RESEARCH ARTICLE

Development of nuclear microsatellite loci for *Pinus albicaulis* Engelm. (Pinaceae), a conifer of conservation concern

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Abstract

Pinus albicaulis (whitebark pine) is a widely-distributed but rapidly declining high elevation western North American tree and a candidate for listing under the U.S. Endangered Species Act. Our objectives were to develop reliable nuclear microsatellite markers that can be used to assess within-population genetic diversity as well as seed and pollen migration dynamics, and to validate markers using two geographically proximal *P. albicaulis* populations. We identified 1,667 microsatellite-containing sequences from shotgun DNA libraries of *P. albicaulis*. Primer pairs were designed for 308 unique microsatellite-containing loci, and these were evaluated for PCR amplification success and segregation in a panel of diploid needle tissue. DNA was extracted with an SDS protocol, and primers were screened through gel electrophoresis. Microsatellites were genotyped through fluorescent primer fragment analysis. Ten novel and 13 transferred loci were found to be reproducible in analyses based on 20 foliage samples from each of two locations: Henderson Mountain, Custer Gallatin National Forest, Montana, and Mt. Washburn, Yellowstone National Park, Wyoming (USA). Transferred loci had higher numbers of alleles and expected heterozygosities than novel loci, but also revealed evidence for a higher frequency of null alleles. Eight of the 13 transferred loci deviated significantly from Hardy-Weinberg Equilibrium, and showed large positive F_{IS} values that were likely inflated by null alleles. Mantel's tests of transferred and novel markers showed no correlation between genetic and geographic distances within or among the two sampled populations. AMOVA suggests that 91% of genetic variability occurs within populations and 9% between the two populations. Studies assessing genetic diversity using these microsatellite loci can help guide future management and restoration activities for *P. albicaulis*.

Introduction

North American forest trees have been exposed to a number of unprecedented health challenges that are likely to be exacerbated by warming temperatures [1]. These challenges include outbreaks of native insects and especially bark beetles [2], mortality of older age classes from drought [3], and the accidental introduction and spread of destructive exotic pests and pathogens (e.g., [4]). In fire-prone communities in the western United States, intervals between fires are projected to decline over the next century, as climate warms [5, 6], and this may lead to metapopulation extirpation and the extinction of some woody plant species [7, 8]. As populations regenerate following disturbance, the recovery of community structure and genetic diversity is extremely important [9, 10].

Pinus albicaulis Engelm. (Pinaceae, whitebark pine) is a widely distributed western North American tree species of subalpine and treeline elevations ranging from about 37˚ to 55˚ N latitude. It functions as an ecological keystone and foundation species in these high elevation communities [11, 12]. The species, however, has experienced range-wide mortality due to insect outbreaks, pathogens, and climate change; consequently, it is a candidate for listing under the U.S. Endangered Species Act [13]. *Pinus albicaulis* is a slow-growing species that does not produce large cone crops until at least 60 years of age [14]. It tolerates cold, dry, and windy environments, forming self-replacing stands on harsh sites. On productive sites, *P. albicaulis* is an early seral species in successional communities, which depend on disturbance, primarily fire, for renewal (e.g., [15]). It relies on the Clark's nutcracker (*Nucifraga columbiana* Wilson) for seed dispersal, which influences its ecology and population biology [16–19]. *Pinus albicaulis* regenerates rapidly from seed caching in burned terrain by nutcrackers following fires, with new seedlings establishing within a few years [20–22]. At treeline elevations on arid sites, it is often dominant and a tree island initiator, facilitating infilling and upward expansion of the alpine treeline ecotone [23–25]. *Pinus albicaulis* treeline communities redistribute and retain snow, regulating downstream flows in summer. In addition, the large seeds comprise an important wildlife food source for many granivorous birds besides Clark's nutcrackers, as well as small mammals and bears [26].

Pinus albicaulis is a host for mountain pine beetles (*Dendroctonus ponderosae* Hopkins), which have erupted in large-scale outbreaks in the Rocky Mountains and elsewhere in the West over the past 20 years. It is also susceptible to white pine blister rust, which is caused by the introduced pathogen *Cronartium ribicola* J.C. Fisch. and continues to spread geographically [22, 27, 28]. Both tree mortality from mountain pine beetles and mortality and canopy damage from blister rust progressively reduce the number of cone-producing individuals, depleting genetic diversity for future generations [2]. Blister rust also infects and kills young trees, thereby reducing regeneration [29]. In *P. albicaulis*, some evidence suggests that heterozygotes are more tolerant of blister rust [30]. However, with declines in cone production and high mortality, heavily impacted *P. albicaulis* stands may be experiencing various degrees of inbreeding or pollen limitation. Given decades of decline, *P. albicaulis* has become a species of management and conservation concern for federal agencies in the U.S. and Canada [27, 31, 32]. Developing cost-efficient methods to identify the magnitude and apportionment of genetic diversity in *P. albicaulis* populations is a high priority, since this can expedite conservation actions. These actions potentially include tracking parentage to search for pest and pathogen resistance, assessment of local genetic diversity to highlight populations in need of additional protection, restoration of post-fire genetic diversity to pre-burn levels, and to inform decisions on seed collections (e.g., [33, 34]).

Nuclear microsatellites (nSSRs) have been a key tool for estimating genetic diversity in species of conservation concern. They have been used to differentiate among populations (pequi,

Caryocar brasiliense Camb.) and monitor hybridization between rare and widespread taxa (Colorado hookless cactus, *Sclerocactus glaucus* [K. Schumann] L.D. Benson) [35, 36]. They have also been used successfully to describe genetic diversity in threatened conifers [37–41]. Genetic diversity in *P. albicaulis* has been previously estimated using genetic markers other than nSSRs, including allozymes [42, 43], organellar simple sequence repeats [34, 44], and single nucleotide polymorphisms (SNPs) [45–47]. However, nSSRs are more accurate and efficient for studies focusing on individual-level differences, including research on sibling reconstruction, linkage disequilibrium, genetic structure, parentage, and individual identification [48]. Thus, nuclear microsatellites should be a useful tool to characterize population genetic diversity and structure in *P. albicaulis* and to monitor spatial and temporal changes in genetic composition within stands and across populations. Despite the utility of nSSRs for surveys of genetic diversity in species of conservation concern, nuclear microsatellites have not yet been successfully developed for *P. albicaulis*. The species has a large, highly repetitive genome that has confounded efforts to develop single locus polymorphic microsatellites [49]. In addition, microsatellite transfer among pine species has been inconsistent [50].

To facilitate finding useful nSSR markers, many investigators look to near-relatives, such as congeneric taxa including sister species for potential transfer [40, 51–53]. *Pinus albicaulis* is one of 21 pines in section *Quinquefoliae* subsection *Strobus* [54, 55], which in the most recent classification includes the stone pines of former subsection *Cembrae* [56, 57]. Divergence dates for subsection *Strobus* (based on nuclear molecular clock estimates; [58]) are estimated at 11–20 mya. The most recently published phylogenies for *Pinus* subgenus *Strobus* constructed from nuclear, mitochondrial, and chloroplast gene sequences variously resolve *P. albicaulis* with species of subgenus *Strobus* native to North America, Asia, and Europe [59].

Here, our first and primary objective was to develop reliable nSSR markers in *P. albicaulis* in order to provide an efficient, informative tool to monitor genetic diversity of populations in light of current and future threats. Our secondary objective was to determine the utility of these nSSR markers by comparing genetic diversity in two populations previously studied in the Greater Yellowstone Ecosystem [43]. To maximize the number of nSSRs, we combined screening of previously identified loci from related pine species for potential transferability with surveying *P. albicaulis* shotgun nuclear genomic sequences for taxon-specific SSR sequences.

Materials and methods

To develop novel microsatellite loci for *P. albicaulis*, we identified di- and tri-nucleotide repeats using the procedure outlined in Jennings et al. [60], a strategy that was designed to identify very short (< 80bp) paired-end sequence data from early-generation Illumina sequencers. Genomic DNA from diploid needle tissue from a single tree (Custer Gallatin National Forest, Montana; 45.443˚N, -110.005˚W, 2,470 m a.s.l.) was used to make a standard Illumina library containing internal 'barcoding' adapters (barcode 5'-CACT; [61]). This library was pooled with 15 additional barcoded libraries from other conifer species, and five pmol were sequenced on an Illumina Genome Analyzer II using 80 bp paired end reads (University of Oregon Genomics Core Facility, Eugene, OR, USA). Libraries were sorted by barcode using a custom Perl script (https://github.com/listonlab/utility-scripts/blob/master/bcsort_v5.pl). For this strategy, paired microreads are concatenated and separated by 50 N's (e.g., forward read + 50 N's + reverse complement reverse read), and identical to nearly-identical sequences (> 95% identity) are filtered to a single representative sequence using cd-hit-454 (Niu et al. 2010). This allowed us to search for dinucleotide (>5 repeats) and trinucleotide (>4 repeats) motifs in the Read 1 or Read 2 sequences using SSR_pipeline [62], and to design flanking

amplification primers in the Read 1 and Read 2 sequences using BatchPrimer3 [63] and synthesized at Integrated DNA Technologies (IDT, Coralville, IA, USA). We used default settings for BatchPrimer3, with the exception of the following parameters: product length (min = 100 bp, max = 200 bp), primer length (min = 17 bp, max = 25 bp, optimum = 19 bp), melting temperature (min = 48˚C, max = 63˚C, optimum = 54˚C); number of primers per sequence = 1. The original sequencing reads are deposited at the NCBI Short Read Archive under accession SRR7944190. Primers for 49 nSSR loci were screened for transferability to *P. albicaulis*, including 14 loci from *P. cembra* L. (Swiss stone pine) [64, 65], three loci from *P. parviflora* (Japanese white pine) [66], 13 loci from *P. koraiensis* Siebold & Zucc. (Korean pine) [67], and 19 loci from *P. strobus* L. (eastern white pine) [68] (S1 Table).

DNA was extracted from silica-dried needles using an SDS-based protocol optimized for *Pinus* species, modified with 1μl β-mercaptoethanol per 404μl of grinding buffer [69]. DNA was eluted in 130μl TE buffer, and concentrations were determined using a NanoDrop 2000 microspectrophotometer (Thermo Scientific, Wilmington, DE, USA). Microsatellite screening used 10μl polymerase chain reaction (PCR) mixtures containing 1X GoTaq G2 Master Mix (Promega, Madison, WI, USA), BSA (1 μM), mixed forward and reverse primers (2.5 μM), and 10ng DNA. Amplifications for novel primers were performed in an Eppendorf vapo.protect Mastercycler proS using the following cycling conditions: denaturation for 5 min. at 95˚C, 35 cycles of 45 s each at 95˚C, 45 s at 58˚C, and 15 s at 72˚C, followed by a final annealing cycle of 72˚C for 5 min. Transferred primers were amplified using the conditions described in the literature [64–68]. PCR products were visualized on 2% agarose gel using GelRed (Biotium, Fremont, CA, USA) and a low molecular weight DNA ladder (New England BioLabs, Ipswich, MA, USA).

Fluorescent primers were designed with 5' tags [70]. Microsatellite locus amplifications for fragment analysis were created using the Fluorescent Tag Microsatellite PCR Protocol, with minor modifications. Reactions consisted of 10.5μl mixtures containing 1X GoTaq G2 Colorless Master Mix (Promega, #M7832), BSA (1 μM), non-tagged primer (5 μM), tagged primer (0.5 μM), fluorescent tag (5 μM; VIC, PET, or FAM), and 10ng DNA using the above conditions. PCR products were mixed in multiplexes of three and sent to the DNA Laboratory at Arizona State University for fragment analysis (School of Life Sciences, Tempe, AZ, USA). Fragment analysis outputs were visualized and genotyped using *GeneMarker* V2.6.2 (SoftGenetics).

In association with an on-going ecological study, and to compare with past population genetic research in this region, two populations were selected from the Greater Yellowstone Ecosystem for evaluation; Henderson Mountain (Custer Gallatin National Forest, MT, USA; letter approving sample collection) and Mt. Washburn (Yellowstone National Park, WY, USA; research permit YELL-2016-SCI-6064), which are separated by 50 km (Fig 1) [11]. Overstory composition in these two subalpine stands consists of cone-bearing *P. albicaulis*, *P. contorta* Douglas, *Picea engelmannii* Parry ex Engelm., and *Abies lasiocarpa* (Hooker) Nuttall. The two stands are 0.5–5.0 km from post-fire study areas established in the Greater Yellowstone Ecosystem in 1990, so the selected stands represent putative seed sources for areas regenerating following the 1988 Yellowstone fires. *Pinus albicaulis* from Mt. Washburn was collected within a 13.3 ha (0.13 km^2) area ranging from 2,810–3,005 m elevation in north-central Yellowstone National Park, on north and west-facing slopes, 7–9 km north of Canyon Junction. Samples from Henderson Mountain were collected within a 15.7 ha (0.16 km^2) area ranging from 2,690–2,785 m elevation on south and east-facing slopes, 3 km northeast of Cooke City, MT, and 5–7 km northeast of Yellowstone Park. For both populations, 20 individuals nearest to randomly generated points were sampled, with five fascicles collected from each individual
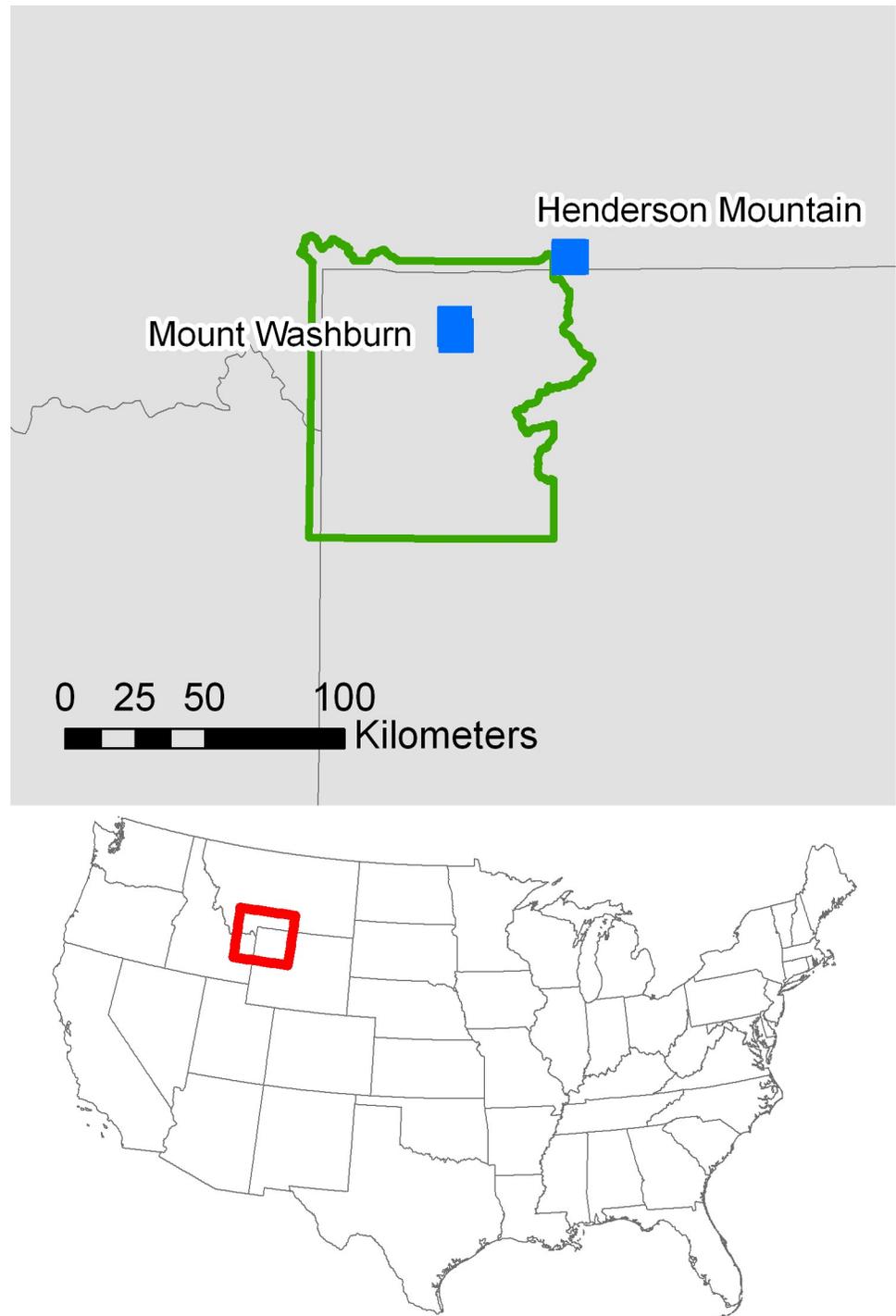
Fig 1. Map of sample collection locations. Top: Sample collection locations for *Pinus albicaulis* Engelm. (Pinaceae). Henderson Mountain, Custer Gallatin National Forest, MT and Mt. Washburn, Yellowstone National Park, WY. Bottom: Geographic location of Yellowstone National Park, Central Rocky Mountains.

and silica-dried for extended storage. Tree diameter was measured at breast height (DBH; S2 Table).

Eight individuals from Henderson Mountain were screened for initial locus testing, and 20 individuals from each population were used for final population genetic assessment. Due to minor amplification failure, a total of 26 individuals from Henderson Mountain and 22 individuals from Mt. Washburn were sampled to bring the total sample size up to 20 individuals for each of the 23 loci selected from the initial screening. Standard genetic diversity measures, Hardy-Weinberg Equilibrium, and inbreeding coefficients were calculated using *GenAlEx* 6.5 [71, 72]. The occurrence and frequency of null alleles was estimated using ML-NullFreq with 10,000 permutations [73]. Histograms of average number of alleles per locus were created in *R* [74] to visualize differences between populations. Population differences in the mean number of alleles per locus and expected heterozygosity are defined as the difference between Mt. Washburn and Henderson Mountain. Differences in means were plotted in *R*, along with 95% confidence intervals. Partitioning of population genetic variation was also evaluated by analysis of molecular variance (AMOVA) using Nei's genetic distances and 9,999 permutations (*GenAlEx* 6.5). Isolation-by-distance within and between populations was tested using Mantel's correlation with 9,999 permutations in *GenAlEx* 6.5 [71, 72, 75].

Results

Of the sequences screened, we identified 1,667 (1,341 di- and 326 tri-nucleotide) microsatellite-containing sequences from shotgun DNA libraries of *P. albicaulis*. Primer pairs were designed for 308 unique microsatellite-containing loci, and these were evaluated for PCR amplification success and segregation in a panel of diploid needle tissue. Of these markers, ten novel *P. albicaulis*-derived loci amplified consistently and cleanly and were polymorphic in the two screened populations (Tables 1 and 2). The total number of alleles (N_A) for these novel loci ranged from 2–9 in the combined populations, with an average of 3.3 alleles per locus (Table 2). Observed heterozygosity (H_O) ranged from 0.02–0.50 ($\bar{x} = 0.18$) and expected heterozygosity (H_E) ranged from 0.02–0.40 ($\bar{x} = 0.18$). All loci conformed with Hardy–Weinberg Equilibrium (HWE) (Table 2).

Although transferability of nSSR loci to the *P. albicaulis* genome transfer was highly variable depending on the source species, 13 of the 42 loci (27% overall) amplified successfully, yielding interpretable results (Tables 2 and 3). Sequencing revealed these nSSRs to be the expected basepair motifs, though they tended to have fewer repeats than listed for the source species. Loci developed for *P. koraiensis* had the highest rate of transferability, with eight of 13 (62%) tested loci amplifying in *P. albicaulis*, followed by *P. parviflora* with one of three (33%), and *P. strobus* with three of 19 (16%). Loci developed for *P. cembra* had the lowest rate of transferability with only one successful locus from a pool of 14 loci (7%). The average number of alleles per locus varied from 5–8 for the transferred microsatellites (Table 3). For transferred loci, N_A ranged from 3–15 ($\bar{x} = 7.2$), H_O ranged from 0.18–0.56 ($\bar{x} = 0.39$), and H_E ranged from 0.22–0.89 ($\bar{x} = 0.60$). Eight loci deviated from HWE (Table 2).

For our survey populations, the global range of alleles per locus was similar, but Henderson Mountain generally had more genetic diversity than Mt. Washburn (Fig 2, S1 Fig). Henderson Mountain had a higher average number of alleles, ranging from 2–9 ($\bar{x} = 4.6$), with an average of 1.30 private alleles per locus. H_O ranged from 0.04–0.56 ($\bar{x} = 0.29$), and H_E ranged from 0.04–0.84 ($\bar{x} = 0.43$) (Table 2). N_A for Mt. Washburn ranged from 1–12 ($\bar{x} = 4.1$), with an average of 0.91 private alleles per locus. H_O ranged from 0–0.65 ($\bar{x} = 0.32$), and H_E ranged from 0–0.90 ($\bar{x} = 0.41$) (Table 4). Eight loci deviated from Hardy–Weinberg equilibrium in both populations (Table 2). Null allele frequency estimates for transferred loci ranged from 0–0.51 at Henderson Mountain, and 0–0.30 at Mt. Washburn (Table 2).

Table 1. Descriptive information for the 10 microsatellite loci isolated from *Pinus albicaulis*.

Locus name	Primer sequence (5'–3')	Repeat motif	Allele size (bp)
ALBI_B08	F: CGTTACAGTAATTGGACG	$(CG)_n$	259–271
	R: GTTTGTAGCAAACAGTAC		
ALBI_063	F: TCTCTTCGAGATTTAAACCCA	$(AT)_n$	197–201
	R: ACCGTTCAGAATTAGACCAT		
ALBI_069	F: GATCTCCGACCGTAATGTGT	$(AG)_n$	195–197
	R: AGTGCGACCTTACAAAGAGG		
ALBI_112	F: ATTTCCCACCATCCCTAATG	$(TC)_n$	284–325
	R: GCGGACATACGGATCAAGT		
ALBI_113	F: ATGAGGACCAGGAGTAAAT	$(AG)_n$	273–276
	R: GCTATTGTGTAAGCCTGCGT		
ALBI_116	F: AGAACATGAAAGGCCTAGGAA	$(AT)_n$	157–159
	R: GGATCCTCTGGCCAAGTTAG		
ALBI_149	F: TAGGATCCCTTCTTTTTGGG	$(AC)_n$	199–226
	R: TTATGTCTTGGCTTGGCAGT		
ALBI_151	F: GAACTTCTGAGGAAGCTTGATG	$(AG)_n$	167–197
	R: TTCTGATCAATCTAAAGGTCAATCT		
ALBI_160	F: TGTTATTGCTAACGGAGATGG	$(TG)_n$	229–233
	R: TTTTCTATTGTAGACAGTGTCGTCTT		
ALBI_171	F: ACAAAGCCACGAACAATCA	$(CT)_n$	267–271
	R: GCCAATTGTTTGTTGGCTCTTTTAAC		

Locus name, primer sequences, repeat motif, and allele size range for the 10 microsatellite loci isolated for *Pinus albicaulis* Engelm. (Pinaceae). Annealing temperature is 58°C for all loci.

Across all markers, the overall inbreeding coefficient (F_{IS}) was 0.32 for Henderson Mountain, and 0.24 for Mt. Washburn. However, loci developed specifically for *P. albicaulis* showed a much lower inbreeding coefficient (0.007) than those transferred from other species (0.291) (Table 4); this difference is likely a consequence of the higher number of null alleles in the transferred SSR markers. An AMOVA identified 91% of variance within populations, and 9% among populations. The spatial distance between samples at Henderson Mountain was larger (range = 8–538 m, $\bar{x}$ = 231) than at Mt. Washburn (range = 7–348 m, $\bar{x}$ = 173) (S3 Table). Mantel's test within populations showed no correlation between genetic and geographic distances at Henderson Mountain ($r_{x,y}$ = -0.120, p = 0.121), indicating that individuals are no more closely related than expected with a random spatial pattern (S2 and S3 Figs). A weakly positive correlation at Mt. Washburn ($r_{x,y}$ = 0.162, p = 0.051) indicated that individuals may be more closely related genetically to nearby individuals than expected under a random spatial pattern (S2 and S3 Figs). Between the two populations, there was no spatial autocorrelation ($r_{x,y}$ = 0.029, p = 0.149) (S3 Table).

Discussion

Our primary objective—the development of reliable nSSR markers useful for assessing genetic diversity in populations of *P. albicaulis*—was accomplished with the identification of ten novel and 13 transferred microsatellite loci. Of the 308 novel microsatellites identified from shotgun sequencing of *P. albicaulis*, only ten loci were sufficiently polymorphic and reliable to be identified for marker development, corresponding to a success rate of ~3%. This is approximately one-fifth the success rate of SSRs developed in *Chamaecyparis lawsoniana* (A. Murray) Parl.

Table 2. Microsatellite loci, source species, and application to two populations of *Pinus albicaulis* in the Greater Yellowstone Ecosystem.

Locus name	Source species	N_a			Henderson Mountain						Mt. Washburn					
			H_o	H_e	N_A	A_P	A_N	H_O	H_E	HWE	N_A	A_P	A_N	H_O	H_E	HWE
ALBI_B08	*P. albicaulis*	2	0.025	0.025	2	1	0.0	0.045	0.044	ns	1	0	0.3	0.000	0.000	-
ALBI_063	*P. albicaulis*	3	0.500	0.404	3	1	0.0	0.455	0.361	ns	2	0	0.0	0.550	0.439	ns
ALBI_069	*P. albicaulis*	3	0.075	0.073	3	2	0.0	0.150	0.141	ns	1	0	0.0	0.000	0.000	-
ALBI_112	*P. albicaulis*	3	0.132	0.169	2	0	0.0	0.105	0.100	ns	3	1	0.2	0.158	0.234	ns
ALBI_113	*P. albicaulis*	3	0.231	0.207	3	1	0.0	0.143	0.135	ns	2	0	0.0	0.333	0.278	ns
ALBI_116	*P. albicaulis*	2	0.024	0.024	2	1	0.0	0.048	0.046	ns	1	0	0.0	0.000	0.000	-
ALBI_149	*P. albicaulis*	9	0.370	0.360	6	2	0.0	0.346	0.308	ns	7	3	0.0	0.400	0.423	ns
ALBI_151	*P. albicaulis*	2	0.023	0.023	2	1	0.0	0.043	0.043	ns	1	0	0.0	0.000	0.000	-
ALBI_160	*P. albicaulis*	3	0.319	0.354	3	0	0.0	0.407	0.381	ns	3	0	0.1	0.200	0.304	ns
ALBI_171	*P. albicaulis*	3	0.150	0.224	3	0	0.3	0.150	0.296	ns	3	0	0.0	0.150	0.141	ns
Mean			**3.3**	**0.184**	**0.184**	**2.9**	**0.9**	**0.00**	**0.189**	**0.185**		**2.4**	**0.4**	**0.06**	**0.179**	**0.182**
PcHJM	*P. cembra*	5	0.390	0.468	4	1	0.1	0.381	0.481	ns	4	1	0.0	0.400	0.446	ns
RPS119	*P. strobus*	7	0.375	0.774	6	1	0.4	0.350	0.794	***	6	1	0.2	0.400	0.710	ns
RPS124	*P. strobus*	4	0.184	0.218	3	0	0.3	0.111	0.202	ns	4	1	0.0	0.250	0.229	ns
RPS127	*P. strobus*	9	0.275	0.804	9	3	0.5	0.238	0.814	***	6	0	0.3	0.316	0.737	***
P5	*P. koraiensis*	7	0.289	0.771	6	2	0.4	0.300	0.743	***	5	1	0.2	0.350	0.745	***
P29	*P. koraiensis*	4	0.450	0.363	3	1	0.0	0.450	0.366	ns	3	1	0.0	0.450	0.359	ns
P37	*P. koraiensis*	7	0.475	0.697	5	1	0.3	0.350	0.690	***	6	2	0.1	0.600	0.699	**
P38	*P. koraiensis*	11	0.353	0.470	8	5	0.2	0.300	0.428	ns	5	2	0.1	0.200	0.271	*
P45	*P. koraiensis*	15	0.563	0.893	13	3	0.2	0.571	0.892	*	12	2	0.2	0.579	0.892	ns
P52	*P. koraiensis*	5	0.512	0.647	4	1	0.2	0.381	0.602	ns	4	1	0.0	0.650	0.681	ns
P62	*P. koraiensis*	3	0.487	0.489	3	1	0.0	0.474	0.517	ns	2	0	0.0	0.500	0.455	ns
P63	*P. koraiensis*	8	0.375	0.714	8	2	0.4	0.350	0.739	***	6	0	0.2	0.400	0.666	**
PisATG0012	*P. parviflora*	8	0.400	0.659	6	2	0.1	0.500	0.648	ns	6	2	0.2	0.300	0.648	**
Mean			**7.2**	**0.394**	**0.602**	**6.0**	**1.8**	**0.25**	**0.369**	**0.614**		**5.3**	**1.1**	**0.12**	**0.420**	**0.591**

Source species for primer development, and genetic diversity for 10 microsatellite loci isolated for *Pinus albicaulis* Engelm. (Pinaceae) and 13 microsatellite loci transferred, based on 20 individuals from each population. (Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park, WY.)

N_a = total number of alleles; H_o = observed heterozygosity, total sample; H_e = expected heterozygosity, total sample; N_A = number of alleles; A_P = private alleles; A_N = null allele frequency; H_O = observed heterozygosity; H_E = expected heterozygosity; HWE = Hardy-Weinberg Equilibrium (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$)

https://doi.org/10.1371/journal.pone.0205423.t002

using these same methods [60]. This conversion rate is extremely low for species of genus *Pinus*, which have already been characterized by low conversion rates, attributed to the

Table 3. Results of microsatellite locus transferability to *Pinus albicaulis* for primers developed in other *Pinus* species.

Source species	Loci tested	Loci transferred	Success (%)	Average N_a
P. cembra	14	1	7.1	5.0
P. koraiensis	13	8	61.5	7.5
P. strobus	19	3	15.8	6.7
P. parviflora	3	1	33.3	8.0

Number of loci tested per species, number that amplified in *Pinus albicaulis* Engelm. (Pinaceae), percent of total loci tested that transferred successfully, and average number of alleles (N_a) per locus. Primers were screened in two populations (Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park, WY).

https://doi.org/10.1371/journal.pone.0205423.t003
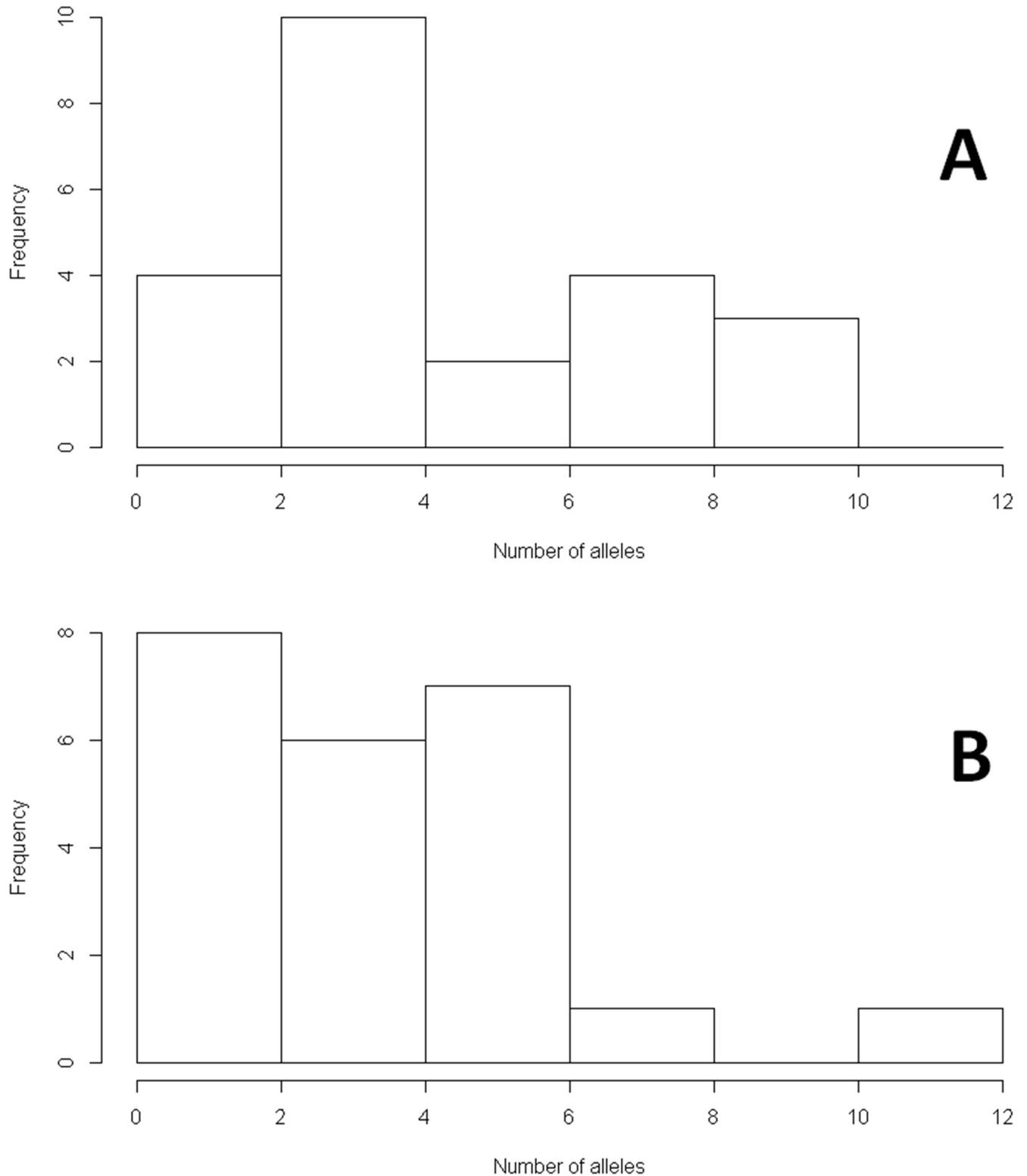
Fig 2. Number of alleles per locus in two *Pinus albicaulis* populations. Histograms of the count frequency per locus for number of alleles for all of 23 microsatellite loci for two populations of *Pinus albicaulis* Engelm. (Pinaceae) **A)** Henderson Mountain, Custer Gallatin National Forest, MT, and **B)** Mount Washburn, Yellowstone National Park, WY.

Table 4. Genetic variability at microsatellite loci in two *Pinus albicaulis* populations.

	Average N_A	Average A_P	Average H_O	Average H_E	F_{IS}
Novel	3.30		0.184	0.184	0.007
Transferred	7.15		0.394	0.602	0.291
Henderson	4.57	1.30	0.291	0.428	0.320
Washburn	4.09	0.91	0.315	0.413	0.236
Overall	4.33	1.11	0.303	0.427	0.168

Average number of alleles (N_A), private alleles (A_P), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) by SSR development (novel developed for *Pinus albicaulis* Engelm. [Pinaceae], transferred developed in other pines) and population (Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park, WY).

https://doi.org/10.1371/journal.pone.0205423.t004

complexity of their genomes (> 20Gbp) and the presence of large numbers of paralogous SSR families [76], [77], [78]. The low yield is likely due to the selection of shorter motifs, which are known to show lower mutation rates than long microsatellite repeats [79]. The success rate of SSR marker transfer from other white pines to *P. albicaulis* was 13 out of 49—almost 27%. The average number of alleles per locus of the transferred microsatellites (7.2) is more than twice that of the novel microsatellites (3.2), suggesting that the transferred loci may be useful in studies requiring high individual identification power, or detecting differences in spatial differentiation. For studies sensitive to the presence of null alleles and error in heterozygote identification, the markers derived from *P. albicaulis* are likely to perform better, albeit at the cost of variability. Notably, transferability of loci from the two east Asian species (*P. koraiensis* and *P. parviflora*) was substantially higher than for the European (*P. cembra*) and North American (*P. strobus*) species tested. This study highlights the opportunity for screening newly published novel microsatellites from closely related species in *P. albicaulis*, such as recently developed for *P. sibirica* [80]. While these were not included in the present study, they also have a high likelihood of transferability, due to the close genetic affinity between *P. albicaulis* and *P. sibirica*.

Our secondary objective was to provide a case example of the utility of these microsatellites for population genetic research by comparing genetic diversity in two populations of *P. albicaulis* previously studied using allozyme analysis [43]. Despite the relatively small geographic distance between these sites (50 km), the nSSR markers revealed that both Henderson Mountain and Mt. Washburn had private alleles, averaging 1.3 and 0.9 per locus, respectively. This is likely a result of the small sample sizes (20 individuals per site) used in this study, though such sample sizes should yield adequate estimates [81]. Compared to other tools for measuring genetic diversity in *P. albicaulis*—including allozymes [43] and SNPs [47]—the microsatellite loci described here have a higher mean number of alleles per locus, as is expected with microsatellite markers. For example, with allozyme analysis these sites showed 1.6–1.7 alleles per polymorphic locus [43]. The surveyed SSRs revealed around three times the number of alleles per polymorphic locus, with 4.1–4.6 (Table 3).

All deviations from HWE are due to an excess of homozygous genotypes (not heterozygotes) and are most likely the result of allelic dropout (null alleles). All loci that violate HWE are transferred loci, which may indicate imperfect conservation of primer binding sites in *P. albicaulis*, or a presence/absence polymorphism in some chromosomal lineages. In these instances, null alleles fail to amplify, leading to decreased observed heterozygosity and apparent reaction failure in null homozygous individuals [82]. The potential impact of null alleles may be revealed from our F_{IS} values, which are large and positive for both populations, leading to potentially erroneous inferences of moderate inbreeding. Six loci—RPS119, RPS127, P5,

P45, P63, and PisATG0012—were estimated to have high null allele frequencies in both populations. Loci that diverge from HWE and show evidence of null alleles should be used with caution as they inflate homozygosity and thus may not be applicable for all analyses (Table 2). Looking at only the novel loci developed for *P. albicaulis*, the inbreeding coefficient is very small, conforming to expectations from past research indicating random mating [43]. For studies sensitive to the presence of null alleles and error in heterozygote identification, the markers derived from *P. albicaulis* are less informative but may yield more accurate estimates.

Mantel's test revealed low genetic differentiation among populations and high gene flow, with no correlation between geographic distance and genetic relatedness among populations. Within populations, Mantel's correlation shows no spatial autocorrelation at Henderson Mountain, with individuals no more or less closely related genetically to those nearby geographically than expected at random. There is weak evidence for isolation by distance in the Mt. Washburn population, with a positive Mantel correlation indicating that genetic distance between individuals increases with increasing physical distances. However, the AMOVA, which revealed that 9% of genetic differentiation occurred between populations, indicates greater population differentiation compared to SNP findings [47]. A larger sample of trees across varying distances will be required to determine the geographic distances required for isolation-by-distances and population differentiation.

Our results with *P. albicaulis* reveal that locus transferability is a viable option for developing microsatellites in five-needle pines, with the caveat that null alleles may show high frequencies in some interspecific comparisons. Other efforts in cross-species amplification in *Pinus*, however, have had mixed results, with some cases appearing successful [50, 83, 84], and others showing far lower success rates than novel marker development [67, 85]. It may be the case that the loci we tested for transfer were located at stable sites in the *P. albicaulis* genome (e.g., near centromeres, regions of low recombination, or in linkage with conserved genes).

These 23 nSSR markers provide a useful low-cost method to survey *P. albicaulis* genetic diversity, population structure, and parentage. Employing high-level PCR multiplexing [48] would further increase screening efficiency. Previous molecular studies have used isozymes and chloroplast DNA microsatellites to support the division of *P. albicaulis* into five "seed zones" in the Inland West [34]. Since molecular markers primarily reflect neutral processes (migration; drift), and the extent of differentiation of neutral markers is usually far lower than differentiation in adaptive traits [34], this approach by itself may be of limited value for developing seed zones that ensure seedlings are well-adapted to growing conditions at specific planting sites. Nevertheless, neutral markers like these SSRs offer a relatively rapid screen that can be used to partition genetic variation into genealogical groups, without the major expense of establishing and measuring multiple test plantations over multiple environments (e.g., [86]). These genealogical groups can be used to prioritize populations for blister rust resistance screening in *P. albicaulis*, and for the exploration of climate change mitigation strategies (e.g., [87, 88]). Since SSRs typically reveal neutral differentiation, these markers can also provide a baseline for identifying candidate SNPs that are associated with adaptive traits, such as cold tolerance [89], aridity traits, and wood property traits [90]. The identification of F_{ST} outliers depends upon accurate estimation of background differentiation and ancestry, and microsatellite markers are often the ideal choice for these estimates [91].

It is imperative to develop a predictive model for how much genetic diversity will remain in *P. albicaulis* populations as they decline in response to white pine blister rust infection and mountain pine beetle outbreaks, particularly with respect to those trees remaining as seed sources after disturbances. The time needed to recover genetic diversity following stand-replacing disturbances will be an essential research question as well, especially as time intervals between fires decrease as a result of climate change. The Greater Yellowstone Ecosystem in

particular is projected to experience reduced intervals between wildfires, decreasing from the historical average of 100–500 years to fewer than 30 years by 2099 [5]. Given this trend, we will need to predict whether *P. albicaulis* will be able to recover genetic diversity rapidly enough to avoid bottlenecking.

Given the imperative conservation goals for *P. albicaulis*, SSR markers offer a complement to SNPs as a multi-allelic marker that can be used to measure the diversity—and changes in diversity—of rare genetic variation. Studies assessing genetic diversity based on these neutral SSR loci, and a more comprehensive sample of potentially-adaptive diversity (e.g., [45, 46]), can help guide future conservation efforts and restoration plans for *P. albicaulis*.

Supporting information

S1 Fig. Difference in number of alleles and heterozygosity between two *Pinus albicaulis* populations. A) difference in mean number of alleles (-0.74) and 95% confidence interval of the difference (-2.29 to 0.81) and **B)** difference in mean expected heterozygosity (-0.015) and 95% confidence interval of the difference (-0.178 to 0.148) of 23 microsatellite loci for two populations of *Pinus albicaulis* Engelm. (Pinaceae): Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park, WY. Plotted points are the difference in means, Washburn minus Henderson, and the lines are the 95% confidence intervals around those points.
(TIF)

S2 Fig. Mantel's test frequency distributions in two *Pinus* albicaulis populations. Frequency distribution of random R_{xy} versus the observed R_{xy} (red line) for 9999 permutations, from Mantel's test on two *Pinus albicaulis* Engelm. (Pinaceae) populations (**A**. Henderson Mountain, Custer Gallatin National Forest, MT, and **B**. Mount Washburn, Yellowstone National Park, WY). R_{xy} is the correlation between geographic and genetic distance between individuals. A positive value indicates individuals are more closely related genetically to those nearby geographically than random, and a negative value indicating individuals are less closely related to those nearby.
(TIF)

S3 Fig. Correlation between geographic distance and genetic distance in two *Pinus albicaulis* populations. Correlation between geographic distance and genetic distance, from Mantel's test on two *Pinus albicaulis* Engelm. (Pinaceae) populations. **A)** Henderson Mountain, Custer Gallatin National Forest, MT showing no correlation between genetic and geographic distances ($r_{x,y}$ = -0.120, p = 0.121) and **B)** Mount Washburn, Yellowstone National Park, WY showing a positive correlation ($r_{x,y}$ = 0.162, p = 0.051).
(TIF)

S1 Table. Descriptive information for screened primers of microsatellites developed for other species of *Pinus*. Locus name, source species of primer development, and allele size range for 49 primers screened for transferability to *Pinus albicaulis* Engelm. (Pinaceae). Bold entries successfully transferred.
(DOCX)

S2 Table. Diameter at breast height of two sampled *Pinus albicaulis* populations. Minimum, maximum, and mean diameter at breast height (DBH) in centimeters compared between *Pinus albicaulis* Engelm. (Pinaceae) sampled at two populations (Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park,

WY).
(DOCX)

S3 Table. Distances between sampled *Pinus albicaulis* within two populations in the Greater Yellowstone Ecosystem. Minimum, maximum, and mean distance between *Pinus albicaulis* Engelm. (Pinaceae) sampled at two populations (Henderson Mountain, Custer Gallatin National Forest, MT, and Mount Washburn, Yellowstone National Park, WY). R_{xy} and $P_{(rxy-rand\,>\,=\,rxy-data)}$ from Mantel's correlation, showing isolation by distance and spatial autocorrelation, testing for each population and for all individuals combined.
(DOCX)

Acknowledgments

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References

1. Millar CI, Stephenson NL. Temperate forest health in an era of emerging megadisturbance. Science. 2015; 349(6250): 823–826. https://doi.org/10.1126/science.aaa9933 PMID: 26293954

2. Raffa KF, Aukema BH, Bentz BJ, Carroll AL, Hicke JA, Turner MG, et al. Cross-scale drivers of natural disturbances prone to anthropogenic amplification: the dynamics of bark beetle eruptions. Bioscience. 2008; 58(6): 501–517. https://doi.org/10.1641/B580607

3. Van Mantgem PJ, Stephenson NL, Byrne JC, Daniels LD, Franklin JF, Fulé PZ, et al. Widespread increase of tree mortality rates in the western United States. Science. 2009; 323(5913): 521–524. https://doi.org/10.1126/science.1165000 PMID: 19164752

4. Lovett GM, Weiss M, Liebhold AM, Holmes TP, Leung B, Lambert KF, et al. Nonnative forest insects and pathogens in the United States: impacts and policy options. Ecol Appl. 2016; 26(5): 1437–1455. https://doi.org/10.1890/15-1176 PMID: 27755760

5. Westerling AL, Turner MG, Smithwick EAH, Romme WH, Ryan MG. Continued warming could transform Greater Yellowstone fire regimes by mid-21st century. PNAS. 2011; 108(32): 13165–13170. https://doi.org/10.1073/pnas.1110199108 PMID: 21788495

6. Moritz MA, Parisien MA, Batllori E, Krawchuk MA, Van Dorn J, Ganz DJ, et al. Climate change and disruptions to global fire activity. Ecosphere. 2012; 3(6): 49. https://doi.org/10.1890/ES11-00345.1

7. Bowman DMJS Murphy BP, Neyland DLJ Williamson GJ, Prior LD. Abrupt fire regime change may cause landscape-wide loss of mature obligate seeder forests. Global Change Biol. 2014; 20: 1008–1015. https://doi.org/10.1111/gcb.12433 PMID: 24132866

8. Enright NJ, Fontaine JB, Bowman DMJS, Bradstock RA, Williams RJ. Interval squeeze: altered fire regimes and demographic responses interact to threaten woody species persistence as climate changes. Front Ecol Environ. 2015; 13: 265–272. https://doi.org/10.1890/140231

9. Mosseler A, Major JE, Rajora OP. Old-growth red spruce forests as reservoirs of genetic diversity and reproductive fitness. Theor Appl Genet. 2003; 106(5): 931–937. https://doi.org/10.1007/s00122-002-1156-1 PMID: 12647069

10. Rajora OP, Pluhar SA. Genetic diversity impacts of forest fires, forest harvesting, and alternative reforestation practices in black spruce (*Picea mariana*). Theor Appl Genet. 2003; 106(7): 1203–1212. https://doi.org/10.1007/s00122-002-1169-9 PMID: 12748771

11. Tomback DF, Arno SF, Keane RE. The compelling case for management intervention. In: Tomback DF, Arno SF, Keane RE, editors. Whitebark pine communities: ecology and restoration. Washington, DC: Island Press; 2001. pp. 3–25.

12. Ellison AM, Bank MS, Clinton BD, Colburn EA, Elliott K, Ford CR, et al. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Front Ecol Environ. 2005; 3(9): 479–486.

13. U.S. Fish and Wildlife Service. Endangered and threatened wildlife and plants; 12-month finding on a petition to list *Pinus albicaulis* as Endangered or Threatened with critical habitat. Federal Register. 2011; 76: 42631–42654.

14. Arno SF, Hoff RJ. *Pinus albicaulis* Engelm. whitebark pine. In: Burns RM, Honkala BH, editors. Silvics of North America. Washington, DC: United States Department of Agriculture; 1990. pp. 268–279.

15. Arno SF. Community types and natural disturbance processes. In: Tomback DF, Arno SF, Keane RE, editors. Whitebark pine communities: ecology and restoration. Washington, DC: Island Press; 2001. pp. 74–88.

16. Tomback DF. Dispersal of whitebark pine seeds by Clark's nutcracker: a mutualism hypothesis. J Anim Ecol. 1982; 51: 451–467. https://doi.org/10.2307/3976

17. Hutchins HE, Lanner RM. The central role of Clark's nutcracker in the dispersal and establishment of whitebark pine. Oecologia. 1982; 55: 192–201. https://doi.org/10.1007/BF00384487 PMID: 28311233

18. Tomback DF, Linhart YB. The evolution of bird-dispersed pines. Evol Ecol. 1990; 4: 185–219. https://doi.org/10.1007/BF02214330

19. Tomback DF, Schoettle AS, Chevalier KE, Jones CA. Life on the edge for limber pine: seed dispersal within a peripheral population. Ecoscience. 2005; 12: 519–529. https://doi.org/10.2980/i1195-6860-12-4-519.1

20. Tomback DF, Hoffmann LA, Sund SK. Coevolution of whitebark pine and nutcrackers: implications for forest regeneration. In: Schmidt WC, McDonald KJ, editors. Proceedings–symposium on whitebark pine ecosystems: ecology and management of a high mountain resource, Bozeman, MT, 1989. USDA INTGTR-270. Ogden, Utah: US Department of Agriculture, Forest Service, Intermountain Research Station; 1990 pp. 118–129.

21. Tomback DF, Sund SK, Hoffmann LA. Post-fire regeneration of *Pinus albicaulis*: height-age relationships, age structure, and microsite characteristics. Can J For Res. 1993; 23: 113–119. https://doi.org/10.1139/x93-018

22. Tomback DF, Anderies AJ, Carsey KS, Powell ML, Mellmann-Brown S. Delayed seed germination in whitebark pine and regeneration patterns following the Yellowstone fires. Ecology. 2001; 82(9): 2587–2600. https://doi.org/10.1890/0012-9658(2001)082[2587:DSGIWP]2.0.CO;2

23. Resler LM, Tomback DF. Blister rust prevalence in krummholz whitebark pine: implications for treeline dynamics, Northern Rocky Mountains, Montana, U.S.A. AAAR. 2008; 40(1): 161–170. https://doi.org/10.1657/1523-0430(06–116)[RESLER]2.0.CO;2

24. Tomback DF, Chipman KG, Resler LM, Smith-McKenna EK, Smith CM. Relative abundance and functional role of whitebark pine at treeline in the Northern Rocky Mountains. AAAR. 2014; 46(2): 407–418. https://doi.org/10.1657/1938-4246-46.2.407

25. Tomback DF, Resler LM, Keane RE, Pansing ER, Andrade AJ, Wagner AC. Community structure, biodiversity, and ecosystem services in treeline whitebark pine communities: potential impacts from a non-native pathogen. Forests. 2016; 7(1): 21. https://doi.org/10.3390/f7010021

26. Tomback DF, Kendall KC. Biodiversity losses: The downward spiral. In: Tomback DF, Arno SF, Keane RE, editors. Whitebark pine communities: ecology and restoration. Washington, DC: Island Press; 2001. pp. 243–262.

27. Tomback DF, Achuff P. Blister rust and western forest biodiversity: ecology, values and outlook for white pines. For Pathol. 2010; 40: 186–225. https://doi.org/10.1111/j.1439-0329.2010.00655.x

28. Schwandt JW, Lockman IB, Kliejunas JT, Muir JA. Current health issues and management strategies for white pines in the western United States and Canada. For Pathol. 2010; 40: 226–250. https://doi.org/10.1111/j.1439-0329.2010.00656.x

29. McDonald GI, Hoff RJ. Blister rust: an introduced plague. In: Tomback DF, Arno SF, Keane RE, editors. Whitebark pine communities: ecology and restoration. Washington, DC: Island Press; 2001. pp. 193–220.

30. Krakowski J, Aitken SN, El-Kassaby YA. Inbreeding and conservation genetics is whitebark pine. Conserv Genet. 2003; 4: 581–593. https://doi.org/10.1023/A:1025667700479

31. COSEWIC. COSEWIC assessment and status report on the Whitebark Pine Pinus albicaulis in Canada. Committee on the Status of Endangered Wildlife in Canada. 2010. Available from: www.sararegistry.gc.ca/status/status_e.cfm Cited 10 February 2018.

32. Keane RE, Tomback DF, Aubry CA, Bower AD, Campbell EM, Cripps CL, et al. A range-wide restoration strategy for whitebark pine (*Pinus albicaulis*). Gen. Tech. Rep. RMRS-GTR-279. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station; 2012. https://doi.org/10.2737/RMRS-GTR-279

33. Leirfallom SB, Keane RE, Tomback DF, Dubrowski S. The effects of seed source health on whitebark pine (*Pinus albicaulis*) regeneration density after wildfire. Can J For Res. 2015; 45: 1597–1606. https://doi.org/10.1139/cjfr-2015-0043

34. Mahalovich MF, Hipkins VD. Molecular genetic variation in whitebark pine (Pinus albicaulis) in the inland west. In: Keane RE, Tomback DF, Murray MP, Smith CM, editors. The future of high-elevation, five-needle white pines in Western North America: Proceedings of the High Five Symposium, Missoula, MT, 2010. Proceedings RMRS-P-63. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station; 2011. pp. 118–132.

35. Collevatti RG, Grattapaglia D, Hay JD. Population genetic structure of the endangered tropical tree species *Caryocar brasiliense*, based on variability at microsatellite loci. Mol Ecol. 2001; 10(2): 349–356. https://doi.org/10.1046/j.1365-294X.2001.01226.x PMID: 11298950

36. Schwabe AL, Neale JR, McGlaughlin ME. Examining the genetic integrity of a rare endemic Colorado cactus (*Sclerocactus glaucus*) in the face of hybridization threats from a close and widespread congener (*Sclerocactus parviflorus*). Conserv Genet. 2015; 16(2): 443–457. https://doi.org/10.1007/s10592-014-0671-3

37. Nurtjahjaningsih IL, Saito Y, Lian CL, Tsuda Y, Ide Y. Development and characteristics of microsatellite markers in *Pinus merkusii*. Mol Ecol Resour. 2005; 5(3): 552–553. https://doi.org/10.1111/j.1471-8286.2005.00984.x

38. Dou JJ, Zhou RC, Tang AJ, Ge XJ, Wu W. Development and characterization of nine microsatellites for an endangered tree, *Pinus wangii* (Pinaceae). APPS. 2013; 1(2):1200134. https://doi.org/10.3732/apps.1200134 PMID: 25202511

39. Thao DV, Widyatmoko AY, Guan L, Gotoh E, Watanabe A, Shiraishi S. Isolation and characterization of tetranucleotide microsatellite markers for *Pinus merkusii*. Conserv Genet Res. 2013; 5(2): 433–436. https://doi.org/10.1007/s12686-012-9821-4

40. Moraga-Suazo P, Orellana L, Quiroga P, Balocchi C, Sanfuentes E, Whetten RW, et al. Development of a genetic linkage map for *Pinus radiata* and detection of pitch canker disease resistance associated QTLs. Trends Ecol Evol. 2014; 28(6): 1823–1835. https://doi.org/10.1007/s00468-014-1090-2

41. Phong DT, Lieu TT, Hien VT, Hiep NT. Genetic diversity of the endemic flat-needle pine *Pinus krempfii* (Pinaceae) from Vietnam revealed by SSR markers. Genet Mol Res. 2015; 14(3): 7727–7739. https://doi.org/10.4238/2015.July.13.19 PMID: 26214454

42. Jorgensen SM, Hamrick JL. Biogeography and population genetics of whitebark pine, *Pinus albicaulis*. Can J For Res. 1997; 27(10): 1574–1585. https://doi.org/10.1139/x97-118

43. Bruederle LP, Tomback DF, Kelly KK, Hardwick RC. Population genetic structure in a bird-dispersed pine, *Pinus albicaulis* (Pinaceae). Can J Bot. 1998; 76(1): 83–90. https://doi.org/10.1139/b97-162

44. Richardson BA, Brunsfeld SJ, Klopfenstein NB. DNA from bird-dispersed seed and wind-disseminated pollen provides insights into postglacial colonization and population genetic structure of whitebark pine (*Pinus albicaulis*). Mol Ecol. 2002; 11(2): 215–227. https://doi.org/10.1046/j.1365-294X.2002.01435.x PMID: 11856423

45. Liu JJ, Sniezko R, Murray M, Wang N, Chen H, Zamany A, et al. Genetic diversity and population structure of whitebark pine (*Pinus albicaulis* Engelm.) in western North America. PloS One. 2016; 11(12): e0167986. https://doi.org/10.1371/journal.pone.0167986 PMID: 27992468

46. Syring J, Tennessen JA, Jennings TN, Wegrzyn J, Scelfo-Dalbey C, Cronn R. Targeted capture sequencing in whitebark pine reveals range-wide demographic and adaptive patterns despite challenges of a large, repetitive genome. Front Plant Sci. 2016; 7. https://doi.org/10.3389/fpls.2016.00484 PMID: 27148310

47. Lind BM, Friedline CJ, Wegrzyn JL, Maloney PE, Vogler DR, Neale DB, et al. Water availability drives signatures of local adaptation in whitebark pine (*Pinus albicaulis* Engelm.) across fine spatial scales of the Lake Tahoe Basin, USA. Mol Ecol. 2017; 26: 3168–3185. https://doi.org/10.1111/mec.14106 PMID: 28316116

48. Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, et al. Current trends in microsatellite genotyping. Mol Ecol Res. 2011; 11(4): 591–611. https://doi.org/10.1111/j.1755-0998.2011.03014.x PMID: 21565126

49. Morse AM, Peterson DG, Islam-Faridi MN, Smith KE, Magbanua Z, Garcia SA, et al. Evolution of genome size and complexity in Pinus. PLoS ONE. 2009; 4:e4332. https://doi.org/10.1371/journal.pone.0004332 PMID: 19194510

50. González-Martínez SC, Robledo-Arnuncio JJ, Collada C, Díaz A, Williams CG, Alía R, et al. Cross-amplification and sequence variation of microsatellite loci in Eurasian hard pines. Theor Appl Genet. 2004; 109: 103–11. https://doi.org/10.1007/s00122-004-1596-x PMID: 14985972

51. Barbara T, Palma-Silva CL, Paggi GM, Bered F, Fay MF, Lexer C. Cross-species transfer of nuclear microsatellite markers: potential and limitations. Mol Ecol. 2007; 16(18): 3759–3767. https://doi.org/10.1111/j.1365-294X.2007.03439.x PMID: 17850543

52. Oreshkova NV, Sedel'nikova TS, Pimenov AV, Efremov SP. Analysis of genetic structure and differentiation of the bog and dry land populations of *Pinus sibirica* Du Tour based on nuclear microsatellite loci. Russ J Genet. 2014; 50(9): 934–941. https://doi.org/10.1134/S1022795414090105

53. Villalobos-Arámbula AR, Pérez de la Rosa JA, Arias A, Rajora OP. Cross-species transferability of eastern white pine (*Pinus strobus*) nuclear microsatellite markers to five Mexican white pines. Genet Mol Res. 2014; 13(3): 7571–7576. https://doi.org/10.4238/2014.September.12.24 PMID: 25222257

54. Gernandt DS, López GG, García SO, Liston A. Phylogeny and classification of *Pinus*. Taxon. 2005; 54(1): 29–42. https://doi.org/10.2307/25065300

55. Syring J, Willyard A, Cronn R, Liston A. Evolutionary relationships among *Pinus* (Pinaceae) subsections inferred from multiple low-copy nuclear loci. Am J Bot. 2005; 92(12): 2086–2100. https://doi.org/10.3732/ajb.92.12.2086 PMID: 21646125

56. Little EL, Critchfield WB. Subdivisions of the genus *Pinus* (pines). MP-1144. Washington, DC: US Department of Agriculture, Forest Service; 1969.

57. Price RA, Liston A, Strauss SH. Phylogeny and systematics of *Pinus*. In: Richardson DM, editor. Ecology and Biogeography of *Pinus*. Cambridge, MA: Cambridge University Press; 1998. pp. 49–68.

58. Willyard A, Syring J, Gernandt DS, Liston A, Cronn R. Fossil calibration of molecular divergence infers a moderate mutation rate and recent radiations for Pinus. Mol Biol Evol. 2006; 24(1): 90–101. https://doi.org/10.1093/molbev/msl131 PMID: 16997907

59. Hao ZZ, Liu YY, Nazaire M, Wei XX, Wang XQ. Molecular phylogenetics and evolutionary history of sect. *Quinquefoliae* (*Pinus*): Implications for Northern Hemisphere biogeography. Mol Phylogenet Evol. 2015; 87: 65–79. https://doi.org/10.1016/j.ympev.2015.03.013 PMID: 25800283

60. Jennings TN, Knaus BJ, Kolpak S, Cronn R. Microsatellite primers for the Pacific Northwest endemic conifer *Chamaecyparis lawsoniana* (Cupressaceae). Am J Bot. 2011; 98(11): e323–e325. https://doi.org/10.3732/ajb.1100317 PMID: 22012929

61. Cronn R, Liston A, Parks M, Gernandt DS, Shen R, Mockler T. Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. Nucleic Acids Res. 2008; 36(19): e122. https://doi.org/10.1093/nar/gkn502 PMID: 18753151

62. Miller MP, Knaus BJ, Mullins TD, Haig SM. SSR_pipeline: A Bioinformatic Infrastructure for identifying microsatellites from paired-end Illumina high-throughput DNA sequencing data. J Hered. 2013; 104(6): 881–885. https://doi.org/10.1093/jhered/est056 PMID: 24052535

63. You FM, Huo N, Gu YQ, Luo M-C, Ma Y, Hane D, et al. BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinformatics. 2008; 9: 253. https://doi.org/10.1186/1471-2105-9-253 PMID: 18510760

64. Salzer K, Sebastiani F, Gugerli F, Buonamici A, Vendramin GG. Isolation and characterization of polymorphic nuclear microsatellite loci in *Pinus cembra* L. Mol Ecol Resour. 2008; 9(3): 858–861. https://doi.org/10.1111/j.1755-0998.2008.02396.x PMID: 21564769

65. Lendvay B, Höhn M, Brodbeck S, Mîndrescu M, Gugerli F. Genetic structure in *Pinus cembr*a from the Carpathian Mountains inferred from nuclear and chloroplast microsatellites confirms post-glacial range contraction and identifies introduced individuals. Tree Genet Genomes. 2014; 10: 1419–1433. https://doi.org/10.1007/s11295-014-0770-9

66. Iwasaki T, Sase T, Takeda S, Ohsawa TA, Ozaki K, Tani N, et al. Extensive selfing in an endangered population of *Pinus parviflora* var. *parviflora* (Pinaceae) in the Boso Hills, Japan. Tree Genet Genomes. 2013; 9: 693–705. https://doi.org/10.1007/s11295-012-0585-5

67. Yu JH, Chen CM, Tang ZH, Yuan SS, Wang CJ, Zu YG. Isolation and characterization of 13 novel polymorphic microsatellite markers for *Pinus koraiensis* (Pinaceae). Am J Bot. 2012; e421–e424. https://doi.org/10.3732/ajb.1200145 PMID: 23028004

68. Echt CS, May-Marquardt P, Hseih M, Zahorchak R. Characterization of microsatellite markers in eastern white pine. Genome. 1996; 39(6): 1102–1108. https://doi.org/10.1139/g96-138 PMID: 8983182

69. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep. 1993; 11(2): 113–116. https://doi.org/10.1007/BF02670468

70. Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF. M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. Biotechniques. 2001; 31(1): 24–26. PMID: 11464515

71. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes. 2006; 6: 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

72. Peakall R, Smouse PE. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics. 2012; 28: 2537–2539. https://doi.org/10.1093/bioinformatics/bts460 PMID: 22820204

73. Kalinowski ST, Taper ML. Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. Conserv Genet. 2006; 7(6): 991–995. https://doi.org/10.1007/s10592-006-9134-9

74. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2016. URL https://www.R-project.org/ Cited 16 October 2017.

75. Mantel N. The detection of disease clustering and a generalized regression approach. Cancer Res. 1967; 27: 209–220. PMID: 6018555

76. Karhu A, Dietrich J-H, Savolainen O. Rapid expansion of microsatellite sequences in pines. Mol Biol Evol. 2000; 17: 259–265. https://doi.org/10.1093/oxfordjournals.molbev.a026305 PMID: 10677848

77. Elsik CG, Williams CG. Low-copy microsatellite recovery from a conifer genome. Theor Appl Genet. 2001; 103(8): 1189–1195. https://doi.org/10.1007/s001220100725

78. Elsik CG, Williams CG. Families of clustered microsatellites in a conifer genome. Mol Gen Genet. 2001; 265(3): 535–542. https://doi.org/10.1007/s004380100443

79. Payseur BA, Jing P, Haasl RJ. A genomic portrait of human microsatellite variation. Mol Biol Evol. 2010; 28(1): 303–12. https://doi.org/10.1093/molbev/msq198 PMID: 20675409

80. Belokon MM, Politov DV, Mudrik EA, Polyakova TA, Shatokhina AV, Belokon YS, et al. Development of microsatellite genetic markers in Siberian stone pine (*Pinus sibirica* Du Tour) based on the de novo whole genome sequencing. Russ J Genet. 2016; 52(12): 1263–1271. https://doi.org/10.1134/s1022795416120036

81. Hale ML, Burg TM, Steeves TE. Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PloS one. 2012; 7(9): e45170. https://doi.org/10.1371/journal.pone.0045170 PMID: 22984627

82. DeWoody J, Nason JD, Hipkins VD. Mitigating scoring errors in microsatellite data from wild populations. Mol Ecol Resour. 2006; 6(4): 951–957. https://doi.org/10.1111/j.1471-8286.2006.01449.x

83. Chagné D, Chaumeil P, Ramboer A, Collada C, Guevara A, Cervera MT, et al. Cross-species transferability and mapping of genomic and cDNA SSRs in pines. Theor Appl Genet. 2004; 109(6): 1204–1214. https://doi.org/10.1007/s00122-004-1683-z PMID: 15448894

84. Liewlaksaneeyanawin C, Ritland CE, El-Kassaby YA, Ritland K. Single-copy, species-transferable microsatellite markers developed from loblolly pine ESTs. Theor Appl Genet. 2004; 109: 361–369. https://doi.org/10.1007/s00122-004-1635-7 PMID: 15045175

85. Echt CS, May-Marquardt P. Survey of microsatellite DNA in pine. Genome 1997; 40: 9–17. https://doi.org/10.1139/g97-002 PMID: 9061909

86. O'Neill GA, Nigh G, Wang T, Ott PK. Growth response functions improved by accounting for nonclimatic site effects. Can J For Res. 2007; 37: 2724–2730. https://doi.org/10.1139/X07-100

87. Sniezko RA, Mahalovich MF, Schoettle AW, Vogler DR. Past and current investigations of the genetic resistance to Cronartium ribicola in high-elevation five-needle pines. In: Keane RE, Tomback DF, Murray MP, Smith CM, editors. The future of high-elevation, five-needle white pines in Western North America: Proceedings of the High Five Symposium, Missoula, MT, 2010. Proceedings RMRS-P-63. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station; 2011. pp. 246–264.

88. Keane RE, Holsinger LM, Mahalovich MF, Tomback DF. Restoring whitebark pine ecosystems in the face of climate change. Gen. Tech. Rep. RMRS-GTR-361. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station; 2017.

89. Eckert AJ, Bower AD, Wegrzyn JL, Pande B, Jermstad KD, Krutovsky KV, et al. Association genetics of coastal Douglas fir (*Pseudotsuga menziesii* var. *menziesii*, Pinaceae). I. Cold-Hardiness related traits. Genetics. 2009; 182(4): 1289–1302. https://doi.org/10.1534/genetics.109.102350 PMID: 19487566

90. Eckert AJ, van Heerwaarden J, Wegrzyn JL, Nelson CD, Ross-Ibarra J, González-Martínez SC, et al. Patterns of population structure and environmental associations to aridity across the range of loblolly pine (*Pinus taeda* L., Pinaceae). Genetics. 2010; 185(3): 969–982. https://doi.org/10.1534/genetics.110.115543 PMID: 20439779

91. Rosenberg NA, Pritchard JK, Weber JL, Cann HM, Kidd KK, Zhivotovsky LA, et al. Genetic structure of human populations. Science. 2002; 298: 2381–2385. https://doi.org/10.1126/science.1078311 PMID: 12493913