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An Economical Approach to Distinguish Genetically Needles of Limber from Whitebark Pine

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Abstract: Whitebark pine is difficult to distinguish from limber pine when seed cones are not present. This is often the case because of young stand age, growth at environmental extremes, or harvesting by vertebrate species. Developing an economical genetic identification tool that distinguishes non-cone-bearing limber from whitebark pine, therefore, could aid many kinds of research on these species. Phylogenetic studies involving limber and whitebark pine suggest that chloroplast DNA sequences differ between these species. We therefore wanted to identify chloroplast loci that could differentiate limber from whitebark pine trees by taking an economical approach involving restriction-site analysis. We generated chloroplast DNA barcode sequences sampled from limber and whitebark pine trees that we identified using attached seed cones. We searched for nucleotide differences associated with restriction endonuclease recognition sites. Our analyses revealed that *matK* and the *psbA-trnH* spacer each readily amplified and harbored multiple DNA-sequence differences between limber and whitebark pine. The *matK* coding sequence of whitebark pine has a BsmAI restriction site not found in limber pine. The *psbA-trnH* spacer of limber pine has two PsiI restriction sites, neither of which is found in whitebark pine. DNA-sequence and restriction-site analysis of the *psbA-trnH* spacer from 111 trees showed complete congruence between visually and genetically identified limber (n = 68) and whitebark (n = 43) pine trees. We conclude that restriction site analysis of the chloroplast *psbA-trnH* spacer and *matK* involves both minimal technical expertize and research funds. These findings should be of value to foresters interested in species identification and distribution modeling, as well as the analysis of fossil pine pollen, given that gymnosperms transmit chloroplast DNA paternally.

Keywords: chloroplast DNA; genetic identification; Greater Yellowstone Ecosystem; haplotypes; *Pinus albicaulis*; *Pinus flexilis*; western North America

1. Introduction

Whitebark pine, *Pinus albicaulis* Engelm., is a candidate species for the Endangered Species Act threatened and endangered list that plays a keystone role in Rocky Mountain coniferous forests and elsewhere in western North America [1]. Understanding the population responses of whitebark pine to climate change is of high interest to forest managers, especially in the northern Rocky Mountain

region encompassing Yellowstone National Park [2]. This is because whitebark pine is ecologically important and its nutrient-rich seeds represent an important food source for grizzly bears and other animals. Understanding whether whitebark pine can tolerate warmer and drier conditions, compared to its current predilection, will depend on being able to model properly its fundamental niche [3].

The issue is that whitebark pine populations can be sympatric with the similar five-needle limber pine, *Pinus flexilis* James, and the geographic ranges of these two pines are highly overlapping in the northern Rocky Mountains, Great Basin, and Southern Sierra Nevada of North America [4]. Limber and whitebark pine differ primarily by seed cone and secondarily by pollen cone morphology (Figure 1). Without seed cones, limber and whitebark pine are difficult to distinguish from each other.

Figure 1. Cones of limber and whitebark pine. Seed cones of limber pine (upper left) and whitebark pine (upper right). Pollen cones of limber pine (lower left) and whitebark pine (lower right). Photos by M. Lavin [5].

Positive identification of trees lacking seed cones, therefore, is critical for field sampling and especially for the development of species distribution models. Current species distribution models suggest that whitebark pine is rarely associated with the warmer and drier conditions at the lower tree line where limber pine predominates in the Greater Yellowstone Ecosystem [6]. Consistent with this observation, United States Department of Agriculture (USDA) Forest Inventory and Analysis field surveys assume that non-cone-bearing five-needle pines are limber pine if occurring below an elevation threshold of about 2440 m (8000 feet) and whitebark pine if occurring above that 2440 m threshold. Buermeyer et al. [2] hypothesized, however, that whitebark pine seedlings can tolerate warm and dry conditions but are excluded competitively by other conifers before becoming cone bearing. Thus,

quantification of the warm and dry tolerances of whitebark pine is needed to evaluate the sensitivity of this keystone species to climate change. We therefore explored an economical approach to the genetic analysis of pine needle samples as a means of distinguishing limber from whitebark pine when seed cones are absent.

Although limber and whitebark pine are similar ecologically, geographically, and vegetatively, they are distant relatives within *Pinus* section *Quinquefoliae* subsection *Strobus*—the white pines—according to the analyses of seed cone morphology [7] and genetic data. Genetic studies showing the distant relationship between limber and whitebark pine include analyses of rapidly amplified monomorphic and polymorphic DNA [8] and isozyme loci [9], and phylogenetic analyses of nuclear and chloroplast DNA data. Phylogenetic analyses resolving the distant relationship of limber and whitebark pine, each represented by multiple conspecific accessions, include that of Hao et al. [10], who analyzed two nuclear and four chloroplast loci; Syring et al. [11], who used the nuclear late-embryogenesis-abundant-(LEA)-like protein encoding locus; and Liston et al. [12], who compared chloroplast *matK* and *trnG* sequences. Other studies resolving a distant relationship of limber and whitebark pine within subsection *Strobus*, but involving only a single accession each of limber and whitebark pine, include Gernandt et al. [13], who analyzed the chloroplast *matK* and *rbcL* sequences from representatives of all the major groups of *Pinus*, and Parks et al. [14], who analyzed the nearly complete chloroplast genomes of 31 representative species of the genus *Pinus*.

These phylogenetic studies resolve limber pine within a North American clade that includes such species as southwestern white pine (*Pinus strobiformis* Engelm.). Limber pine and closely related species have been in residence in western North America for millions of years [15]. In contrast, the above phylogenetic studies resolve whitebark pine within a primarily Eurasian clade and it is closely related to such species as Swiss stone pine (*Pinus cembra* L.) and Siberian pine (*Pinus sibirica* Du Tour). Whitebark pine is a recent Pleistocene immigrant into western North America from a Eurasian source area [12].

The above biogeographic and genetic evidence suggests that finding a marker to distinguish genetically limber from whitebark pine should be technically straightforward and economically feasible. We targeted chloroplast loci due to their high likelihood of revealing fixed genetic variation between species, which is predicted because of the low effective population size of haploid genomes (e.g., [16]). In addition, chloroplast loci are readily amenable to PCR amplification because primer sites usually are conserved among land plants [17,18]. Analyses of chloroplast DNA sequences even show differences between closely related parapatric pine species from the northern Rocky Mountain region, as exemplified by *Pinus ponderosa* and *Pinus scopulorum* [16,19]. We thus targeted commonly used chloroplast loci [18] to identify a genetic locus that is readily PCR-amplified and DNA-sequenced (e.g., <1000 bps in length) and that harbors nucleotide site differences involving restriction endonuclease recognition sites.

Our overall objective was to find a genetic identification method that can be both economically feasible and involve minimal lab equipment and modest technical expertise. With such a method identified, field crews can sample needles from the trees in question and confirm or assign species identification in the lab with minimal cost and effort.

2. Materials and Methods

We sampled needles from 111 limber and whitebark pine trees, which we visually identified as species using attached seed cones. We targeted our sampling primarily in the Greater Yellowstone Ecosystem, the region where we are developing species distribution models for limber and whitebark pine. All samples came from U.S. Forest Service lands and included elevation gradients where limber and whitebark pine were likely to overlap. We sampled two five-needle fascicles per tree where a fascicle contained uniformly green needles. We secured individual samples in plastic or glassine envelopes, which were stored in dry and dark conditions at room temperature until analysis. Sampling occurred

during the summer and fall of 2017–2019. We recorded latitude, longitude, elevation, and species for each needle sample.

We distinguished limber pine by its open and intact seed cones that measure 7–15 cm long and have persistent woody scales [20,21]. Seed cones of limber pine often persist on the tree and on the ground around the tree. We distinguished whitebark pine by its closed seed cones that measure 4–8 cm long and have tightly overlapping scales (Figure 1) [20,21]. Seed cones of whitebark pine can be common on the tree before birds and squirrels harvest them, but rarely occur intact on the ground around the tree. Pollen cones of limber pine range from yellow to pale red, whereas pollen cones of whitebark pine are usually scarlet (Figure 1) [20]. We used the color of pollen cones as a secondary visual identification marker because the color in limber pine pollen cones ranges from yellowish to reddish, because subtending scales in both species obscure color differences during the early season, and because the color of whitebark pollen cones fades by late summer or fall.

We isolated total genomic DNA from a 1–2 mm segment of a single pine needle using the Sigma Aldrich Extract-N-Amp™ Plant PCR Kit (St. Louis, Missouri) and secondarily the Qiagen DNeasy Plant Mini Kit (Valencia, California). Using the same Extract-N-Amp Plant PCR Kit or the Qiagen PCR Core kit, we PCR-amplified some of the more commonly targeted chloroplast loci described in Taberlet et al. [17] and Shaw et al. [18]. We selected loci that potentially differed in DNA sequence between limber and whitebark pine, as evinced by phylogenetic studies such as Liston et al. [11] that focused on limber and whitebark pine, and close relatives. These included the *matK* coding region, the *psbA-trnH* spacer, the *trnG* intron, the *trnT-trnF* region, and the *trnD-trnT* region. We used primarily the more economical Sigma Aldrich Extract-N-Amp™ Plant PCR Kit and then the Qiagen kits on samples that did not amplify with the Sigma Aldrich kit.

PCR primers and reaction conditions followed Shaw et al. [18]. We used GENEWIZ (Boston, Massachusetts) to Sanger sequence our unpurified PCR templates. We used Sequencher 4.1 (Gene Codes, Ann Arbor, MI, USA) to contig forward and reverse sequence reads. For sequence alignment, we used MUSCLE with default parameter settings [22], and manually aligned sequences using PhyDE [23]. We analyzed a random 10% subset of the samples, stratified across species and geographical distributions, to determine the ease and consistency of PCR amplification and the presence of DNA-sequence variation before expanding the analysis to all needle samples. We accessioned DNA-sequence data and associated metadata, including geographical coordinates and PCR primers, with GenBank (Supplemental Table S1). The protocol for the analysis of restriction endonuclease recognition sites followed Parks et al. [24].

3. Results

Of the chloroplast loci evaluated, the *trnD-trnT* region rarely PCR-amplified and so was not analyzed further. The *trnT-trnF* region readily PCR-amplified but DNA sequences did not differ between limber and whitebark pine (GenBank accessions MN537000–MN537018; Appendix A; Supplemental Table S1). The *matK* coding sequences of limber and whitebark pine (GenBank accessions MN542807–MN542818; Supplemental Table S1) differed at eight nucleotide sites out of the approximately 1630 total sites. One of these involved a BsmAI restriction site that cleaved the whitebark pine *matK* amplicon into two fragments, about 623 and 1000 base pairs (bps; Figure 2, top panel; Appendix A). Notably, this BsmAI restriction site found in whitebark pine also occurred in most white pine species but was absent from the white pine group in just limber pine and its closest relatives, *Pinus ayacahuite* Ehreng. ex Schlecht. and *P. strobiformis* Engelm. (Appendix A).

The *psbA-trnH* spacer sequences of limber and whitebark pine (GenBank accessions MN603507–MN603617; Appendix A; Supplemental Table S1) differed at three nucleotide sites out of approximately 662 bps. The length of the spacer in whitebark pine is 535 bps and in limber pine 534 bps because of a deletion at site 116 within the spacer region (Figure 3; Supplemental Figure S1, panel A). The other nucleotide differences at sites 250 and 377 within the spacer involved PsiI restriction sites found in limber but not in whitebark pine. The most common limber pine haplotype ("flexilis" in

Figure 3) has both PsiI restriction sites, which results in cleaving the PCR amplicon into three fragments of, 5′ to 3′, 327, 126, and 209 bps (PsiI is a blunt-end restriction endonuclease; Figure 2, middle panel; Figure 3; Supplemental Figure S1, panels B and C). The much less-common limber pine haplotype ("flexilis-i" where "i" indicates "intermediate"; Figure 3) has one PsiI restriction site, which results in cleaving the PCR amplicon into two fragments of, 5′ to 3′, 453 and 209 bps (Figure 2, middle panel; Figure 3; Supplemental Figure S1, panel C; Appendix A). The downstream PsiI restriction site is found among white pines only in *Pinus flexilis* and close North American relatives, *Pinus ayacahuite*, *Pinus lambertiana* Dougl., and *Pinus strobus* L. (Appendix A). We found the upstream PsiI restriction site in only limber pine and then in most limber pine samples (Supplemental Table S1; Appendix A).

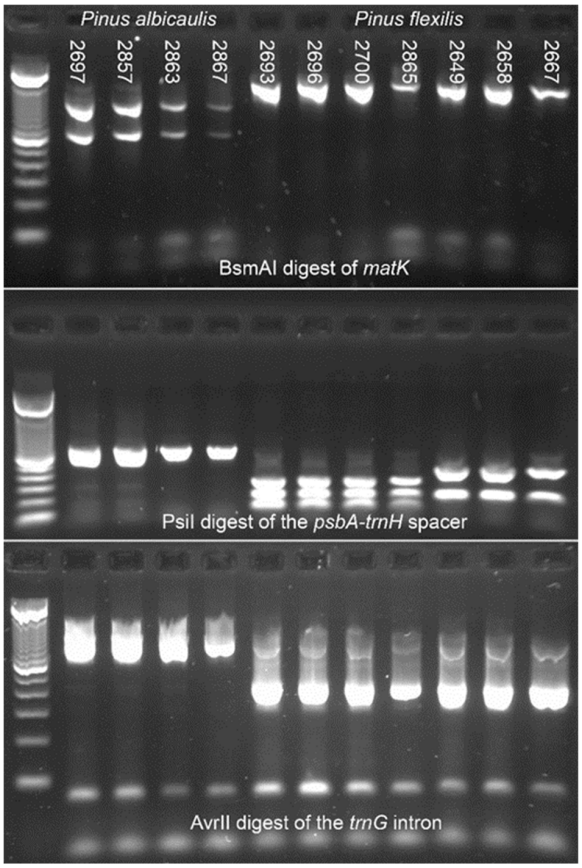

Figure 2. Restriction enzyme digests of whitebark (*Pinus albicaulis*, lanes 2697 to 2867) and limber pine (*Pinus flexilis*, lanes 2693 to 2667) PCR amplicons. The 12 lanes in each of the three panels represent the same DNA sample. The left-hand lane in each panel represents a 100 bps DNA ladder. Subsequent lanes are labeled by the DNA isolate number, from 2697 to 2667, the information for which is reported in the Supplemental Table S1.

```
                  0      111       121       241       251          371       381
                  |      |    *    |         |         *|          |     *     |
albicaulis ... TAA ... TCCCCCATTCCATC ... ATACTCATTCTATAATAT ... CATTTTGATAATAA
flexilis-i ... TAA ... TCCCC-ATTCCATC ... ATACTCATTCTATAATAT ... CATTTTTATAATAA
flexilis   ... TAA ... TCCCC-ATTCCATC ... ATACTCATTTTATAATAT ... CATTTTTATAATAA
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Figure 3. Three chloroplast *psbA-trnH* spacer haplotypes detected in this study. The numbers across the top represent nucleotide site positions beginning after the TAA stop codon in the 5′ flanking *psbA* gene. Asterisks and bold font indicate site numbers 116, 250, and 377, where differences in nucleotides mark each haplotype. Nucleotides in italic mark the PsiI restriction sites (250–255, 376–381), which are found in the two haplotypes sampled from limber pine but not in the single haplotype sampled from whitebark pine. The PsiI restriction site encompassing sites 250–255 is unique to limber pine (Supplemental Figure S1, panel B).

The *trnG* intron sequences of limber and whitebark pine (GenBank accessions MN542795–MN542806; Appendix A; Supplemental Table S1) differed at six nucleotide sites out of approximately 821 bps. One of these differences involves an AvrII restriction site present in limber pine and absent in whitebark pine, which results in cleaving the trnG intron amplicon into fragments of 5′ to 3′; 401 and 422 bps (AvrII is a sticky-end restriction endonuclease; Figure 2, bottom panel; Appendix A). The AvrII restriction site is common among the white pines and occurs in limber pine and all its closest relatives, *Pinus ayacahuite*, *Pinus chiapensis* (Martínez) Andresen, *P. lambertiana*, *P. strobiformis*, and *P. strobus*, as well as *Pinus gerardiana* Wall. ex D. Don and *Pinus peuce* Griseb. (Appendix A). The absence of this restriction site thus is a derived condition in *Pinus albicaulis* and its closest and mostly Eurasian relatives (Appendix A).

Because the "flexilis" haplotype of the *psbA-trnH* spacer, with two PsiI restriction sites (Figure 2, lanes 2693–2865; Figure 3), is unique to *Pinus flexilis* among white pine species, we DNA-sequenced this chloroplast locus for all 111 of our seed-cone identified limber and whitebark pine samples. Species identifications derived from seed-cone morphology and from haplotypes, which we identified by analysis of both DNA sequences and restriction sites, were completely congruent (Supplemental Table S1). The "flexilis" haplotype was most common and distributed at every site where we sampled limber pine (Figure 4). We sampled the "flexilis-i" haplotype from only five limber pine trees, all from within Montana, and these were sympatric with limber pine trees having the "flexilis" haplotype (Figure 4).

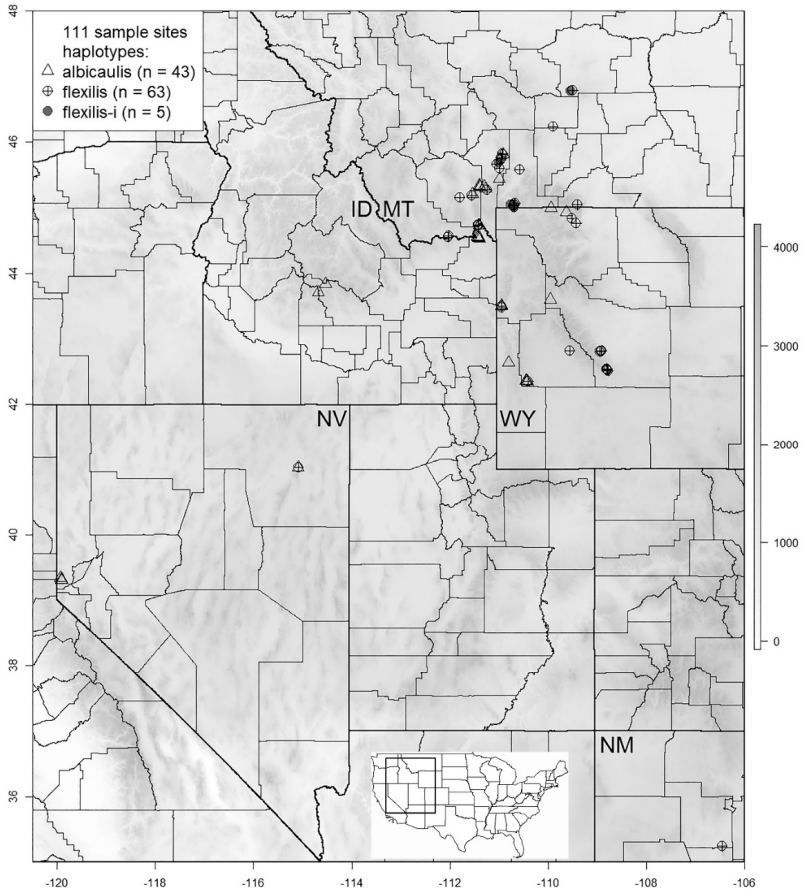

Figure 4. Geographic distribution in Idaho (ID), Montana (MT), Nevada (NV), New Mexico (NM), and Wyoming (WY) of the 111 sites where we sampled trees with attached seed cones. The rectangle on the inset map of the USA provides a larger geographical context of sample sites. Symbols marking each site indicate the sampled *psbA-trnH* haplotype. The shading represents elevation (m). Overlapping cross-circles and open triangles indicate where limber and whitebark pine are sympatric (e.g., Madison County, Montana; Elko County, Nevada; and Teton County, Wyoming).

4. Discussion

In agreement with DNA barcode studies [25–27], we find that the chloroplast *matK* and *psbA-trnH* loci represent optimal genetic barcodes for distinguishing limber from whitebark pine. Indeed, *matK* and the *psbA-trnH* spacer were identified as a particularly informative combination for distinguishing pine species [26]. A PsiI digest of the *psbA-trnH* spacer that remains uncut would suggest that the needles belong to whitebark pine, and a BsmAI digest of *matK* (Figure 2, upper and middle panels) could verify this. Although the *trnG* intron is useful, restriction-site analysis of the *trnG* intron is redundant to that of the *psbA-trnH* spacer in that both result in a cut of the respective amplicon of limber pine, but not that of whitebark pine (Figure 2, middle and bottom panels). In addition, the cost of the AvrII restriction endonuclease, which cuts the *trnG* intron, is $0.70/unit, whereas the cost of the PsiI enzyme, which cuts the *psbA-trnH* spacer, is $0.29/unit (see the caption to Table 1).

Table 1. Costs for analyzing 200 needle samples—the number that can be analyzed with an order of minimal quantities of items needed for DNA isolation, PCR amplification, and restriction-site analysis (using the *trnG* intron combined with the AvrII restriction endonuclease would reduce 200 possible samples to 100). After protocol optimization and troubleshooting performed during our study, we estimate labor at about 40 h.

Category	Item	Cost	Total
DNA extraction and PCR kit	Extract-N-Amp (1 kit)	$244.00	
	Shipping	$58.47	
PCR primers	*psbA* (50 nmol)	$7.48	
	trnH (50 nmol)	$7.48	
	ORF515 (50 nmol)	$6.46	
	matK (50 nmol)	$6.80	
	Shipping	$12.00	
Restriction endonuclease analysis	PsiI (400 units)	$115.00	
	BsmAI (1000 units)	$68.00	
	BSA (12 mg)	$30.00	
	shipping	$29.00	$584.69
Labor (undergraduate student)	40 h ($15/h)	$600.00	$1184.69

Our results, derived mainly from sampling the region of the Greater Yellowstone Ecosystem, should be general to the entire range of limber and whitebark pine in western North America. This prediction stems from the well-established phylogenetic relationships that resolve limber pine as an ancient North American resident and the distantly related whitebark pine as a Pleistocene immigrant into North America (e.g., [12,14,15]).

We do not expect introgressive hybridization to confound our results. Reports of introgression between limber and whitebark pine are uncommon and cursory [7,28]. Indeed, introgression between limber and whitebark pine is not expected given their distant relationship (e.g., [8–14]). Other potentially relevant reports of introgression are also not confounding to our findings. Introgression between *Pinus albicaulis* and *Pinus lambertiana* Dougl. is limited to a northeastern California contact zone with gene flow directed from whitebark into sugar pine [12]. Introgression between *Pinus flexilis* and *Pinus strobiformis* is limited to a contact zone in Arizona and New Mexico [15]. Our whitebark pine samples came from sites that were often sympatric with limber pine (Figure 4, where the cross-circles and open triangles overlap) or sugar pine, *Pinus lambertiana* (Figure 4, the Lake Tahoe area, Washoe County, Nevada). Even at such sites, we found complete congruence between haplotype and seed-cone identification of the tree samples (Supplemental Table S1).

Forest ecologists, paleoecologists, and managers with an interest in genetically distinguishing limber and whitebark pine should be able to accomplish this with minimal research funds and modest technical expertise. With access to a lab having some genetics expertise, plastic disposables (e.g., pipette tips and reaction tubes), a heat block, a microfuge, a PCR machine, a gel rig, and agarose, the cost

for materials of genetically identifying 200 pine needle samples using restriction-site variation at the chloroplast and *psbA-trnH* loci could be as low as $585 (Table 1). Including undergraduate student labor would only double this cost estimate (Table 1). Our suggested genetic identification approach also would be amenable to the analysis of a single or few needle samples, which could be analyzed within a few hours and with little cost in supplies.

5. Conclusions

The restriction-site analysis of the chloroplast *matK* and *psbA-trnH* sequences potentially extends to studies of fossil pollen of limber and whitebark pine [29–31]. The environmental conditions of pollen fossilization would have to be favorable to DNA preservation but given that chloroplasts are inherited paternally in gymnosperms [32], isolation of chloroplast DNA from fossil pollen would be amenable to the approach we detail in our study.

The conventional assignment of non-cone-bearing limber pine occurring below an approximate 2440 m elevation threshold and whitebark pine above that 2440 m threshold is problematic given that our limber pine samples often exceeded this elevation threshold (Figure 5). Although few of our whitebark pine samples came from below the 2440 m threshold (Figure 5), we did genetically identify a five-needle tree without seed cones as whitebark pine, which came from a 1990 m site in the area of Anaconda–Pintler Wilderness of western Montana and we know that this area harbors whitebark pine below the 2440 m threshold. Thus, a questionable elevation threshold warrants the development of an economically feasible genetic identification tool to develop species distribution models that can predict with the greatest accuracy the fundamental niches of limber and whitebark pine. We think that settling on *matK* and *psbA-trnH* loci achieves our goal of finding an economically and technically feasible approach to identify genetically limber and whitebark pine throughout the geographic range of where they co-exist.

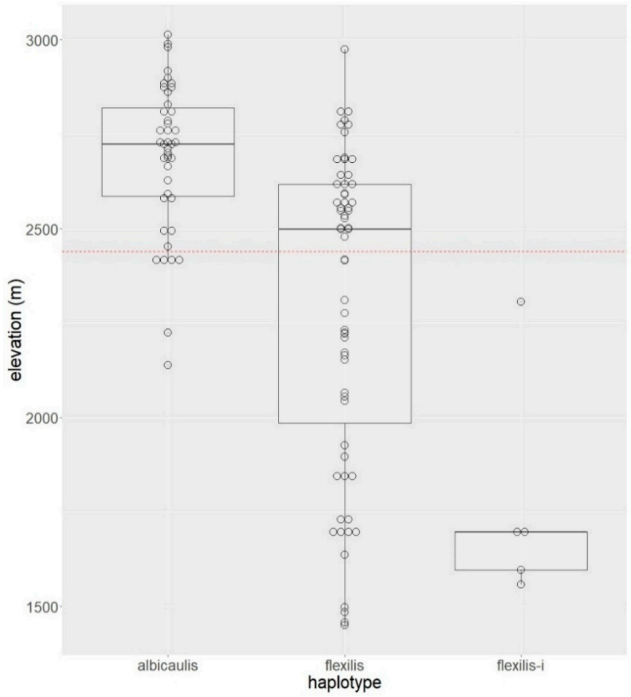

Figure 5. Dotplots with boxplot overlays showing the range of elevation for each of the three chloroplast DNA *psbA-trnH* haplotypes (n = 43, 63, and 5 for the "albicaulis", "flexilis", and "flexilis-i" haplotypes, respectively). The box captures 50% of the data points and the thick horizontal bar represents the median. Data points above and below the whiskers represent outliers. The red dashed line represents the 2440 m threshold above which five-needle pines lacking cones conventionally are assigned to whitebark pine.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/12/1060/s1, Figure S1: Chromatograms showing the difference in the nucleotide sequence of the three psbA-trnH spacer haplotypes, Table S1: Metadata for the 111 five-needle pine samples from which we comprehensively DNA sequenced and PsiI-restriction-site analyzed the *psbA-trnH* spacer (Appendix A).

Author Contributions: Conceptualization, A.J.H., R.E.K., K.L., and M.L.; data curation, F.A. and M.L.; investigation, A.J.H., D.L., and M.L.; methodology, A.J.H. and M.L.; resources, A.J.H. and M.L.; validation, A.J.H. and M.L.; writing—original draft preparation, A.J.H. and M.L.; writing—review and editing, F.A., A.J.H., D.L., R.E.K., K.L., and M.L.

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

Appendix A

Chloroplast DNA sequences of the *psbA-trnH* spacer region, the *matK* coding region, the *trnG* intron, and the *trnL* intron region of limber pine (*Pinus flexilis*), whitebark pine (*P. albicaulis*) that we generated during this study, along with selected sequences of pine species represented in GenBank. The numbers above the DNA sequences represent the nucleotide site number. The numbers following the species name refer to DNA isolate (Supplemental Table S1) and GenBank accession number. Annotations include locations of restriction endonuclease recognition sites. Wide formatting requires posting the Appendix to http://www.montana.edu/mlavin/data/Appendix.txt (copy and paste Appendix.txt contents into text editor without text wrapping.)

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