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cpSSR and High-Resolution Melting Analysis (HRM) for *Pinus pseudostrobus* Lindl. Variety Genotyping and Discrimination

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Abstract: The unambiguous identification of varieties within the *Pseudostrobus* complex is a key step to facilitate tree selection and monitoring in the wild as well as in plantations. Molecular tools provide a powerful approach for species delimitation; however, the use of DNA barcodes in this group has met limited success due to widespread haplotype sharing from lineage sorting, hybridization and introgression. Here, we evaluate the utility of real-time PCR coupled with high-resolution melting (HRM) to discriminate among *Pinus pseudostrobus* Lindl. var. *pseudostrobus*, *apulcensis* and *oaxacana*, from wild populations in central and southern Mexico, using chloroplast DNA sequence variants located within the *clpP*, *ycf2*, *trnL*(UAA)–*trnT*(UGU) and t*rnI*(CAU)–*trnF*(GAA) loci. The markers *ycf2*/*trnL*(UAA)–*trnT*(UGU) produced clear melting patterns that separated the varieties *pseudostrobus* and *oaxacana* from type var. *apulcensis*, whereas *clpP* discriminated over 60% of var. *oaxacana* individuals. This assay underlines the usefulness of these less-used DNA regions as potential biological markers and exhibits the effect of geography on allele distribution and the likely presence of hybrids among the species and varieties.

Keywords: chloroplast DNA; conifers; field populations; genotyping; real-time PCR; high-resolution melting curves

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1. Introduction

Pinus pseudostrobus Lindley is an economically important timber species distributed in Mexico and Central America throughout a wide altitude range (1600–3300 m above sea level) [1]. The species grows under a broad range of environmental conditions and exhibits considerable phenotypic variability and plasticity [2,3]. This has led to the recognition of different varieties or ecotypes, because of either adaptive processes [4,5] or hybridization events with *Pinus montezumae* Lambert and possibly other related species, particularly in populations within the Trans-Mexican Volcanic Belt [4,6–8]. As a result, the taxonomy of the species remains controversial; while Martínez (1948) [9] recognized up to five morphologically distinct varieties (var. *pseudostrobus* (typical), *oaxacana*, *apulcensis*, *coatepecensis* and *estevezii*), other authors have considered only two or three with different types and var. *oaxacana* as *Pinus oaxacana* Mirov [1,3,6]. The problem is further complicated by the fact that the distribution of the different varieties shows considerable overlap [3,6], which can create confusion when carrying out collections, since they are not always readily distinguishable in the field. The morphological differentiation among varieties is mostly based on apophysis elongation, peduncle length and cone size and shape [1,3].

Notwithstanding, the *Pseudostrobus* varieties exhibit important differences regarding their habitat preference and production potential; except for var. *estevezii*, which grows in northeastern Mexico, the other four varieties grow in central and southern Mexico. While var. *pseudostrobus* grows at higher altitudes and prefers deep and fertile soil, var. *apulcensis* grows at lower elevations and lower humidity [6] and var. *coatepecensis* also grows at low elevations, but only in the state of Veracruz, and var. *oaxacana* shows preference for dry areas. Provenance trials comparing growth traits, survival and frost damage in var. *pseudostrobus* and var. *apulcensis* show these to have important differences in growth and adaptive characteristics [5]. Therefore, the establishment of a fast and precise system to discriminate among species and varieties, that ease evaluation and monitoring, is important for tree breeding efforts and the establishment of seed and clonal orchards.

Chloroplast DNA (cp), such as intergenic spacers and genes, for example *trnK–matK*, *atpB–rbcL*, *ndhF–rpl32*, *psbJ–petA*, *trnL–trnF* and *matK–ycf1*, has been used in conifer species for identification, phylogenetic and population genetic studies [7,10,11]. In particular, simple sequence repeats (SSRs or microsatellites) are often informative for finer-scale patterns within and between populations because they are highly polymorphic; therefore, they are applied to visualize intraspecific genetic variability [12]. The advantages of using chloroplast microsatellite sequences (cpSSRs) in *Pinus* spp. include its highly conserved genome sequence, low (if any) recombination and paternal inheritance [13–15]. However, the description of cpSSRs for *Pinus* is limited, when compared to angiosperms [16–19].

Nevertheless, studies within the *P. pseudostrobus* complex and other pines of the *Montezumae* clade have shown that they exhibit low levels of sequence divergence and minimal phylogenetic structure [11,12,20], indicating that the taxonomic and phylogenetic issue is far from resolved. However, the identification and testing of new genetic markers to discriminate different varieties in a fast and inexpensive manner could provide a useful tool for forestry research in these species.

High-resolution melting analysis (HRM) is a highly sensitive, rapid and relatively inexpensive PCR-based method used for mutation scanning and genotyping. HRM detects SNPs (single-nucleotide polymorphisms) and small insertions or deletions in amplified DNA fragments by comparing changes in the dissociation rate of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) with the increase in temperature [21]. To monitor these changes, a fluorescent dye is added to the PCR mix that intercalates into DNA and fluoresces only when bound in dsDNA; changes in fluorescence occur as the temperature rises and the DNA strands separate. The thermally induced melting behavior depends on the thermodynamic characteristics of the PCR product, defined by sequence length, nucleotide order, GC content and complementarity [21].

HRM is used widely for allelic discrimination of well-characterized SNPs, or to screen for the existence of unknown variants [22], mainly in clinical and diagnostic studies [23,24]. When combined with short nuclear or chloroplast sequences, DNA barcodes (Bar-HRM), it provides a powerful tool for species and variety identification and authentication [25]. EST, SSR and characterized SNP markers have been used to discriminate among different varieties of cherry [26], olive [27] and lentil [25], among others. Likewise, distinguishing the origin of *Pinus radiata* D.Don and *Pseudotsuga menziesii* (Mirb.) Franco timber with the *trnL*(UAA) locus was accomplished, even by using wood samples [28].

Few studies have attempted to incorporate HRM analysis into wild population studies. Using nuclear DNA, HRM analyses allowed the identification of swordfish populations [29], whereas the internal transcribed spacer region 2 ribosomal DNA (*ITS2* rDNA) was tested for genotyping monotypic dinoflagellates [30] and strain identification and polymorphism characterization in field endosymbiotic *Wolbachia* was achieved by targeting a specific surface protein gene [31]. Examples in plants include chloroplast haplotype identification in *Arenaria* spp. [32], as well as the delimitation of different Mediterranean *Pinus* species using the *trnL*(UAA) locus [33]. This technology was also tested to screen for haplotype variation in the genus *Cyclopia* by using twelve non-coding cpDNA and applied to *C. subternata* wild populations with three markers within the *atpI–atpH, trnG–trnG2G* and *trnQ–5′rps16*

intergenic spacers [34]. As part of a program to establish *P. pseudostrobus* provenance trials and seed orchards in central and southern México, the aim of this study was to test various chloroplast simple sequence repeats (cpSSRs) to be used in combination with HRM for the rapid detection and differentiation of four *P. pseudostrobus* varieties (var. *pseudostrobus*, var. *apulcensis*, var. *oaxacana* and var. *coatepecensis*) obtained from wild populations growing in the region.

2. Materials and Methods

2.1. Plant Material

Needle tissue from 195 *P. pseudostrobus* trees growing in different populations in the central and southern part of the natural range was collected (Figure 1, Table S1). Individuals were phenotypically identified in the field by the collectors and selected based on their superior traits for the establishment of provenance trials and seed orchards. Ninety-seven samples were classified as var. *pseudostrobus* (provenances: Mexico State, Chiapas, Puebla, Tlaxcala, Oaxaca, Veracruz, Michoacán and Jalisco), 53 belonged to var. *oaxacana* (provenances: Oaxaca, Veracruz and Puebla), 31 were var. *apulcensis* (provenances: Hidalgo, Chiapas and Veracruz) and 16 were var. *coatepecensis* (provenance: Veracruz) (Figure 1). Trees selected from the same locality grew at least 100 m apart. From two to three small branches from each tree were collected and stored at −80 °C until used. Eight *P. montezumae* tissue samples were obtained from seedling needles from a commercial nursery in Puebla (four), needles from wild mature trees in Mexico State (two) and megagametophytes from seeds (Colpos seed collection) (two).

2.2. DNA Extraction

Total DNA was extracted and purified from 200 mg of needle tissue using a modified CTAB based method [35]. DNA concentration and quality were verified spectrophotometrically with NanoDropTM ND-1000 (Nanodrop Technologies Inc., Wilmington, DE, USA) and visually by standard agarose gel electrophoresis (0.8% agarose in TBE 1×). An aliquot of DNA was eluted in nuclease-free water to a final working stock of 10 ng/µL.

2.3. Primer Design and Selection

We researched the literature to identify known DNA regions that exhibited adequate polymorphism to perform a high-resolution melting (HRM) analysis. Furthermore, we aligned the complete chloroplast sequences for *P. montezumae* (JN854183.1) and *P. pseudostrobus* (JN854178.1), obtained from the National Center for Biotechnology Information (NCBI) with ClustalW [36]. Additionally, we included a consensus sequence obtained from an alignment of *Pinus oocarpa* Shiede ex Schltdl (KY963969.1), *Pinus taeda* Linnaeus (FJ899561.2) and *Pinus greggii* Engelm. ex Parl. 1867 (JN854198.1), that targeted regions of commonly used chloroplast markers in *Pinus* genetic diversity studies [7,10,12,37] (Figure 1).

To ensure detection by HRM, we established the following criteria for primer selection: (1) the targeted region exhibited polymorphism between *P. pseudostrobus* and *P. montezumae*; (2) region length was of less than 200 bp; (3) polymorphisms consisted mainly of G/C for T/A, $(TT/AA)_2$; (4) insertions or deletions of at least 2 bp. The tested primer pairs are summarized in Table 1. Amplification by each primer pair was verified using DNA from *P. pseudostrobus* var. *pseudostrobus* and *P. montezumae*. A standard PCR was performed in 10 µL reactions, using 1 µL of DNA at 10 ng/ µL and Taq Platinum® (Invitrogen), according to the manufacturer's instructions. The cycling parameters were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 5 min. Gel electrophoresis was performed to confirm amplification and to initially screen for variation in amplicon size. Only primers that amplified for both species were used.

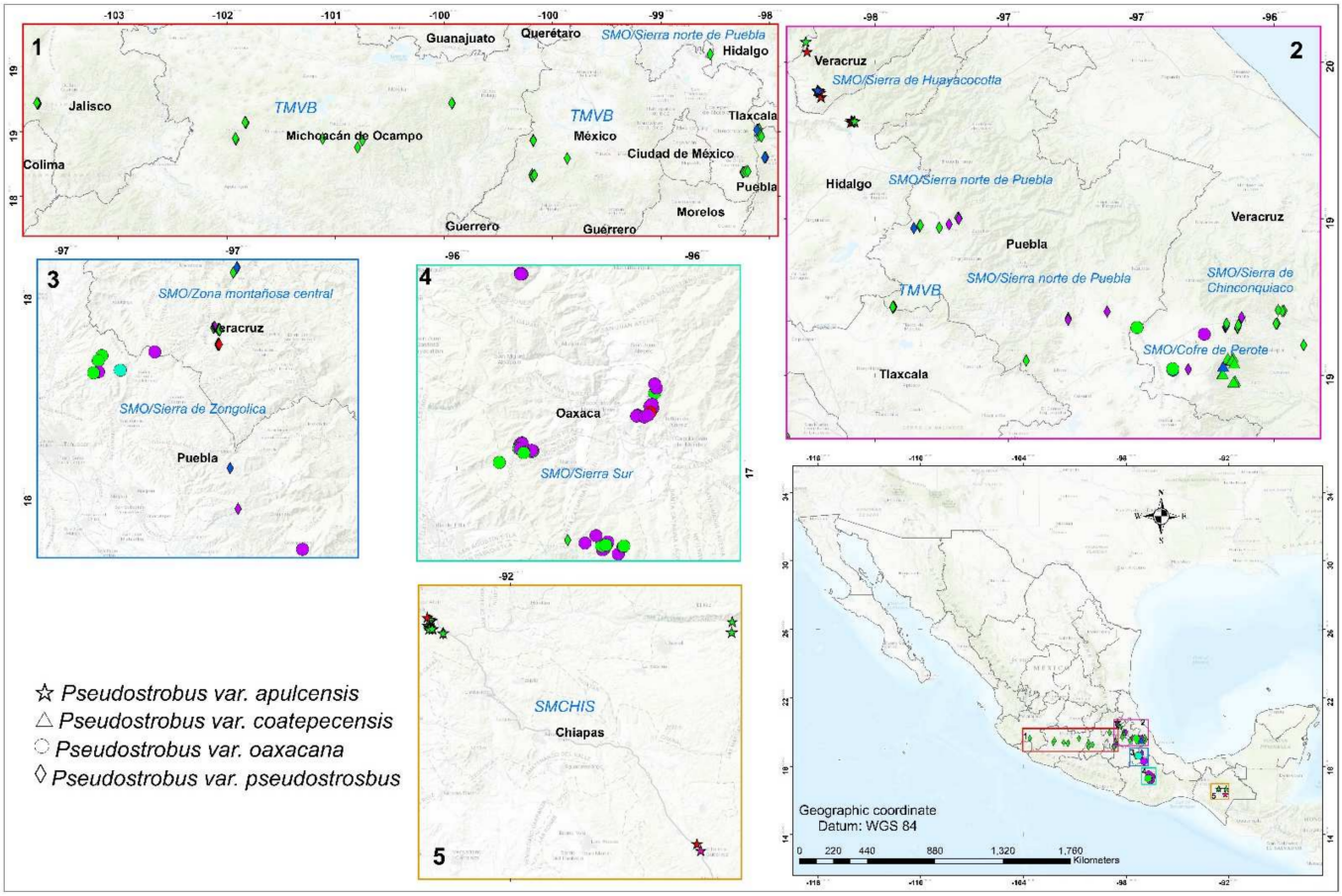

Figure 1. Geographic distribution of sampled populations of *Pinus pseudostrobus* varieties in central and southern Mexico. Different shapes represent varieties: *pseudostrobus* (diamond), *apulcensis* (star), *oaxacana* (circle) and *coatepecensis* (triangle). Box outline color and number (1–5) matches region indicated in the map, whereas colors reflect different haplotypes: A (green), B (purple), C (blue), D (red), E (magenta), F (turquoise), G (black), H (pink) and I (yellow). *TMVB*, *T*rans-*M*exican *V*olcanic *B*elt; *SMO*, *S*ierra *M*adre *O*riental; *SMCHIS*, *S*ierra *M*adre de *Chi*apas.

Table 1. Candidate microsatellite primers tested for high-resolution melting analysis.

Locus	Polymorphism	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
clpP (30204) Wofford et al. (2014)	Indel 4G	CGTAGCGGAAGGTTGATCTCAT	TTCGGATTGATCCTAACCATACCA
trnS–trnG IGS Dong et al. (2012)	G–T; T–G	TTGTCTTCTTCGGACTCCTACCCA	GAAGGGTTCTCTATCTATTTAGGGT
cpDNA5 *	indel TACAG	ACCCGCAACTTCCGTCTTGA	CCTATCTAGCGCCTATCATGGA
cpDNA6 *	indel AAATTAT; G–T	CCACATTAACATATTGACCCATACCT	AGATCTTTCAATTGCAGAACAGATAGG
cpDNA7 *	A–G; indel CGAAT	CCAGAGCTTTGGCTCCCATT	GATCGGATCCAAGTATCTTCCCA
cpDNA8 *	indel TCCCCTTCT	TGCTCAATATTGGGTGGGAGAAA	TCGATTGGGGTAGAGATAGAGAAGG
cpDNA9 *	indel GG	TCCTATGCGGGAACTGGAAT	TACTCGCAGTGTATTGTGGC
cpDNA10 *	indel (CTT)4	GCACAATCCGTTCAACTCTCTT	TCCCGTGCCTAATAGCTTTCTC

* primer pairs developed for this research study.

2.4. Real-Time PCR and HRM Analysis

To standardize the HRM conditions and test the discriminative power of primer pairs, three individuals from different populations of *P. pseudostrobus* var. *pseudostrobus* from Jalisco and Michoacán, three of var. *oaxacana* from Oaxaca, three of var. *apulcensis*

from Hidalgo, two of var. *coatepecencis* from Veracruz and four of *P. montezumae* from a commercial nursery were tested. Once the conditions were established, the HRM assay was performed using DNA from 184 additional *P. pseudostrobus* individuals. All experiments were carried out on a CFX96 cycler Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions (final volume of 20 µL) were set up in triplicate in transparent PCR plates (96 wells), with a 1× Type-it HRM PCR Kit (Qiagen®) that contained EvaGreen®, 800 µM of each primer and 1 µL of DNA at 10 ng/µL. The cycling parameters were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 10 s. After the PCR amplification steps, the melting curves for the products were generated by heating at 0.2 °C increments, at a rate of 10 s/step, with temperatures ranging from 65 to 95 °C. Plate set-up, PCR parameters, data acquisition and monitoring the amount of fluorescence for each sample was performed with CFX-Manager Software v1.6 (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

A post-PCR HRM analysis of the melting curves was carried out using Precision Melt Analysis™ software (Bio-Rad), which automatically clustered samples according to their melting profile (temperature and shapes of the melting curves). The confidence threshold for a sample to be included in a cluster was 99%. The melting curves were normalized using the pre- and post-melting temperature ranges automatically, given by software, or manually to achieve better results. Derivative plots were also generated to assess the different melting peaks. PCR products with different melting profiles were sequenced (Macrogen) in two directions to validate the results. The sequences were aligned with SeaView software [38] and MUSCLE (MUltiple Sequence Comparison by Log-Expectation) [39] a multiple sequence alignment tool. After sequencing, the amplified chloroplast DNA fragments were concatenated because they were not subject to recombination events. With this information, the haplotype identity was assigned in each *P. pseudostrobus* sample according to the method shown by Galuszynski and Potts [34].

3. Results

To evaluate the effectiveness of high-resolution melting (HRM) as a tool to discriminate among the four *Pseudostrobus* varieties sampled, we first standardized the conditions using a small number of "type" trees of each taxon (var. *pseudostrobus*, *oaxacana*, *apulcensis* and *coatepecensis*), according to morphological data provided by the collectors. As a first criterion, we selected the targeted region based on in silico sequence polymorphisms between *P. pseudostrobus* var. *pseudostrobus* and *P. montezumae*, considering the need to differentiate between these and potential hybrids. PCR analyses were performed and amplicons were separated using gel electrophoresis to screen the different primer pairs and those that produced noticeable changes in size were prioritized for qPCR testing. The amplicons were then subjected to the HRM curve analysis and the most promising primers were chosen considering whether they clustered the different varieties; those that exhibited little inter-varietal polymorphisms (*trnS–trnG* IGS, *cpDNA5* and *cpDNA9*) were not followed. No single target region allowed the simultaneous distinction of all varieties to be performed, although the sequential use of two primer pairs allowed us to discriminate among the type individuals of var. *pseudostrobus*, *oaxacana* and *apulcensis*. However, we were unable to detect a marker that could separate var. *coatepecensis* from var. *pseudostrobus*.

The HRM curves for the target region *clpP*(30204) revealed that *P. pseudostrobus* var. *oaxacana* could be visually separated from var. *pseudostrobus/coatepecensis/apulcensis* and *P. montezumae*, as these were highly characteristic for each group, indicating two potentially polymorphic amplicons (red and green curves in Figure 2A). Sanger sequencing confirmed the amplicons of 162 bp for var. *pseudostrobus/coatepecensis/apulcensis/P. montezumae* (allele *clpP*(30204)-1) and 157 bp for var. *oaxacana* (allele *clpP*(30204)-2), in which the difference in size was the result of an indel of (G)5 at position 99 (Figure 2B).

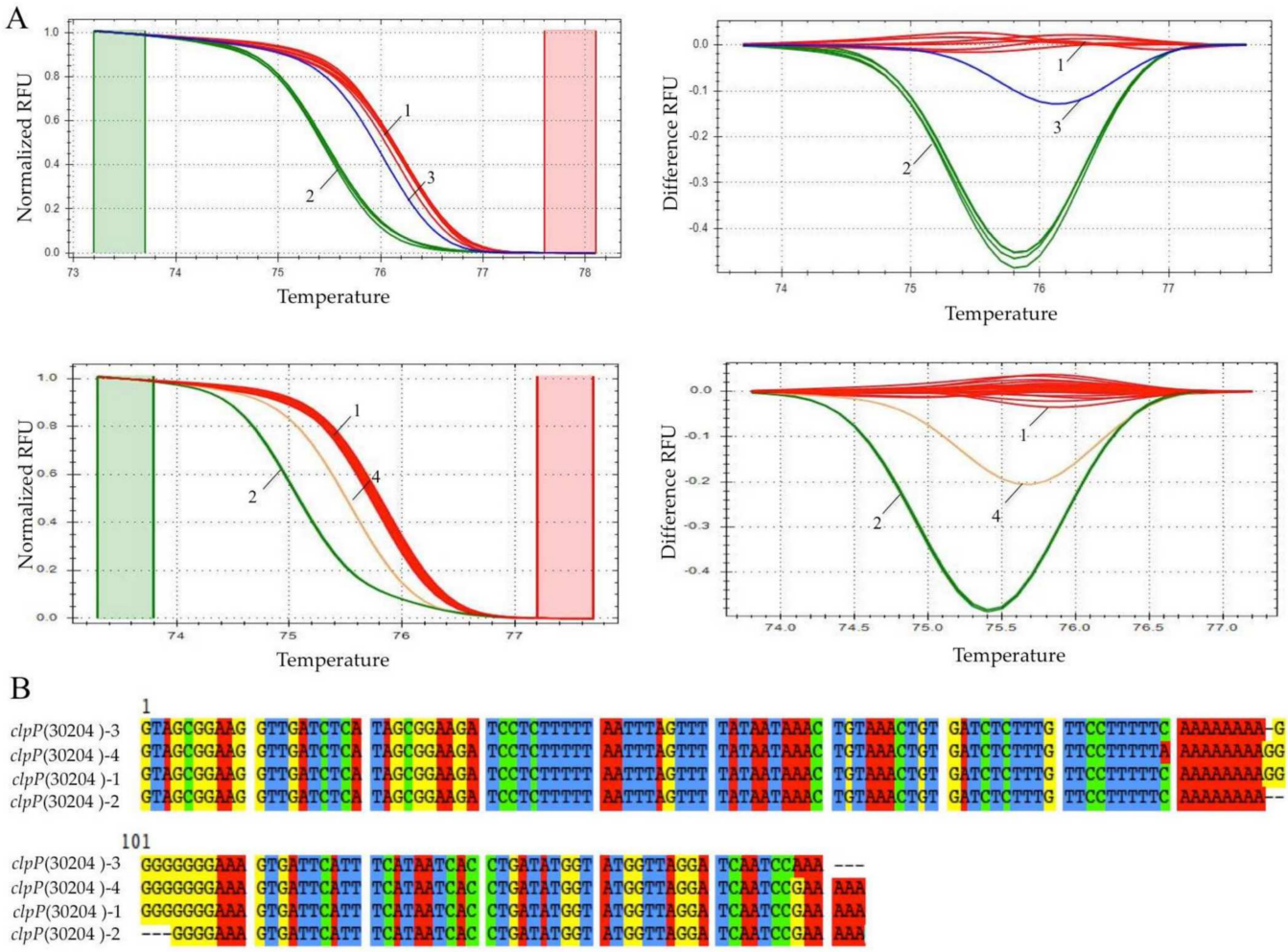

Figure 2. Real-time PCR-HRM analysis with primer set *clpP*(30204). (**A**) Representative normalized (left) and difference curves (right) depicting different alleles: *clpP*(30204)-1 (red), *clpP*(30204)-2 (green), *clpP*(30204)-3 (blue) and *clpP*(30204)-4 (yellow). (**B**) Sequence alignment comparing the amplicons of the different alleles including primers.

In the interest of further discriminating among var. *pseudostrobus*, var. *coatepecensis*, var. *apulcensis* and *P. montezumae*, various primer pairs were tested (Table 1); of these, *cpDNA10* and *cpDNA8* produced HRM patterns that allowed us to perform the most consistent grouping of the taxa, in accordance with their morphological identification. The *cpDNA10* primer produced two discernible HRM melting profiles (Figure 3A), corresponding to a 175 bp amplicon for the var. *pseudostrobus/coatepecensis/oaxacana* (allele *cpDNA10*-1) and a 163 bp amplicon for var. *apulcensis/P. montezumae* (allele *cpDNA10*-2) that resulted from a (TCTTCC)2 at position 60 (Figure 3B). Likewise, the *cpDNA8* primer clustered samples of var. *pseudostrobus/coatepecensis/oaxacana* (*cpDNA8*-1) in a different group from *P. montezumae* (*cpDNA8*-2); however, it subdivided the five different var. *apulcensis* samples from Hidalgo into three groups, i.e., one with var. *pseudostrobus/coatepecensis/oaxacana* (*cpDNA8*-1), two with *P. montezumae* (*cpDNA8*-2) and a final two that were unique to the individuals (*cpDNA8*-3) (Figure 3C). Sequencing showed that allele *cpDNA8*-1 was 111 bp long, whereas *cpDNA8*-2 had a length of 94 bp and they differed by indels at positions 35 (CTCCCCTTTT) and 60 (TCTCTA) (Figure 3D). The unique HRM profile found for *cpDNA8*-3 in two specimens was due to the amplification of a double band and could not be adequately sequenced (data not shown).

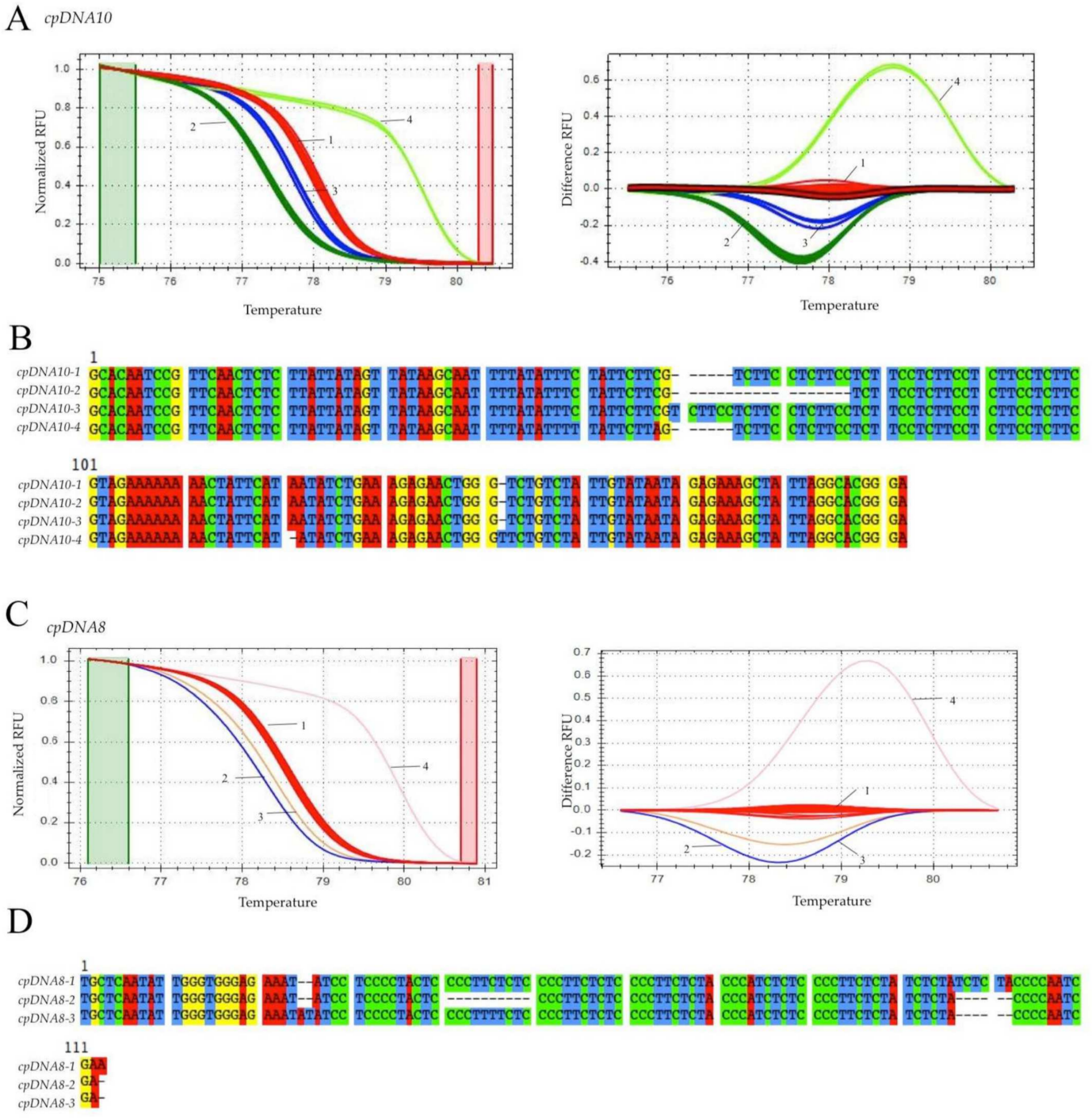

Figure 3. Real-time PCR-HRM analysis with primer sets *cpDNA10* and *cpDNA8*. (**A**) Representative normalized (left) and difference curves (right) depicting different alleles: *cpDNA10*-1 (red), *cpDNA10*-2 (green), *cpDNA10*-3 (blue) and *cpDNA10*-4 (light green). (**B**) Sequence alignment comparing the amplicons of the different *cpDNA10* alleles. (**C**) Representative normalized (left) and difference curves (right) depicting different alleles: *cpDNA8*-1 (red), *cpDNA8*-2 (blue), *cpDNA8*-3 (yellow) and *cpDNA8*-4 (pink). (**D**) Sequence alignment comparing the amplicons of alleles from *cpDNA8*-1 to -3; *cpDNA8*-4 was not sequenced.

We further used *clpP*(30204), *cpDNA8* and *cpDNA10* to investigate their convenience in distinguishing among the varieties of *Pseudostrobus*, as well as to detect other potential polymorphisms (alleles) in these chloroplast regions, in 184 additional adult trees growing throughout Mexico State, Puebla, Chiapas, Tlaxcala, Oaxaca, Veracruz, Michoacán and

Jalisco, as well as in four additional *P. montezumae* specimens. Screening with the *clpP*(30204) marker showed that 64% of var. *oaxacana* specimens (34 of 53 trees) collected in Oaxaca, Puebla and Veracruz exhibited the *clpP*(30204)-2 variant, which increased to 71% when only populations from regions within the Sierra Madre del Sur in Oaxaca were considered. The remainder grouped with var. *pseudostrobus* (allele *clpP* (30204)-1). Of the other analyzed taxa, most exhibited *clpP*(30204)-1, as expected, although eleven var. *pseudostrobus* obtained from Puebla, Oaxaca and Veracruz, as well as a single var. *apulcensis* individual, shared the *clpP*(30204)-2 allele (Table S1). Two unique melting curve profiles were distinguished for a single var. *pseudostrobus* specimen from Michoacán (Figure 2A) and a var. *apulcensis* specimen from Veracruz. The sequencing of the first revealed the deletion of G in position 99 (allele *clpP*(30204)-3, sequence length of 162 bp) (Figure 2B) and the substitution of an A/G at position 90 (haplotype *clpP*(30204)-4, sequence length of 163) in the second (Figure 2B).

Screening populations with *cpDNA8* and *cpDNA10* showed that the HRM profiles of most var. *pseudostrobus, oaxacana* and *coatepecensis* clustered together and the sequencing of random samples showed that they exhibited the *cpDNA8*-1 and *cpDNA10*-1 alleles, as expected. Twelve representatives of var. *pseudostrobus,* two from var. *oaxacana* and two from var. *coatepecensis* formed a different cluster together with *P. montezumae* and sequencing verified that they carried the *cpDNA8*-2 and *cpDNA10*-2 alleles. Although, in most cases, both markers clustered samples similarly, of the 26 trees identified as var. *apulcensis, cpDNA10* distinguished a greater number of individuals and clustered them accordingly with *P. montezumae*, which was further confirmed by sequencing (Table S1); specifically, eight specimens from Chiapas and Veracruz clustered in a typical pattern shown by samples obtained from Hidalgo (type locality, *cpDNA10*-2). Additional melting profiles with *cpDNA10* reflecting different alleles were detected and confirmed by sequencing (Figure 3B); two specimens from Jalisco showed an insertion (TCTTCC) at position 59 (sequence length 181 bp; *cpDNA10*-3). A single specimen from Veracruz presented an allele (*cpDNA10*-4) with various base changes throughout its sequence (T/C at position 50, A/C at 58, a deletion of A at 121 and an insertion of a T at 142) that had greater homology (98%) with *P. devoniana* Lindley 1839 rather than *P. pseudostrobus* or *P. montezumae*. This last sample also exhibited a different allele with *cpDNA8* (106 bp; *cpDNA8*-4) that resulted from the insertion of AT at position 23, the insertion at position 35 present in the allele *cpDNA8*-1 with a T/C at position 46; however, it also exhibited the deletion characteristic of *cpDNA8*-2 at position 60.

Considering the three markers, we detected 11 alleles in the *Pseudostrobus* populations, which conformed to 10 haplotypes (A–J). Haplotypes A–D were the most widely distributed, whereas F–I were each represented by one or two individuals (3.5% of the total samples); thus, they may be considered rare and are unlikely to help differentiate among varieties, even though, at this stage, they did evidence the usefulness of HRM in detecting allelic variants. The J haplotype was unique to individuals of var. *apulcensis* from Hidalgo and was not found in specimens of this variety from other regions. Haplotype A clustered most of the representatives of the species—73% of var. *pseudostrobus*, 47% of var. *apulcensis*, 32% of var. *oaxacana* and 88% of var. *coatepecensis* (Figure 4)—and was clearly distinguished from haplotype C, which conformed to *P. montezumae* (Figure 4). Interestingly, members of var. *apulcensis* showed the most variation; specimens from Hidalgo exhibited the C and J haplotypes; in Sierra Huayacocotla (Veracruz), the majority conformed to a unique D haplotype (56%), although other less frequent haplotypes were detected (A, B, H and I), while most specimens from Sierra Madre of Chiapas conformed to haplotype A and sporadically D or F (Figure 4). Haplotype B was more common in var. *oaxacana* samples from southern Oaxaca (70%), which contrasted with those obtained further north and from Veracruz and Puebla, where the frequency of haplotype A increased to nearly 50%.

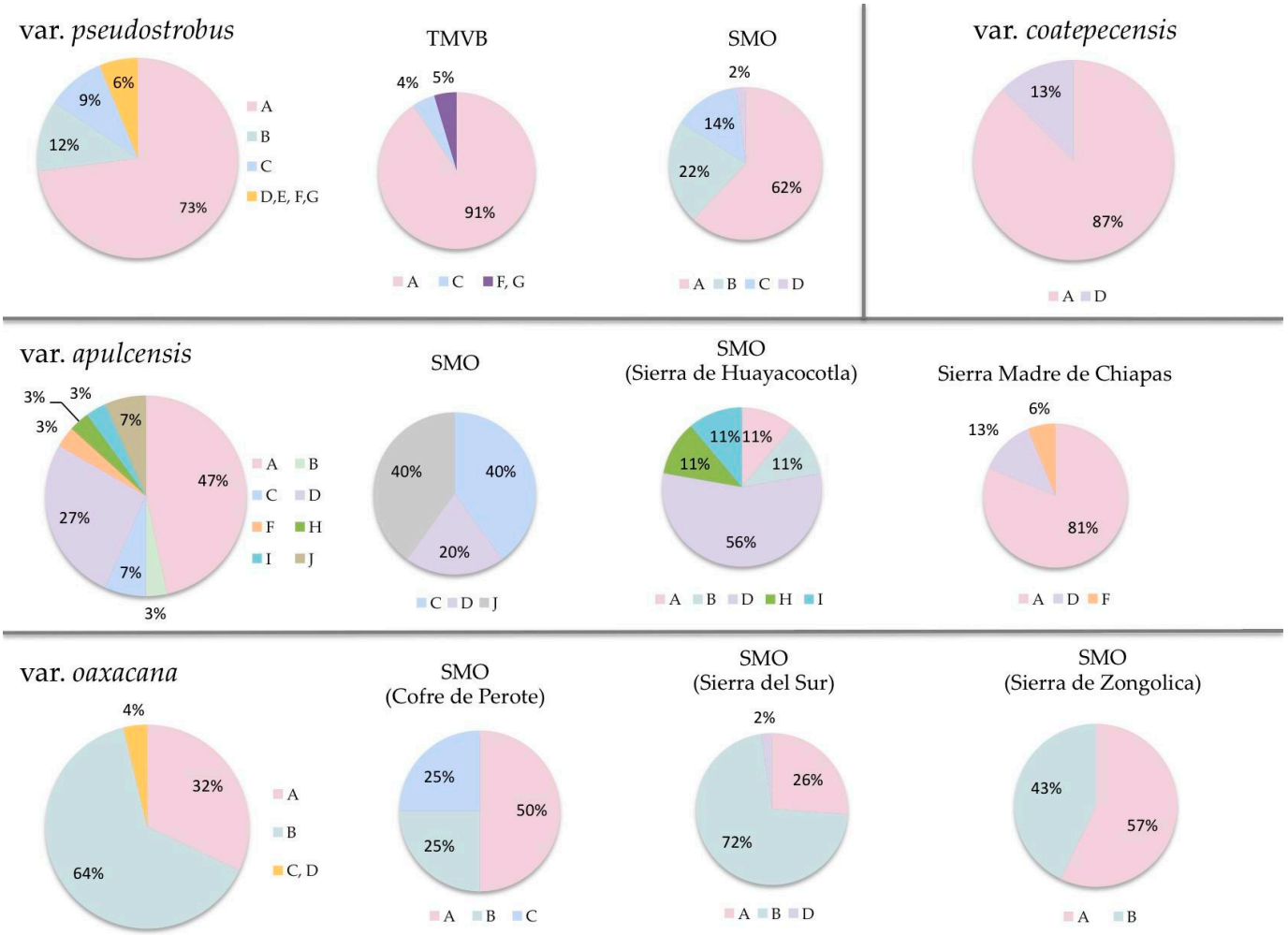

Figure 4. Haplotype frequencies found for the different varieties in the Trans-Mexican Volcanic Belt (TMVB), different regions of the Sierra Madre Oriental (SMO) and Sierra Madre de Chiapas.

4. Discussion

The unambiguous identification and classification of members of the subsection *Ponderosae* and, particularly, of the *Pseudostrobus* complex has generally been a challenge for researchers and for collectors in the field. The group exhibits a mosaic of morphological variation throughout its wide distribution range [4,40], which is further enhanced by its tendency to form natural hybrids with *P. montezumae and*, possibly, *P. hartwegii* [7]. The use of HRM coupled with SSR markers represents a means to direct sampling of representative individuals from natural populations, as well as the potential to explore genetic diversity for breeding and selection purposes. In order to assign individuals morphologically classified as *P. pseudostrobus* var. *pseudostrobus*, var. *apulcensis*, var. *oaxacana* or var. *coatepecensis*, we reviewed both the literature and sequences from NCBI to identify candidate markers to test them as barcodes for HRM analysis.

Our search showed that many of the reported chloroplast regions commonly used in phylogenetic studies, including the *trnL*(GAU)–*trnA*(UGC) spacer, *rbcL, matK, ycf1*, *trnK*(UUU) spacer, *trnG*(UCC) intron, *rps2*, *psbJ–petA* spacer, downstream *trnS*(UGA) and *psbA–trnH* intergenic spacer, were identical between *P. pseudostrobus* and *P. montezumae*; therefore, they were ineffective in discerning between the species, or varieties. This is consistent with reports of their limited applicability to assess phylogenetic relationships at lower taxonomic levels [20,41,42]. The low genetic variation exhibited at these sites is consistent with the lack of phylogenetic resolution among the taxa within the Montezumae clade (which includes *P. pseudostrobus*, *P. montezumae*, *P hartwegii* and *Pinus maximinoi* H.E. Moore,

1967) and is attributed to interspecific gene flow, introgression and incomplete lineage sorting that could explain the sharing of plastid haplotypes among pine species [7,11,43]. Other markers, such as *IRF169* intron 1 and *rpoB* [44], which showed a single base pair change, were also eliminated from the study, since we wanted to ensure a clear-cut HRM profile to differentiate among varieties. The type of polymorphism these regions presented (single base pair change of T and A) produced a subtle temperature shift (0.1 °C) that could potentially pass unnoticed in the HRM analysis due to the detection limit of the technique. Although the base change in *trnS–trnG* IGS (G/T) [45] produced a clear shift in the melting pattern with HRM that could be useful to distinguish *P. pseudostrobus* from *P. montezumae*, a preliminary assay indicated this was not the case for *Pseudostrobus* varieties (data not shown), suggesting low or no intraspecific polymorphisms.

The full chloroplast sequences reported for *P. pseudostrobus*, *P. montezumae* and other species of *Pinus* were compared to identify candidate loci that were polymorphic among these taxa, which could potentially produce contrasting melting patterns with HRM. Primer pairs *cpDNA10* and cpDNA8 unambiguously separated 88% of var. *pseudostrobus* from *P. montezumae*. Primer pair *cpDNA10* is located within the *ycf2* locus; this region has been proposed as a candidate or supplementary region for phylogenetic analysis at lower taxonomic levels in *Pinus* [46] and shown its potential as a barcode to discriminate among species of the *Pinus mugo* complex [47]. Although, in *Pinus ycf2*, it is less variable than *ycf1* [46], our in silico analysis showed it allowed us to distinguish *P. pseudostrobus* from *P. montezumae*, reinforcing the view that this chloroplast locus is worth further exploration. Primer pair *cpDNA8* is located in the *trnL*(UAA)–*trnT*(UGU) intergenic spacer, similar to the *PcL2T1* [12], although it encompasses a shorter region in order to facilitate HRM detection. When tested to explore their potential to discriminate among *Pseudostrobus* varieties, both *cpDNA10* and *cpDNA8* produced two main alleles that clustered var. *pseudostrobus*/var. *oaxacana*/var. *coatepecensis* and var. *apulcensis* with *P. montezumae*. The grouping of different individuals produced by these primer pairs differed only in 15 of 196 specimens, with *cpDNA10* showing greater resolution to separate var. *apulcensis/P. montezumae* from the larger var. *pseudostrobus* cluster. This general melting pattern posed the additional challenge of identifying other chloroplast regions that could separate the taxa within each cluster.

The locus *clpP*(30204) was tested on the basis that it was substantially polymorphic and has proved useful to discriminate among species in *Ponderosae* [12]; our assay showed that it correctly grouped 64% of the specimens identified as var. *oaxacana* from the rest of the analyzed taxa and was present in over 70% of the trees sampled from Oaxaca (Sierra del Sur, SMO). This represents an important achievement of the assay, since identification based on apophysis length and cone size provides variable results. Moreover, this variety grows in intermixed populations with var. *pseudostrobus* in southern Mexico and with var. *apulcensis* in Veracruz [3], allowing potential gene flow to occur among the taxa, further evidenced by the presence of this haplotype in var. *pseudostrobus* individuals from Puebla and Veracruz. Single-locus barcodes have revealed variable levels of success from 26% to 31% in *Inga* and *Araucaria* that increased to 32% and 69% when two or three loci were used [41] and up to seven loci have been used to discriminate up to 60% of *Picea species* [48]. Interestingly, the *clpP*(30204)-2 allele was detected sparingly in var. *apulcensis* only in one specimen from Veracruz and was never found in individuals of this taxon from Chiapas. The reasons for this are not clear; however, they may be related to limited sampling [49].

Here, we were unable to identify markers that could differentiate var. *coatepecensis* from the other taxa. Because *P. pseudostrobus* is very polymorphic, to date, the acceptance of var. *coatepecensis* is subject to controversy. In the 1940s, taxonomists recognized this variety following Martínez' [9] classification of the *Pseudostrobus* group; later, Stead [2] grouped this variety into subsp. *apulcensis*, based on needle anatomy and micromorphological characteristics. Since then, the recognition of the variety has remained discretional and specimens from regions in Veracruz, where this taxon is found, have not been included in more recent phylogenetic analyses, or are considered synonyms of var. *pseudostrobus* [7,11,20]. Our results are suggestive of the latter; yet, it is possible that var. *coatepecensis* consists of a

particular ecotype with limited distribution and of recent diversification, so the discrimination from the other members of the complex may require the use of faster evolving loci, or exhibit subtler changes than those selected for this study (such as changes in single nucleotides).

Although establishing taxonomic limits and relationships within the *Pseudostrobus* complex is beyond the scope of this work, in the attempt to identify loci that associate with particular taxa for barcoding purposes, we provide further evidence of the usefulness of these less explored chloroplast regions to analyze relationships at lower taxonomic level in *Pinus* [12,44,47,50,51]. They also reflected the difficulties faced when attempting to assign a single locus for the discrimination of taxa, due to extensive sharing of alleles. Notwithstanding, our results show emerging patterns that are worthy of further consideration. The mapping of the different *Pseudostrobus* varieties showed a geographical effect on the prevalence of the different haplotypes (Figures 1 and 4). Over 85% of the var. *pseudostrobus* from Michoacán and Jalisco and most of the Trans-Mexican Volcanic Belt (TMVB) identified morphologically corresponded closely to the type specimens and shared the predominant haplotype A and, in a few cases, rare alleles that gave rise to unique haplotypes (E, F and G). However, as the TMVB spreads east and comes together with the Sierra Madre Oriental (SMO) in Puebla and Veracruz, the observed haplotype frequencies changed and there was a notorious increase in haplotypes B and C, that reached nearly 50%. This is suggestive of increased gene flow among taxa in these transitional zones where different populations converge.

A similar geographical effect was also seen for var. *apulcensis*, where half of the specimens from eastern Hidalgo, near Apulco, where the type specimen was collected, shared haplotype C and appeared to be more closely related to *P. montezumae*, whereas the other half showed a unique haplotype J. In contrast, in the Sierra Madre del Sur, in Chiapas, the A haplotype predominated, whereas those from northern Veracruz were conformed mostly by the D haplotype, although various other less-represented variants were also present. This is consistent with other reports analyzing morphological traits [3] and could provide further explanation for the lack of resolution observed in most molecular phylogenetic analyses including these taxa [11,20]. This discontinuous pattern may indicate different geographic lineages for the varieties, with greater introgression or hybridization between *P. pseudostrobus* and *P. montezumae* occurring in the TMVB [7] and possibly var. *estevezii* from the northern range of the SMO, which was not included in this study.

The real-time PCR-HRM approach presented here also provided a means to identify different or rare alleles in wild-growing *Pseudostrobus* populations. The technique was sensitive enough to allow us to perform the discrimination of amplicons based on changes in a single nucleotide (*clpP*(30204)-3 and *clpP*(30204)-4), which speaks of its potential to perform accurate large-scale screenings. These results are particularly important for low-budget labs without sequencing capacity, because PCR-HRM substantially reduces costs. Real-time PCR equipment with adequate sensitivity is becoming standard in many laboratories with molecular biology capabilities; furthermore, there is an increase in the number of companies that develop and supply reagents, so costs have lowered in consequence. The main caveat is the cost of specialized software for the HRM analysis, although freeware developed by universities or as part of R-based resources can circumvent this limitation if needed. Thus, the identification of informative markers for a non-model tree species remains the most relevant hurdle that can limit implementation and represents the most expensive phase during the establishment of a reproducible protocol. An important accolade to reduce costs and make the overall analysis more efficient would include the development of a multiplex assay.

5. Conclusions

Here, we describe the application of an HRM curve analysis for the inter- and intraspecific discrimination of individuals from wild populations of *P. pseudostrobus* and *P. montezumae,* using chloroplast SSP markers. Although none of the tested markers was able

to produce clusters exclusive to a single variety, performing sequential real-time PCRs with a second marker usually allowed us to perform the discrimination of most of the taxa. The marker *cpDNA10* allowed us to conduct a relatively straightforward discrimination of two groups, *P. pseudostrobus* var. *pseudostrobus*/var. *oaxacana* and *P. pseudostrobus* var. *apulcesis/P. montezumae.* Afterward, the analysis with *clpP*(30204) permit us to perform the separation of 64% of the individuals identified as var. *oaxacana.* Discerning between *P. pseudostrobus* var. *apulcensis* and *P. montezumae* was successful in 83% of the cases. However, these markers did not discriminate between var. *pseudostrobus*/var. *oaxacana* from var. *coatepecensis*, reflecting the existing challenge encompassed in identifying phylogenetically informative molecular markers to establish taxonomic limits within the *Pseudostrobus* complex and other closely related species. However, the geographical effect on the distribution of eleven haplotypes, evidenced by this study, may support further research into the biogeography of the group. Even with its limitations, HRM shows great potential as a useful, fast and economical molecular tool for high-throughput intraspecific, as well as allelic, discrimination of wild populations of *Pinus* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13020200/s1, Table S1: Location of collected specimens of different *Pseudostrobus* varieties and grouping according to haplotype as obtained by HRM analysis.

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