, Petit RJ. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor Appl Genet.* 1995;91(8):1253–6. [[CrossRef](#)].

26. Żukowska WB, Wójkiewicz B, Lewandowski A, Łászló R, Wachowiak W. Genetic variation of Scots pine (*Pinus sylvestris* L.) in Eurasia: Impact of postglacial recolonization and human-mediated gene transfer. *Ann For Sci.* 2023;80(1):42. [CrossRef].
27. McPherson H, van der Merwe M, Delaney SK, Edwards MA, Henry RJ, McIntosh E, et al. Capturing chloroplast variation for molecular ecology studies: A simple next generation sequencing approach applied to a rainforest tree. *BMC Ecol.* 2013;13:8. [CrossRef].
28. Keith JA. Committee on the status of endangered wildlife in Canada (COSEWIC). In: *Endangered species in the prairie provinces*. Edmonton, AB, Canada: Alberta Culture Historical Resources Division;1986. p. 17.
29. Ambrose JD. Status report on the red mulberry, *Morus rubra*, in Canada. Gatineau, QC, Canada: Committee on the Status of Endangered Wildlife in Canada; 1990.
30. Sullivan J. *Morus rubra*. In: *Fire effects information system*. Fort Collins, CO, USA: USDA Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory; 1993.
31. Stone KR. *Morus alba*. In: *Fire effects information system*. Fort Collins, CO, USA: USDA Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory; 2009.
32. Nepal M, Wichern D. Taxonomic status of red mulberry (*Morus rubra*, Moraceae) at its northwestern boundary. *Proc South Dak Acad Sci.* 2013;92:19.
33. Aggarwal RK, Udaykumar D, Hendre PS, Sarkar A, Singh LI. Isolation and characterization of six novel microsatellite markers for mulberry (*Morus indica*). *Mol Ecol Notes.* 2004;4(3):477–9. [CrossRef].
34. Tani N, Kawahara T, Yoshimaru H. Development and diversity of microsatellite markers for endangered species, *Morus boninensis* Koidz., to establish conservation program. *Mol Ecol Notes.* 2005;5(2):398–400. [CrossRef].
35. Thompson J, Higgins D, Gibson T. ClustalX. 2001 [cited 2026 Sep 16]. Available from: <https://www.theclustal.com/download/current/>.
36. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol Bioinform Online.* 2007;1:47–50. [CrossRef].
37. Goudet J. Fstat, in A program to estimate and test gene diversities and fixation indices, release. 2002 [cited 2026 Sep 16]. Available from: <https://www2.unil.ch/popgen/softwares/fstat.htm>.
38. Nei M. Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci U S A.* 1973;70(12):3321–3. [CrossRef].
39. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics.* 2000;155(2):945–59. [CrossRef].
40. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol Ecol.* 2005;14(8):2611–20. [CrossRef].
41. Earl DA, VonHoldt BM. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour.* 2012;4(2):359–61. [CrossRef].
42. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and clustal X version 2.0. *Bioinformatics.* 2007;23(21):2947–8. [CrossRef].
43. Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics.* 2009;25(11):1451–2. [CrossRef].
44. Varshney RK, Graner A, Sorrells ME. Genic microsatellite markers in plants: Features and applications. *Trends Biotechnol.* 2005;23(1):48–55. [CrossRef].
45. Pazy B, Zohary D. The process of introgression between *AEGILOPS* polyploids: Natural hybridization between *A. variabilis*, *a. OVATA*, and *A. BIUNCIALIS*. *Evolution.* 1965;19(3):385–94. [CrossRef].
46. Zohary D, Feldman M. Hybridization between amphidiploids and the evolution of polyploids in the wheat (*Aegilops-Triticum*) group. *Evolution.* 1962;16(1):44–61. [CrossRef].
47. Yang CX, Liu SY, Zerega NJC, Stull GW, Gardner EM, Tian Q, et al. Phylogeny and biogeography of *Morus* (Moraceae). *Agronomy.* 2023;13(8):2021. [CrossRef].
48. Wang M, Zhu M, Qian J, Yang Z, Shang F, Egan AN, et al. Phylogenomics of mulberries (*Morus*, Moraceae) inferred from plastomes and single copy nuclear genes. *Mol Phylogenet Evol.* 2024;197:108093. [CrossRef].
49. Burgess KS, Husband BC. Maternal and paternal contributions to the fitness of hybrids between red and white mulberry (*Morus*, Moraceae). *Am J Bot.* 2004;91(11):1802–8. [CrossRef].

50. Müller E, Eidesen PB, Ehrich D, Alsos IG. Frequency of local, regional, and long-distance dispersal of diploid and tetraploid *Saxifraga oppositifolia* (Saxifragaceae) to Arctic glacier forelands. *Am J Bot.* 2012;99(3):459–71. [[CrossRef](#)].
51. Curtis SE, Clegg MT. Molecular evolution of chloroplast DNA sequences. *Mol Biol Evol.* 1984;1(4):291–301. [[CrossRef](#)].
52. Palmer JD. Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am Nat.* 1987;130:S6–29. [[CrossRef](#)].
53. Adhikari B, Parajuli S, Nepal MP. Reporting complete chloroplast genome of endangered red mulberry, useful for understanding hybridization and phylogenetic relationships. *Sci Rep.* 2025;15(1):13403. [[CrossRef](#)].
54. De Kort H, Prunier JG, Ducatez S, Honnay O, Baguette M, Stevens VM, et al. Life history, climate and biogeography interactively affect worldwide genetic diversity of plant and animal populations. *Nat Commun.* 2021;12(1):516. [[CrossRef](#)].
55. Levin DA, Francisco-Ortega J, Jansen RK. Hybridization and the extinction of rare plant species. *Conserv Biol.* 1996;10(1):10–6. [[CrossRef](#)].
56. Huxel GR. Rapid displacement of native species by invasive species: Effects of hybridization. *Biol Conserv.* 1999;89(2):143–52. [[CrossRef](#)].
57. Haygood R, Ives AR, Andow DA. Consequences of recurrent gene flow from crops to wild relatives. *Proc R Soc Lond Ser B Biol Sci.* 2003;270(1527):1879–86. [[CrossRef](#)].
58. Ellstrand NC. Gene flow by pollen: Implications for plant conservation genetics. *Oikos.* 1992;63(1):77. [[CrossRef](#)].
59. Rieseberg LH, Zona S, Abernomb L, Martin TD. Hybridization in the island endemic, *Catalina* mahogany. *Conserv Biol.* 1989;3(1):52–8. [[CrossRef](#)].
60. Ambrose JD, Kirk D. National recovery strategy for red mulberry (*Morus rubra* L.). Guelph, ON, Canada: Ontario Ministry of Natural Resources; 2004.
61. Adhikari B, Ringgenberg E, Smith G, Johnson R, Parajuli S, Carstens JD, et al. Mitochondrial genome of the North American *Morus rubra* L. (red mulberry) reveals plastome gene transfer and essence of RNA editing. *Discover Plants.* 2026;3(1):28. [[CrossRef](#)].